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Original Article

Combination of CD157 and FLAER to Detect Peripheral Blood Eosinophils by Multiparameter Flow Cytometry

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The identification of eosinophils by flow cytometry is difficult because most of the surface antigens expressed by eosinophils are shared with neutrophils. Some methods have been proposed, generally based on differential light scatter properties, enhanced autofluorescence, lack of CD16 or selective positivity of CD52. Such methods, however, show several limitations. In the present study we report a novel method based on the analysis of glycosylphosphatidylinositol (GPI)-linked molecules. The combination of CD157 and FLAER was used, since FLAER recognizes all GPI-linked molecules, while CD157 is absent on the membrane of eosinophils and expressed by neutrophils. Peripheral blood samples from normal subjects and patients with variable percentages of eosinophils (n = 31), and without any evidence for circulating immature myeloid cells, were stained with the combination of FLAER-Alexa Fluor and CD157-PE. A FascCanto II cytometer was used. Granulocytes were gated after CD33 staining and eosinophils were identified as CD157^{-}/FLAER^{+} events. Neutrophils were identified as CD157^{+}/FLAER^{+} events. The percentages of eosinophils detected by this method showed a very significant correlation both with automated counting and with manual counting (r = 0.981 and 0.989, respectively). Sorting assays were carried out by a S3 Cell Sorter: cytospins obtained from CD157^{-}/FLAER^{+} events consisted of 100% eosinophils, while samples from CD157^{+}/FLAER^{+} events were represented only by neutrophils. In conclusion, this method shows high sensitivity and specificity in order to distinguish eosinophils from neutrophils by flow cytometry. However, since CD157 is gradually up-regulated throughout bone marrow myeloid maturation, our method cannot be applied to cases characterized by immature myeloid cells. 〔*J Clin Exp Hematop 55 (2) :* 55-60*, 2015*〕

Keywords: CD157, eosinophils, FLAER, multiparameter flow cytometry

INTRODUCTION

A recent, interesting paper by Muroi *et al*.[1] emphasizes the difficulty in identifying eosinophils by multiparameter flow cytometry (MFC). In fact, most of the surface antigens expressed by eosinophils are shared with neutrophils.[2,3]

The methods so far proposed to identify eosinophils in human whole blood by MFC are generally based on differential light scatter properties, enhanced autofluorescence, lack of CD16 or selective positivity of CD52.[2,4-8] When present in sufficient numbers, eosinophils may be identified as a granulocytic population with higher SSC and lower FSC than granulocytes, and with slightly brighter CD45 expression.[2] However, such properties are not expressed in all cases and are not easily detectable when eosinophils are present in low percentages. In addition, CD16 can also be expressed by normal eosinophils,[9,10] and CD52 can be expressed by neutrophils as well.[11]

We report novel observations concerning a simple method to identify peripheral blood eosinophils by means of MFC, using the association of CD157 plus FLAER. These markers are used to detect clones of paroxysmal nocturnal hemoglobinuria (PNH),[12] which can be identified because they lack glycosylphosphatidylinositol (GPI)-anchored proteins. According to either partial or total absence of GPI-linked molecules, PNH clones are termed PNH2 and PNH3, respectively. Modern routine detection of PNH clones is carried out by MFC on peripheral blood granulocytes and monocytes, and the most used markers are CD66b, CD16, and CD24 for granulocytes, and CD14 for monocytes.[13]

CD157 is a member of the CD38 NADase/ADP-ribosylcyclase gene family and is expressed on the surface of neutrophils and

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monocytes, but not by eosinophils.[14,15] FLAER is a fluorochrome-conjugated derivative of the bacterial toxin aerolysin, which specifically recognizes the GPI molecule. FLAER binds to but does not lyse normal cells and for this reason is the best marker for recognizing PNH2 and PNH3 clones. FLAER-based methods are used to detect and enumerate PNH clones non only in classic PNH, but also in cases of aplastic anemia (termed AA/PNH) and in some cases of myelodysplastic syndromes (MDS).[16-18]

Recent studies have provided evidence about high sensitivity of MFC assays based on FLAER plus CD157 combination.[19] For this reason, we added CD157 in routine examination of patients with known or suspected PNH, with suspect of AA/PNH or MDS, or with thrombosis in unusual sites (which can be a peculiar presentation of PNH), or with Coombs-negative hemolysis. In the course of analysis of cytograms, we noticed the presence of variable percentages of granulocytes which were positive for FLAER and CD66b, but lacked CD157 (Fig. 1). Since this molecule is not expressed by eosinophils, we hypothesized that the simultaneous analysis of CD157 and FLAER might be able to distinguish eosinophils from neutrophils.

MATERIAL AND METHODS

Patients and normal subjects

We studied a series of 31 consecutive subjects with either normal or high percentage and/or absolute number of circulating eosinophils: PNH, undergoing periodical control (n = 2); AA/PNH (n = 2); MDS, with the characteristics of refractory anemia (n = 4); reactive eosinophilia (n = 13), Coombs-negative hemolysis (n = 6), normal subjects (n = 4). The general characteristics of the subjects studied are shown in Table 1. This study was approved by our internal scientific committee and informed consent was always obtained.

