

Comparative study of the complement-activating and specific IgE-binding properties of ragweed pollen allergen

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SUMMARY

Previous reports have defined the capacity of ragweed pollen extract (RWA) to activate human complement (C) in fluid phase through the classical pathway and have ascertained a strong correlation between the extent of complement activation and the severity of symptoms of allergic rhinoconjunctivitis during the ragweed blooming season. In the present study the complement-activating and specific IgE-binding capacities of various ragweed allergen preparations were compared. Elimination of physically adsorbed (flavonoid) pigments from the allergenic proteins had no significant effect on their complement-consuming capacity, although the process strongly diminished specific IgE binding. Removal of an IgE-binding trypsin inhibitor from RWA significantly enhanced RWA-induced complement activation, whereas it did not change IgE binding. These findings indicate that neither the physically adsorbed pigments nor the trypsin inhibitor are involved in complement activation by ragweed pollen allergens, and suggest that complement activation and specific IgE binding are distinct molecular properties of ragweed pollen allergen.

Keywords complement ragweed pollen allergens flavonoids trypsin inhibitor

INTRODUCTION

During previous studies we have observed dose-dependent complement (C) activation upon incubation of sera of ragweed pollen-allergic patients and of non-allergic controls with ragweed pollen allergenic extract (RWA) [1]. Complement activation by RWA was initiated through the classical pathway since no C3 activation and cleavage occurred in the sera of three homozygous C2-deficient patients or in a C2-depleted serum preparation [1]. The formation of C3a (C3-anaphylatoxin) and C3bBbP, a protein complex generated during activation of the alternative pathway (AP), was found to be significantly more pronounced in sera of allergic patients compared with non-allergic individuals. C3a was generated in nanomolar amounts, i.e. at concentrations in which this complement activation product is known to have a potent proinflammatory effect (e.g. reactive oxygen radical species production in polymorphonuclear neutrophil leucocytes and eosinophils, reviewed in [1]). In a prospective clinical study, a significant positive correlation was observed between the extent of RWA-induced C3bBbP formation and the severity of symptoms of allergic rhinoconjunctivitis developing during the ragweed blooming season in a 4 week period subsequent to blood sampling [1].

More recently, a similar association was observed between the extent of *in vitro* complement activation and clinical symptom scores in RW-allergic adolescents [2]. Since this association could also be detected in serum samples obtained from the same patients 6 months before the RW blooming season, it seems that this clinically relevant extent of specific complement activation represents a long-lasting characteristic of allergic individuals [2]. These observations indicate that complement activation may play a role in the development of allergy to ragweed pollen. Similar results have been reported with purified house dust allergen [3].

Since RW did not generate C3bBbP in homozygous C2-deficient or C2-depleted sera [1], it seems most probable that complement activation by RW is triggered through the classical pathway (CP). The same mechanism of complement activation had already been described by Berrens *et al.* [4] using purified house dust allergens and by Nagata & Glovsky for several other inhalant allergens [5].

In the present study we have further investigated the mechanism of RW-induced complement activation. Since complement activation was found [1,2] to influence clinical symptoms of allergy generally understood to be primarily triggered by IgE-dependent type I reactions, it seemed important to study the relationship between IgE-binding and complement-activating properties of RWA. Traditional high molecular weight >10-kD ragweed pollen allergens were treated with two different

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procedures, i.e. removal of adsorbed pigments and of a trypsin inhibitor, respectively, and the IgE-binding and complement-activating potencies of the untreated and modified extracts were compared.

Chemical and spectroscopic analysis of the conventional >10-kD ragweed pollen allergens have shown that a sizeable proportion of plant pigments, mainly flavonoids, remains tightly adsorbed to the proteins by physical forces [6,7]. At the same time, low-molecular weight flavonoid glycosides or their aglycones isolated from whole ragweed pollen extracts were found inactive in skin tests in sensitized individuals [8–10]. However, the possible contribution of polyphenolic plant pigments to the phenomenon of complement activation by allergenic extracts has not been investigated so far.

Trypsin inhibitors of the serpin family have recently been described in various species of allergenic pollen by Berrens & Marañón, including those of *Ambrosia elatior* [11]. The trypsin inhibitor from ragweed pollen was found to bind specific human IgE antibodies in the serum of allergic patients [11]. Examination of the possible interference of this inhibitor with the process of complement activation *in vitro* was a further objective of the present studies.

