を認めた場合などでは禁忌で無い限り抗凝固療法を行う。またできる限り肺血流シンチグラフィーで肺塞栓症の有無を確かめることが望ましい。またこの場合に造影 CT は行わない。

６．　エコー検査で膝窩静脈を含む中枢性 DVT が見つかった場合は禁忌でない限り抗凝固療法を行う。また大腿静脈より中枢の DVT では造影 CT で腸骨静脈血栓などの有無と肺動脈血栓の有無を確かめることが望ましい。

（参考）新潟県中越地震被災者の DVT 検診においてエコー検査で DVT を認めなくても D ダイマーが高値の場合では慢性肺塞栓症、膠原病、癌、リンパ線維筋症(LAM)などが病院受診で発見されているので注意が必要である。

研究成果の刊行に関する一覧表

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研究成果の刊行物・別冊

Thrombosis Research 128 (2011) 169–173

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

Epitope analysis of autoantibodies to ADAMTS13 in patients with acquired thrombotic thrombocytopenic purpura ☆

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ARTICLE INFO

Article history:
Received 28 January 2011
Received in revised form 5 March 2011
Accepted 17 March 2011
Available online 14 April 2011

Keywords:
ADAMTS13
Thrombotic thrombocytopenic purpura
Autoantibody
Phage surface display system
Von Willebrand factor

ABSTRACT

Introduction: Autoantibodies to ADAMTS13 have a pivotal role in the pathogenesis of acquired thrombotic thrombocytopenic purpura (TTP). By decreasing the function of ADAMTS13, autoantibodies impair the cleavage of ultra-large von Willebrand factor (UL-VWF) multimers into smaller sizes, leading to lethal platelet-VWF thrombi in the microcirculation. We therefore aimed to determine the sites of autoantibody recognition on ADAMTS13.

Materials and Methods: In this study, IgG purified from 13 acquired TTP patients were examined to determine their binding sites on ADAMTS13. Immobilized IgG on microtiter plate or proteinG beads was screened by phage library expressing various peptides of ADAMTS13.

Results: In screening, diverse peptide sequences were obtained from almost all of the ADAMTS13 domains, including the spacer domain, which is considered a major binding site. In particular, we detected an identical amino-acid sequence in the C-terminus of the spacer domain from Gly662 to Val687 that was recognized by autoantibodies from 5 TTP patients. The specific autoantibody was expected to be associated with the plasma levels of the ADAMTS13 antigen or activity, and with the quantity of ADAMTS13 autoantibodies or the inhibitory autoantibody titer in TTP patient plasma. These measurements, however, did not seem to be related to the presence or absence of the specific autoantibody.

Conclusions: These findings indicate that the specific autoantibody might be a feature of acquired TTP, although its clinical significance remains to be elucidated.

Introduction

Thrombotic thrombocytopenic purpura (TTP) is a life-threatening disease characterized by microvascular platelet-rich thrombi leading to multiple organ failure [1]. The main clinical features are thrombocytopenia, hemolytic anemia, renal failure, neurological dysfunction, and fever. The plasma of TTP patients contains ultra-large von Willebrand factor (UL-VWF) multimers, which are highly reactive with platelets [2,3]. UL-VWF multimers are secreted into the plasma and rapidly processed into smaller and less reactive multimers [4] by cleavage at position Tyr^{1605}-Met^{1606} in the A2 domain. The VWF-cleaving protease is a member of the ADAMTS family, ADAMTS13 [5–8]. The proximal N-terminal domains, consisting of a metalloprotease domain, a disintegrin-like domain, a thrombospondin-1 repeat (TSP1), a cysteine-rich domain, and a spacer domain, are considered essential for the specific binding and subsequent cleavage of VWF [9–12], and seven additional distal C-terminal TSP1 repeats and two CUB domains have significant roles in the recognition of VWF, especially under flow conditions [13,14]. Loss of ADAMTS13 function leads to the accumulation of UL-VWF, resulting in microvascular platelet aggregation.

Autoantibodies to ADAMTS13 are detected in the majority of patients with acquired TTP and are considered to be strongly involved in the pathogenesis. The inhibitory autoantibodies are usually the IgG isotype and non-inhibitory autoantibodies are usually the IgG, IgM [15,16] and IgA [17] isotypes. IgG4-subtype autoantibodies are detected in 90% of patients with acquired TTP [18], although the clinical significance remains unknown. Clinically, high inhibitory autoantibody titers at the onset or during remission are associated with a high risk of relapse in patients with acquired TTP [17,19,20] and lower survival rates [21]. Interestingly, IgG autoantibodies are also detected in 13% of patients with systemic lupus erythematosus and 5% of patients with

☆ This manuscript was presented at 50th American Society of Hematology Annual Meeting (San Francisco, FL on 7/12/2008).

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doi:10.1016/j.thromres.2011.03.010

antiphospholipid antibody syndrome, and IgM autoantibodies are also detected in 18% of patients with systemic lupus erythematosus or antiphospholipid antibody syndrome. Moreover, 4% of healthy individuals are reported to have anti-ADAMTS13 IgG autoantibodies [22].

The pivotal epitopes of anti-ADAMTS13 autoantibodies reside in the spacer domain [23–28]. In the present study, to clarify the precise peptide sequences recognized by anti-ADAMTS13 IgG autoantibodies, we constructed a random cDNA fragment library expressing various peptides of ADAMTS13 on the surface of the lambda phage (ADAMTS13 phage library). We then screened the library using purified IgG immobilized on a microtiter plate or protein G beads in solution, and detected the specific peptide sequence in the spacer domain in 5 of 13 TTP patients. We next assessed the association of the specific autoantibody with the plasma levels of the ADAMTS13 antigen or activity, and the amounts of anti-ADAMTS13 IgG autoantibodies or the inhibitory autoantibody titer.

Materials and methods

Patient samples

Plasma IgG from 13 patients with acquired TTP were used for this screening. These samples were collected at the Mie University (P1~10) and Nara Medical University (P11~13) according to the guidelines of the Ethics Committees of each facility. The clinical features of the TTP patients are described in Table 1.

Construction of ADAMTS13 phage library

The ADAMTS13 phage library was constructed according to previously described methods [29]. Briefly, human wild-type ADAMTS13 cDNA cloned in pcDNA3.1/ myc-His (Invitrogen, Carlsbad, CA) was digested with DNase I, blunted with T4 DNA polymerase and attached to SfiI adaptors. The ligated fragments were fractionated by 3% agarose gel electrophoresis and agarose-containing cDNA fragments from 80 to 160 base pairs were excised, purified, and inserted into the lambda fooDc phage vector digested with SfiI. The phage particles were then created with packaging mixtures (MaxPlax™ Lambda Packaging Extracts; EPICENTRE Biotechnologies, Madison, WI). The phage library was amplified using the *Escherichia coli* strain Q447 to approximately 1×10^7 plaque forming units and stored at 4 °C until screening was performed.

The library was grown with the *E. coli* strain TG1 until complete lysis. After centrifugation, the supernatant was incubated with DNase I and RNase. After another centrifugation, the phage particles were precipitated with polyethylene glycol solution on ice, collected by centrifugation, and resuspended in blocking buffer #1 (0.25% bovine serum albumin, 5% skim milk, 0.1% Tween20) or blocking buffer #2 (2.5% bovine serum albumin, 0.1% Tween20).

Library screening using IgG purified from TTP patients

The ADAMTS13 phage library screening and DNA sequence analysis were performed according to previously described methods [29]. The library was screened using IgG purified from TTP patients, either immobilized or in solution. For screening in the immobilized condition, serially diluted IgG was immobilized on the wells of a microtiter plate overnight at 4 °C. The wells were preblocked with blocking buffer #1 for 1 h at room temperature, then 50 μl of the ADAMTS13 phage library was added to the wells and incubated overnight at 4 °C. The wells were then washed three times with blocking buffer #1, twice with washing buffer #1 (5% skim milk, 0.5% Tween20), and once with washing buffer #2 (10 mM Tris-HCl pH7.4, 5 mM $MgSO_4$, 0.2 M NaCl, 10 mM $CaCl_2$). Bound phages were eluted with 50 μl of washing buffer #2 containing collagenase for 1 h at 37 °C. After the panning procedure was repeated five times, phages were randomly selected and subjected to DNA sequence analysis.