MATERIALS AND METHODS

Ragweed pollen allergen preparations

Pure and dried pollen of *A. elatior* (BioPol Labs, Spokane, NJ; lot no. 89-389-B) was defatted in a Soxhlet apparatus with dry diethylether and then extracted twice with distilled water for 2 h at room temperature. The combined and centrifuged extracts were dialysed against distilled water from Visking membranes of 10 kD nominal cut off, and the retentate high mol. wt allergen preparation, untreated (HMW-N) was obtained by lyophilization in a yield of 11% by weight of the original dry pollen powder. For desorption of electrostatically or physically adsorbed pigments, a portion of HMW-N was redissolved in water and brought to pH 2 with HCl, whereupon the solution was dialysed overnight against acidified water pH 2. After neutralization and a further 6 h dialysis step against three changes of distilled water, the retentate was dried by lyophilization to obtain the high mol. wt allergen preparation, depigmented (HMW-D). Quantitative evaluation showed that the adsorbed pigment material comprised 37% of the dry weight of the HMW-N preparation. The outer liquid containing the desorbed pigments was neutralized with NaOH and then dried by lyophilization (LMW-D).

Serum samples

Most experiments were performed using sera of normal individuals not allergic to ragweed pollen, as established by the allergen-specific IgE Quick test (Dexall Biomedical Labs, Gaithersburg, MD) and by skin prick testing using Pharmacia allergens. For IgE-inhibition assays the defibrinated plasma of a highly ragweed-allergic patient was obtained from PlasmaLab Int. (Everett, USA). Sera of dust mite (*Dermatophagoides pteronyssinus*)-sensitive patients were obtained from various hospitals in Spain.

Trypsin inhibitor RW-TI and inhibitor-depleted HMW preparation

The trypsin inhibitor RW-TI from ragweed pollen was isolated by column chromatography steps and preparative isoelectric focusing as described elsewhere [11]. The inhibitor RW-TI was removed from a bulk extract HMW-N by a series of steps involving

molecular sieving on Sephadex G100 columns monitored by inhibition of the enzymatic activity of bovine trypsin on specific chromogenic substrates [11]. The combined and inhibitor-depleted HMW-protein allergens were recovered by lyophilization.

Interaction of complement with various RW preparations

Serum samples were incubated in 300- μ l aliquots with 30 μ l of RW preparations in veronal-buffered saline (VBS) or with 30 μ l VBS as control for 30 min at 37°C. Activation of complement in the RW-treated and control sera was then immediately evaluated using the enzyme immunoassay and radioimmunoassay (RIA) methodologies detailed below. For rapid screening, the consumption of whole haemolytic C was in some cases established by CH₅₀ assay as described before [3].

ELISA methods for complement activation

Activation of the CP was quantified by determining the extent of C1rC1sC1inh complex formation based on the method of Nilsson & Bäck [12]. Plates were coated with goat anti-C1inh antibodies (ATAB, Stillwater, MN). Normal human serum (NHS) incubated with heat-aggregated human IgG was used as a standard. Samples were treated with polyethylene glycol (PEG) 6000 (Fluka, Buchs, Switzerland) in 6% final concentration after incubation with RW or VBS, and the supernatants in 1:20 dilution were used for analysis. The proportion of bound C1rC1sC1inh complexes was determined using peroxidase-labelled goat anti-human C1s (ATAB) and hydrogen peroxide/tetramethylbenzidine substrate.

Activation of the AP was measured according to the method of Zilow *et al.* [13]. Plates were coated with goat anti-human properdin (ATAB) and then blocked with PBS containing 2% low-fat cow's milk. Serum samples after incubation with RW preparations were applied to the wells in 1:20 dilution in PBS–Tween (PBS–T) containing 0.01 M EDTA. The quantities of captured C3bBbP complexes were determined with rabbit biotinylated anti-C3c (Dako, Glostrup, Denmark) and streptavidine-peroxidase, followed by hydrogen peroxide and tetramethylbenzidine.

Levels of C1rC1sC1inh and C3bBbP complexes were expressed in arbitrary units (U)/ml.

C3a determination

For measurement of human C3a, a commercially available RIA test kit for C3a-des-Arg was applied according to the manufacturer's instructions (Pharmacia, Aylesbury, UK).

Binding of ragweed-specific IgE antibodies

The capacity of the ragweed allergen extracts for binding specific human anti-ragweed IgE antibodies was determined by microtitre enzyme immunoassay in inhibition format, as described [14]. Briefly, the ragweed allergen preparation HMW-N was insolubilized by coupling to paper discs with cyanogen bromide according to Ceska *et al.* [15]. A series of concentrations of soluble allergen preparations was incubated with 1:4 diluted ragweed IgE-positive patient's serum for 3 h at room temperature. The preincubated allergen–serum mixtures were then reacted with the allergen-coated discs in order to capture residual unbound specific IgE. After washing with PBS–T, peroxidase-labelled monoclonal anti-IgE (Ingenasa, Madrid, Spain) was added to the discs. After an additional 3 h, the discs were washed repeatedly, whereupon the degree of specific IgE binding was evaluated by colorimetric determination of bound peroxidase at 450 nm, using hydrogen peroxide and tetramethylbenzidine for colour development.

Statistical analysis

Differences between the allergen-treated and control sera were calculated by the paired *t*-test.

RESULTS

Generation of complement activation products in sera of non-allergic individuals

Serum samples from eight non-allergic subjects were incubated with different concentrations (10–320 µg/ml) of ragweed pollen preparation HMW-N for 30 min at 37°C, and the amounts of C3bBbP, C1rC1sC1inh and C3a were determined. The RW allergens induced a dose-dependent generation of all three products, whereby the highest values were obtained for C3bBbP formation (Fig. 1). The levels of C3bBbP measured in serum samples incubated with ≥ 20 µg/ml HMW-N were significantly higher than those detected in control samples treated with VBS ($P < 0.01$). At a dose of 40 µg/ml, C3bBbP complexes were generated at an amount more than twice the control values, while at 320 µg/ml the level reached over three times the control value.

In contrast, incubation with ragweed pollen HMW-N induced a significant increase in the formation of C1rC1sC1inh complexes by CP activation only at a maximal dose of 320 µg/ml. Ragweed HMW-N also caused appreciable C3a formation ($P < 0.05$), although the differences between allergen-treated and non-treated serum samples were less pronounced than observed for C3bBbP formation (data not shown). This latter observation is in agreement with our previous finding that the formation of C3bBbP complex may serve as the most sensitive *in vitro* indicator of ragweed allergen-induced complement activation [1].

Complement activation and IgE binding by depigmented ragweed pollen allergens

Although only few publications have mentioned it, the > 10 -kD components in dialysed aqueous extracts of ragweed pollen retain a considerable proportion of pigments, mainly flavonoid glycosides. They remain tightly adsorbed to the allergenic proteins by physical

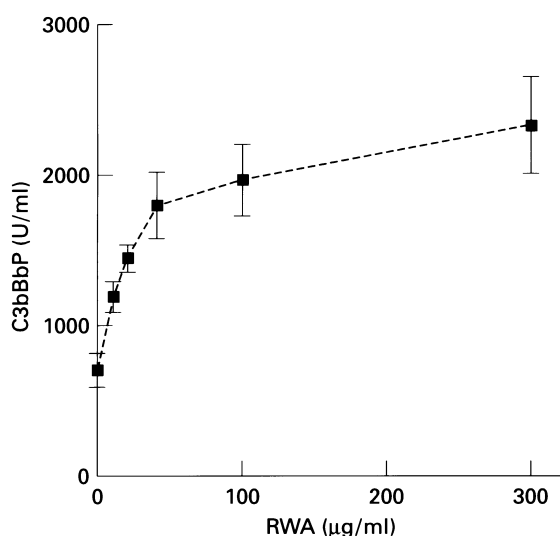


Fig. 1. Formation of C3bBbP (alternative pathway activation) in sera of ragweed non-allergic subjects incubated for 30 min at 37°C with different doses of ragweed pollen extract. Mean $\pm$ s.e.m. values for eight sera of non-allergic individuals.