For the screening in solution, 10 μg of purified IgG from TTP patients was mixed with 25 μl of protein G beads (Dynabead® Protein G; Invitrogen) for 40 min at room temperature. The beads were then preblocked with blocking buffer #2 for 1 h at room temperature, mixed with 50 μl of the phage library, and incubated overnight at 4 °C. The beads were washed three times with blocking buffer #2, twice with washing buffer #1 (2.5% bovine serum albumin, 0.5% Tween20), and once with washing buffer #2 (10 mM Tris-HCl pH7.4, 5 mM $MgSO_4$, 0.2 M NaCl, 10 mM $CaCl_2$). The same procedure was performed for DNA sequence analysis as above.

ADAMTS13 antigen and activity levels, and IgG autoantibody titer in TTP plasma

The ADAMTS13 antigen level in the plasma of patients with TTP was measured using an enzyme linked immunosorbent assay (ELISA) kit (IMUBIND® ADAMTS13 ELISA; American Diagnostica, Stamford, CT) according to the manufacturer's protocol.

ADAMTS13 activity was measured using a FRETS-VWF73 assay (Peptide Institute, Inc., Osaka, Japan) [30] for P1~10 or an ADAMTS13 act-ELISA (Kainos Inc., Tokyo, Japan) [31] for P11~13.

The level of anti-ADAMTS13 IgG autoantibody was examined using an ELISA kit (IMUBIND® ADAMTS13 Autoantibody ELISA; American Diagnostica) according to the manufacturer's protocol. The inhibitory effect of the autoantibody was titrated using Bethesda units (BU), where one BU was defined to reduce the ADAMTS13 activity to 50% that in normal human plasma. Patient plasma was serially diluted and mixed with the same volume of normal human plasma. After incubation for 2 h

Table 1
Clinical characteristics and laboratory data of TTP patients. ADAMTS13 antigen and anti-ADAMTS13 IgG autoantibody titer are indicated as mean ± SD (n = 3). M, male; F, female; SLE, systemic lupus erythematosus; ND, not done.

Patient	Age	Sex	Context	ADAMTS13 antigen (ng/ml)	ADAMTS13 activity (%)	Anti-ADAMTS13 IgG autoantibody titer (μg/ml)	Inhibitory autoantibody titer (BU/ml)
1	28	M	SLE	203.4 ± 21.6	45.3	30.1 ± 5.0	not detected
2	61	M	SLE	86.7 ± 3.4	<3.0	40.5 ± 5.3	1.6
3	16	F	Idiopathic	12.6 ± 2.5	<3.0	43.3 ± 5.3	6.6
4	34	F	Idiopathic	51.9 ± 9.0	<3.0	30.4 ± 8.3	3.5
5	43	F	Idiopathic	4.3 ± 6.6	<3.0	41.4 ± 10.0	2.1
6	59	M	Idiopathic	24.1 ± 12.4	10.5	21.9 ± 5.7	2.5
7	79	M	Idiopathic	13.1 ± 5.6	<3.0	25.9 ± 4.6	0.6
8	45	F	SLE	1.9 ± 6.9	<3.0	37.4 ± 9.0	2.2
9	75	M	Idiopathic	11.6 ± 4.0	<3.0	25.6 ± 6.8	8.2
10	34	F	Idiopathic	76.0 ± 19.4	<3.0	27.1 ± 7.0	2.2
11	21	F	Idiopathic	ND	<3.0	ND	14
12	15	M	Idiopathic	ND	<3.0	ND	64
13	25	M	Idiopathic	ND	<3.0	ND	1.4

Table 2
Summary of results from the screening for ADAMTS13 peptide sequences binding to IgG from TTP patients. Functional domains of ADAMTS13 are shown with numbers of the first and last amino acid residue of each domain on top. Peptide sequences encoded by phage clones are listed with residue numbers of the N- and C-termini. The number in parenthesis indicates the number of identical phage clones obtained independently from one screening.

	Signal peptide, Propeptide	Metalloprotease	Dis-integrin	TSP1-1	Cysteine-rich	Spacer	TSP1-2-8	CUB1-2
	(1–74)	(75–289)	(290–385)	(386–439)	(440–555)	(556–685)	(686–1191)	(1192–1427)
P1				389-402	491-519	662-687 (2)	1023-1062	
P2			286-322		438-472	662-687 (2)	1067-1080	
					503-538			
P3		96-121					722-735	
P4			332-364			681-688		
P5								
P6						620-659	754-773	
						662-687 (4)		
P7		252-259				662-687 (4)	856-873	
							923-930	
P8		281-299					815-837 (2)	1215-1233
							1159-1182	
P9	1-11					662-687		
P10		96-121						
P11	1-9					617-657	690-709	
							927-943 (2)	
P12								
P13		202-218						
		224-244						

at 37 °C, residual ADAMTS13 activity in the mixture was measured using a FRETS-VWF73 assay.

Results

Binding sites of anti-ADAMTS13 autoantibodies

To define the epitopes of anti-ADAMTS13 IgG autoantibodies, the phage library expressing approximately 30 to 50 amino acids of the ADAMTS13 peptide sequence on the surface, was screened with IgG purified from 13 patients with acquired TTP. After 5 rounds of panning, 40 phage clones were picked from each screening and subjected to DNA sequence analysis. Results of the epitope mapping are summarized in Table 2. We detected various ADAMTS13 peptide sequences possibly recognized by IgG from 11 of the 13 TTP patients. The sequences came from almost entire domains except TSP1-6 and CUB2, and there seemed to be at least 2 to 4 recognition sites in each TTP patient. In the case of P1, for example, we obtained 4 peptide sequences, Ser389-Gly402 (TSP1-1), Gly491-Leu519 (cysteine-rich), Gly662-Val687 (spacer) and Pro1023-Glu1062 (TSP1-7). In particular, Gly662-Val687 in the spacer domain (designated as sp662-687) was detected independently from two different phage clones in the screening (the numbers of clones obtained are shown in parenthesis in Table 2). Moreover, the identical peptide sequence was repeatedly obtained from 4 other patients (P2, 6, 7, and 9), suggesting that sp662-687, the carboxyl-terminal sequence of the spacer domain, is one of the specific sites recognized by IgG obtained from patients with acquired TTP.

ADAMTS13 antigen and activity levels, and IgG titer in TTP plasma

Plasma ADAMTS13 antigen level and the anti-ADAMTS13 IgG autoantibody titer were measured using ELISA (Table 1). The antigen levels were all markedly low (1.9 to 86.7 ng/ml) except P1 (203.4 ng/ml). Of 13 samples, 11 had severely low levels of ADAMTS13 activity (< 3%), whereas P1 had 45.3% and P6 had 10.5%.

All the samples were anti-ADAMTS13 IgG titer positive (cut off: 9.6 μg/ml), with values ranging from 21.9 to 43.3 μg/ml (mean 32.4 μg/ml). The inhibitor assay revealed that 12 samples had positive inhibitory autoantibody titers, ranging from 0.6 to 64 BU/ml (mean 9.1 BU/ml), and P1 was negative.

Association of autoantibody to sp662-687 with ADAMTS13

The results of screening indicated an autoantibody bound to Gly662-Val687 in the C-terminus of spacer domain (sp662-687) was detected repeatedly in 5 of 13 patients (P1, P2, P6, P7, and P9). We thus speculated that sp662-687 would affect ADAMTS13 activity. Accordingly, we compared the antigen or activity levels and anti-ADAMTS13 IgG autoantibody or inhibitory autoantibody titers in sp662-687 positive or negative samples. The mean ADAMTS13 antigen, and anti-ADAMTS13 IgG autoantibody and inhibitory autoantibody titers of positive and negative samples were, respectively, 67.8 vs. 29.3 (ng/ml), 28.8 vs. 35.9 (μg/ml) and 2.6 vs. 12 (BU/ml) (Table 3). No significant differences were detected by Mann-Whitney's U-test ($p>0.05$; SPSS version 17.0, SPSS Inc, Chicago, IL). We could not evaluate the association of the ADAMTS13 activity and the sp662-687 autoantibody because 11 of 13 samples showed severely decreased ADAMTS13 activity (<3%).