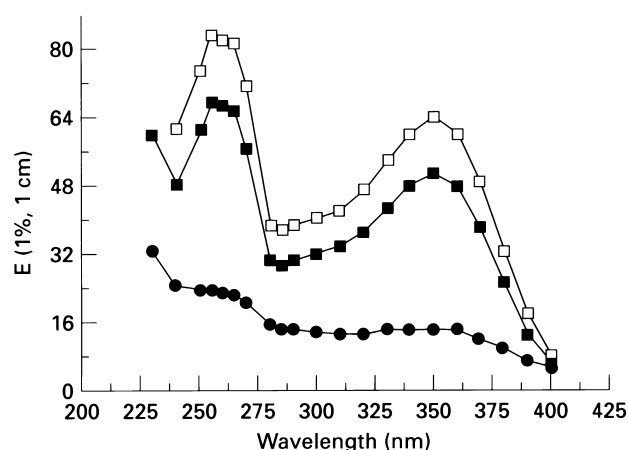


Fig. 2. Ultraviolet absorption spectra (1 cm light-path) of 0.025% solutions of the >10 -kD allergenic proteins HMW in 0.01 M phosphate buffer pH 6.9 before and after removal by dialysis of adsorbed pigments in 0.01 N HCl. The absorption maximum at 350 nm is due to flavonol (-glycosides) of the quercetin (quercitrin) type. □, Allergenic proteins (mol. wt $>10\,000$ D) before depigmentation; ●, allergenic proteins (mol. wt $>10\,000$ D) after depigmentation; ■, desorbed pigments (mol. wt $<10\,000$ D).

and electrostatic forces even after extensive neutral dialysis [6,9,16]. The largest part of these pigments may be desorbed at pH 2 and eliminated by further dialysis steps [7]. This process of 'cleaning up' the allergenic proteins is illustrated by the UV-absorption spectra given in Fig. 2.

In order to investigate whether the removal of adsorbed pigments had any effect on the complement-activating ability of pollen allergens, sera of four non-allergic subjects were incubated with the traditional (HMW-N) and the depigmented (HMW-D) ragweed pollen preparations (see Materials and Methods). As shown in Table 1, both preparations induced significant C3bBbP formation at concentrations of 80–320 µg/ml, but no significant difference was observed in their complement-activating potencies. Similar results were obtained during measurements of C3a generation (Fig. 3). The isolated pigment did not cause a significant increase in C3bBbP formation even at a dose of 80 µg/ml (620 ± 75 U/ml versus 590 ± 75 U/ml measured in the buffer control).

In a total haemolytic assay [3], and using sera of patients allergic to *D. pteronyssinus* but not to ragweed, the normal and depigmented ragweed preparations HMW-N and HMW-D assayed in the range of 80–800 µg/ml were of equal potency (50% haemolytic complement activation at 246 µg/ml), while the capacity of HMW-D to cause 50% reduction of haemolytic complement in the (more sensitive) serum of a ragweed-allergic patient decreased by a factor of only 1.6 relative to HMW-N (189 µg/ml and 118 µg/ml, respectively).

In sharp contrast to the above observations, the binding of specific human anti-ragweed IgE antibodies to RWA dramatically diminished after removing the protein-adsorbed flavonoid pigments. As shown by the IgE inhibition curves of Fig. 3, the depigmentation process caused an ≈ 100 times loss of potency for 50% inhibition, indicating a heavy loss of IgE-binding epitopes.

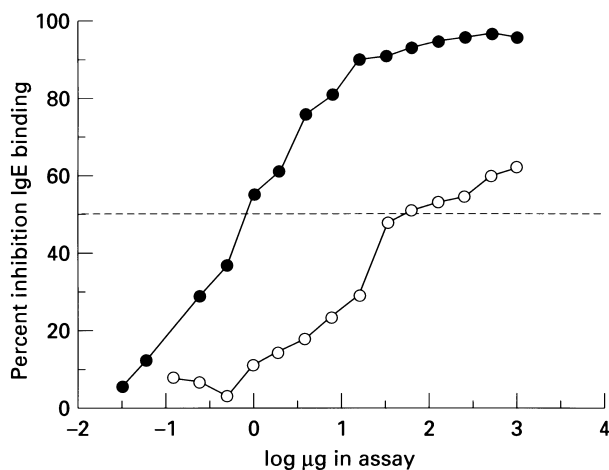
Effect of ragweed pollen trypsin inhibitor on complement activation