Discussion

Both inhibitory and non-inhibitory autoantibodies to ADAMTS13 are associated with the pathogenesis of TTP [15–17,22]. The cysteine-rich/spacer domains are the main and common targets of the autoantibodies [9,23,28]. The spacer domain contains major antigenic sites, such as amino acid regions 572–579 and 657–666 [27], and the sites are recognized by specific autoantibodies produced by B cell clones [26]. Furthermore, one autoantibody type binds an epitope comprising

Table 3
Comparison of laboratory findings between autoantibody to sp662-687 positive and negative samples in TTP patients. ADAMTS13 antigen level or anti-ADAMTS13 IgG autoantibody titer were compared in 10 patients, while ADAMTS13 activity or inhibitory autoantibody titer were compared in all patients. The range of values is indicated in parenthesis.

Autoantibody to sp662-687	Positive	Negative
Mean value of ADAMTS13 antigen level (ng/ml)	67.8 (11.6-203.4)	29.3 (1.9-76.0)
Severe ADAMTS13 activity (<3%)	3/5	8/8
Mean value of anti-ADAMTS13 IgG autoantibody titer (μg/ml)	28.8 (21.9-40.5)	35.9 (27.1-43.4)
Mean value of inhibitory autoantibody titer (BU/ml)	2.6 (0–8.2)	12 (1.4-64)

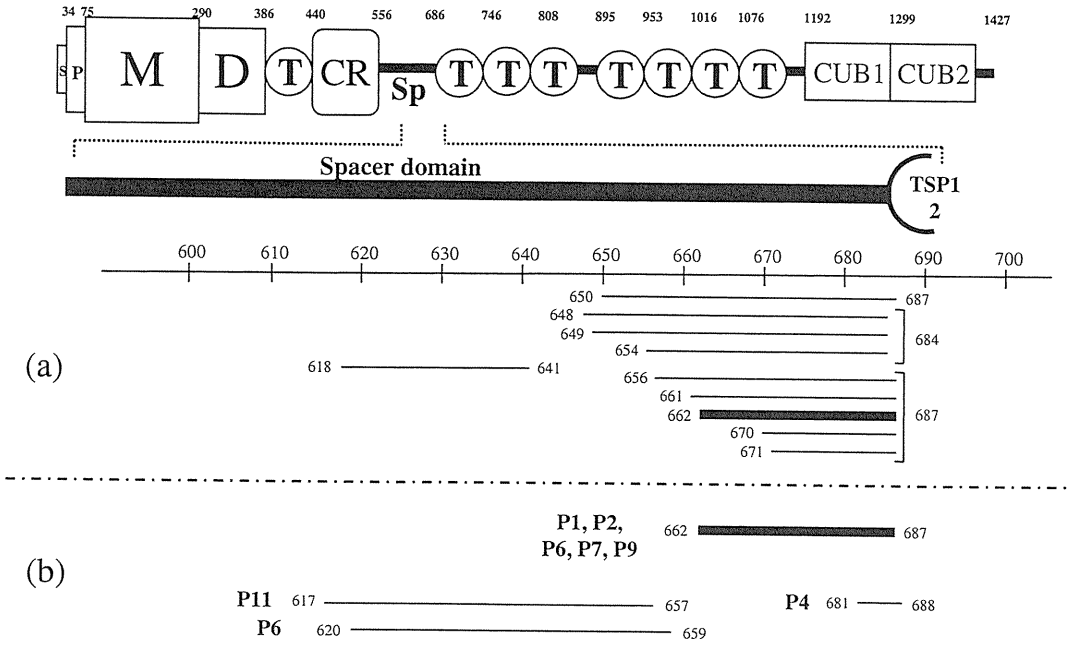

Fig. 1. Overview of peptide sequences in the ADAMTS13 spacer domain encoded by phage clones obtained from screenings for VWF binding sites and the current study. Molecular structure of ADAMTS13 is depicted as a chain of domains with the number of the first amino acid residue of each domain on top. Peptide sequences obtained from the screenings are indicated by horizontal lines with residue numbers of the N- and C-termini. Bold lines indicate an identical peptide sequence. The following abbreviations are used: S, signal peptide; P, propeptide; M, metalloprotease domain; D, disintegrin-like domain; T, thrombospondin-1 repeat; CR, cysteine-rich domain; Sp, spacer domain; and the CUB domain. (a) ADAMTS13 peptide sequences in the spacer domain binding to immobilized VWF [29]. (b) Results of the current screening for peptide sequences in the spacer domain binding to IgG from TTP patients. Of 13 TTP patients, 8 harboured IgG that bound to a peptide sequence that overlapped with VWF binding sequences. Of note, an identical sequence from Gly662 to Val687 (sp662-687) included in the VWF binding sequences was repeatedly detected in 5 patients (P1, P2, P6, P7, and P9).