RW-TI was isolated in homogeneous form as described [11]. Both the inhibitor and the inhibitor-depleted preparation were examined

Table 1. Formation of C3bBbP and C3a in the sera of ragweed non-allergic subjects incubated for 30 min at 37°C with different doses of ragweed allergenic preparations HMW-N and HMW-D, respectively

	None	RWA 80 µg/ml	RWA 200 µg/ml	RWA 320 µg/ml
		C3BbP, U/ml (mean ± s.e.m.)		
Buffer	590 ± 191			
HMW-N		828 ± 104*	1025 ± 161*	1329 ± 168*
HMW-D		810 ± 97*	1154 ± 204*	1293 ± 182**
C3a, ng/ml (mean ± s.e.m.)				
Buffer	1233 ± 339			
HMW-N		ND	1698 ± 354**	ND
HMW-D		ND	1808 ± 225**	ND

Significant difference (calculated by paired *t*-test) compared with the buffer control: **p* < 0.05; ***p* < 0.01; ND, not done.
Mean ± s.e.m. values for four sera.

**Fig. 3.** Dose-response relationship in inhibition format for IgE binding by >10-kD ragweed pollen allergens before and after the depigmentation process. The point of 50% inhibition is representative of the *in vitro* allergenic potency. ●, Normal; ○, depigmented.

for complement activation by measuring C3bBbP formation. As shown in Table 2, AP activation at 16–320 µg/ml final concentrations was found to be significantly higher with the inhibitor-depleted allergenic proteins than with the full extract HMW-N.

Table 2. Formation of C3bBbP in the sera of ragweed non-allergic subjects incubated for 30 min at 37°C with different doses of ragweed allergenic preparations HMW-N and trypsin inhibitor-depleted HMW-N (TID-HMW-N), respectively

	None	RWA 16 µg/ml	RWA 80 µg/ml	RWA 320 µg/ml
		C3BbP, U/ml (mean ± s.e.m.)		
Buffer	981 ± 167			
HMW-N		1068 ± 38	1425 ± 125*	1538 ± 157**
TID-HMW-N		1361 ± 58*	1645 ± 52**	1813 ± 116***

Significant difference (calculated by paired *t*-test) compared with the buffer control: **P* 0.05 < 0.1; ***P* < 0.05; ****P* < 0.01.
Mean ± s.e.m. values for five sera.

Using a mixture of 16 ng of purified RW-TI and 320 µg/ml of HMW-N added to the sera of eight non-allergic persons, it was found that addition of trypsin inhibitor lowered the complement-activating potency of the full pollen extract: RWA significantly increased C3bBbP formation compared with the buffer control in the absence of TI, while in the presence of TI no significant increase in C3bBbP formation occurred (Fig. 4).

By contrast, when the IgE-binding capacity of the full and trypsin inhibitor-depleted ragweed pollen extracts were compared, no significant difference was measured in IgE antibody binding by the two extracts (data not shown).

DISCUSSION

It has previously been reported [1] that ragweed pollen allergens are capable of consuming human C *in vitro* by activating C1 of the classical pathway, similar to what has been observed at high serum dilutions for other inhalant allergens at much lower doses [3–5]. Since CP activation under our experimental conditions of high allergen concentration in virtually undiluted serum allows a positive feedback at the C3 amplification loop, we found it advantageous in the present work to employ a sensitive enzyme immunoassay [13] for the C3bBbP complex assembled during activation of the AP. Under these conditions, the removal of a substantial proportion (37% w/w) of adsorbed (flavonoid) pigment material from the > 10-kD proteins was found to have no influence