Arg660, Tyr661, and Tyr665, which interacts with the A2 domain of VWF [32].

In the present study, we aimed to find major ADAMTS13 peptide sequences recognized by IgG autoantibodies in patients with acquired TTP. For this purpose, we constructed a lambda phage library expressing various peptide sequences of ADAMTS13 on its surface and screened it with IgG purified from 13 patients with acquired TTP. Several short peptide sequences of ADAMTS13 were detected from 11 patients. Screening IgG from P5 and P12 revealed no significant peptide sequences (Table 2), however, because other unrelated peptide sequences that were derived from plasmid, frame-shifted, or reversed DNA sequences bound predominantly to the patient IgG, resulting in a loss of targeted peptide sequences.

Multiple autoantibody binding sites were detected in almost all of the domains obtained from 10 TTP samples. Most of the sites were between the metalloprotease and spacer domains, which are the essential regions for the recognition and catalysis of VWF [9–12]. In particular, the peptide sequence from Gly662 to Val687 in the C-terminus of the spacer domain (sp662-687) was repeatedly detected in 5 patients (P1, P2, P6, P7, and P9, Table 2). Interestingly, sp662-687 was included in one of the VWF-binding epitope sequences that we reported previously [29] (Fig. 1). The spacer domain is considered essential for the specific binding of VWF. The recently published crystal structure of ADAMTS13 from the disintegrin-like domain to the spacer domain suggests that the peptide sequence from Tyr661 to Leu668 between the β9 and β10 sheets forms one of the loop structures that interact with the C-terminal α6 helix of the VWF A2 domain [33]. Arg659, Arg660, and Tyr661 are also critical for the cleavage of VWF [34], and Arg660, Tyr661, and Tyr665 are recognized by an autoantibody derived from patients with TTP [32]. Taken together, these findings indicate that the C-terminal portion of the spacer domain, especially the peptide sequences comprising the β9-β10 loop, is a major antigenic site for the production of autoantibodies. It is uncertain why, in the present study, sp662-687 contained the structure of the following β10 sheet and the initial peptide sequences of the TSP1-2 domain in addition to the β9-β10 loop. We speculate that the structure subsequent to the β9-β10 loop is concealed under the steady state, although exposed to the surface by a flexible conformation, leading to its recognition as an antigenic site.

Therefore, we assessed the impact of an autoantibody to sp662-687 on the plasma levels of ADAMTS13 antigens, ADAMTS13 activity, anti-ADAMTS13 IgG autoantibody and inhibitory autoantibody titers. Unfortunately, none of these measurements was associated with the presence or absence of a specific autoantibody. This result may indicates that the autoantibody itself does not affect the function of ADAMTS13, although it is produced as a result of ADAMTS13 degradation in the antigen-presenting cells in patients with TTP, suggesting that this autoantibody is a feature of TTP; however, other autoantibodies contribute to the inhibitory effect on the catalytic function. Because the phage surface display system used in this study could detect only limited peptide sequences recognized by the autoantibodies, there are likely more peptide sequences that are blocked by other autoantibodies, resulting in inhibition of the protein function.

In conclusion, we identified multiple binding sites of autoantibodies to ADAMTS13 in 11 of 13 patients with acquired TTP. In particular, an autoantibody to the C-terminal sequence of the spacer domain was repeatedly detected from 5 TTP patients, although the autoantibody was not likely associated with the inhibitory effect on the catalytic function. Further studies are required to determine other crucial binding sites recognized by the autoantibodies that directly block the protease function.

Conflict of interest statement

All authors have no conflict of interest.

Acknowledgements

This study was supported in part by a grant from Keio Gijuku Academic Development Funds (T.M.) and a grant from the Ministry of Health, Labour, and Welfare of Japan (M.M).

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