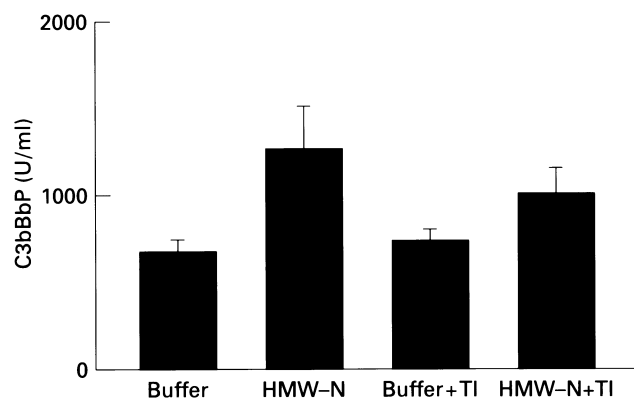


Fig. 4. Formation of C3bBbP in the sera of ragweed non-allergic individuals incubated for 30 min at 37°C with ragweed allergenic extract HMW-N in the presence or absence of 16 ng of purified trypsin inhibitor RW-TI. Mean $\pm$ s.e.m. values for eight experiments performed with serum samples of eight individuals. Significant difference ($P < 0.05$) between samples incubated with HMW-N and buffer, no significant difference between samples incubated with buffer $\pm$ TI and HMW-N $\pm$ TI.

on the complement-activating potential of the ragweed protein allergens. Since the recovered low molecular pigment material was virtually devoid of complement-activating properties (data not shown), it may be concluded that the ability of ragweed pollen extracts to activate complement *in vitro* is an intrinsic property of the (glyco)protein allergens themselves. In view of the residual absorption peak at 350 nm in the UV spectrum even of the depigmented proteins (Fig. 2), the participation of chemically conjugated flavonol side-chains cannot be entirely ruled out, however.

In contrast to the negligible effect of depigmentation on the complement-activating potential of ragweed pollen allergens, the removal of protein-adsorbed (flavonoid) pigments caused an impressive loss of IgE-binding potency (Fig. 4). This effect was not observed with the pollen proteins of other plant families [7]. However, during separate experiments we also noted that measurable IgE binding by the < 10 -kD low molecular components from ragweed pollen extracts as well as by the desorbed pigments only occurred at unrealistic doses, i.e. 500–1000 times higher than required with the > 10 -kD protein allergens (data not shown). The decrease of IgE binding by the depigmented ragweed pollen proteins is probably due to the lability of IgE-specific peptide epitopes at low pH values, as already reported by King *et al.* for ragweed antigen E [17].

The traditional aqueous extracts of allergenic plant pollens comprise a vast array of allergenic proteins differing in their capacity of binding specific human IgE antibodies. Berrens & Marañón have recently isolated IgE-binding trypsin inhibitors (RW-TI) from ragweed pollen [11]. Removal of trypsin inhibitor from RWA did not significantly affect IgE-binding ability of the traditional RW pollen extracts. In view of the fact that, like trypsin, activated C1r and C1s belong to the superfamily of the serine proteases [18], it was assumed that the presence of trypsin inhibitors might interfere with complement activation by pollen allergens, especially those from the Gramineae [4,19]. As shown in Fig. 4, ragweed inhibitor RW-TI indeed partially blocked complement activation, as evidenced by the diminished formation of the C3bBbP complex under the conditions of assay. Accordingly, removal of TI resulted in a significant increase of the

complement-activating ability of the HMW-N (Table 2). However, given the low content of trypsin inhibitor of about 0.5% of the dry weight of whole pollen proteins [11], it appears unlikely that inhibition of the C1-proteases could have greatly influenced our previous results regarding the mechanism of ragweed-induced complement activation [1,2].

In conclusion, marked differences were observed when the effects of two different procedures on the IgE-binding and complement-activating abilities of ragweed pollen allergen were compared. Depigmentation dramatically decreased IgE binding, while it did not affect complement activation. Removal of trypsin inhibitor did not influence IgE binding, whereas the same procedure significantly enhanced complement activation. These observations indicate that the phenomena of complement activation and IgE binding represent distinct and independent molecular properties of ragweed pollen allergen. Complement activation was found by us to be a clinically relevant process [1,2] that can significantly amplify the development of the symptoms of ragweed allergy. Andersson *et al.* [20] demonstrated dose-dependent local formation of complement-activation products (C3a-des-arg, C5a-des-arg) in nasal mucosa of individuals allergic to birch or timothy pollen after specific nasal provocation. Considering these observations, together with our present findings on the independence of IgE binding and complement activation by ragweed allergen, it seems most probable that complement activation does not directly affect allergen–IgE interactions. Most probably complement activation contributes to the development of the symptoms of ragweed allergy by amplifying mast cell degranulation or by other pathological processes that are triggered by the IgE-dependent type I hypersensitivity reactions.

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