Eosinophil counting

Peripheral blood was collected in K_3EDTA pre-treated tubes. Complete blood counts were carried out by an ADVIA 2120 (Siemens, Munich, Germany), and both percentages and absolute numbers of eosinophils were recorded. In addition, manual counting on smears stained with May-Grünwald-Giemsa was performed under a light microscope, in order to confirm the presence of eosinophils and to exclude the presence of circulating immature myeloid cells. Leukocyte differential counts were carried out following the guidelines developed by the Clinical and Laboratory Standards Institute.[20]

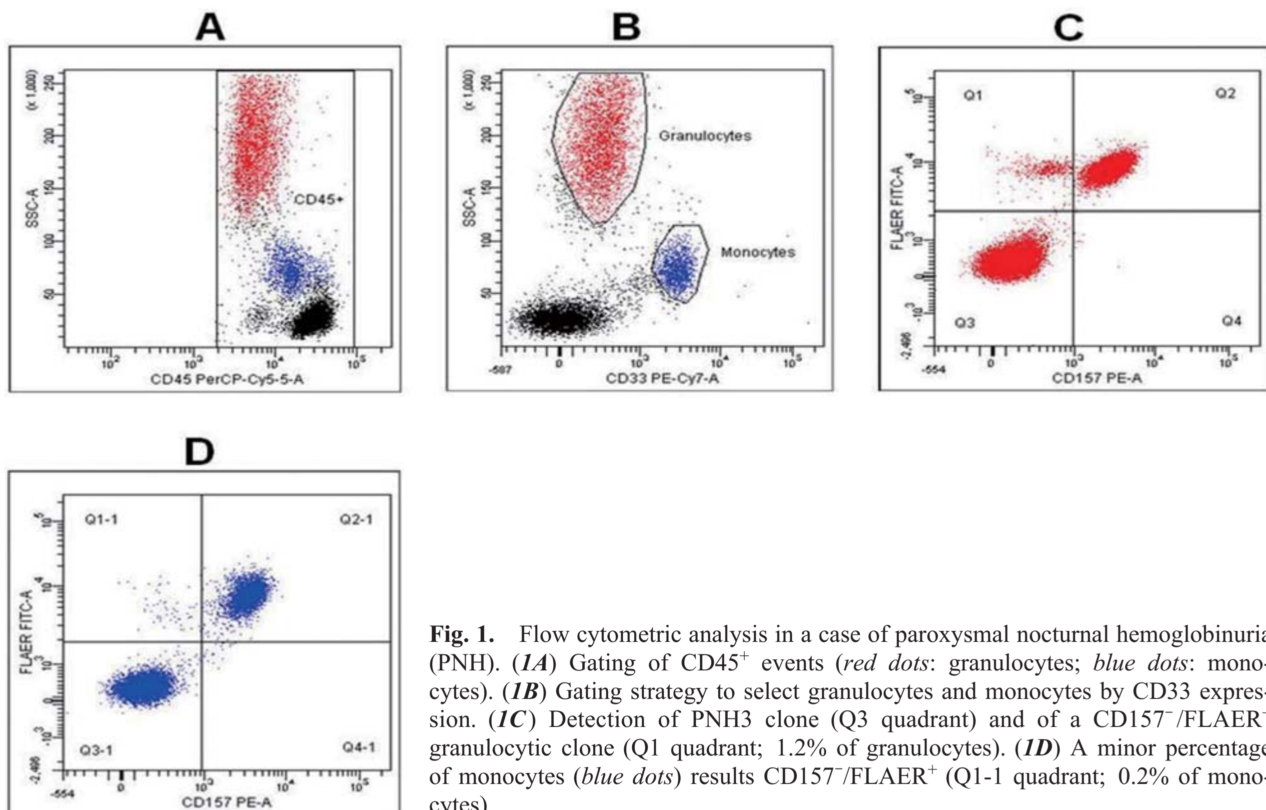

Fig. 1. Flow cytometric analysis in a case of paroxysmal nocturnal hemoglobinuria (PNH). (***1A***) Gating of CD45$^+$ events (*red dots*: granulocytes; *blue dots*: monocytes). (***1B***) Gating strategy to select granulocytes and monocytes by CD33 expression. (***1C***) Detection of PNH3 clone (Q3 quadrant) and of a CD157$^-$/FLAER$^+$ granulocytic clone (Q1 quadrant; 1.2% of granulocytes). (***1D***) A minor percentage of monocytes (*blue dots*) results CD157$^-$/FLAER$^+$ (Q1-1 quadrant; 0.2% of monocytes).

Table 1. Subjects under study: general characteristics

Subjects	n	WBC (× 10^9/L) Means ± SD (range)	Eosinophils (%) Means ± SD (range)	Eosinophils (× 10^9/L) Means ± SD (range)
Healthy subjects	4	4.96 ± 0.75 (4.33−6)	5.4 ± 2.6 (3−9)	0.26 ± 0.11 (0.13−0.39)
PNH and aplastic anemia	4	2.98 ± 1.14 (1.85−4.5)	1.32 ± 1.6 (0.1−4)	0.034 ± 0.033 (0.002−0.074)
Reactive eosinophilia	13	10.53 ± 3.18 (5.04−15.98)	15.98 ± 9.72 (6−39)	1.64 ± 1.16 (0.65−4.9)
Coombs-negative hemolysis	6	8.23 ± 2.43 (4.63 ± 10)	1.55 ± 0.75 (0.8−2.6)	0.13 ± 0.078 (0.075−0.24)
Myelodysplastic syndrome	4	3.45 ± 0.56 (3.07−4.10)	0.98 ± 0.73 (0.15−9)	0.035 ± 0.028 (0.005−0.06)

WBC, white blood cell; SD, standard deviation; PNH, paroxysmal nocturnal hemoglobinuria

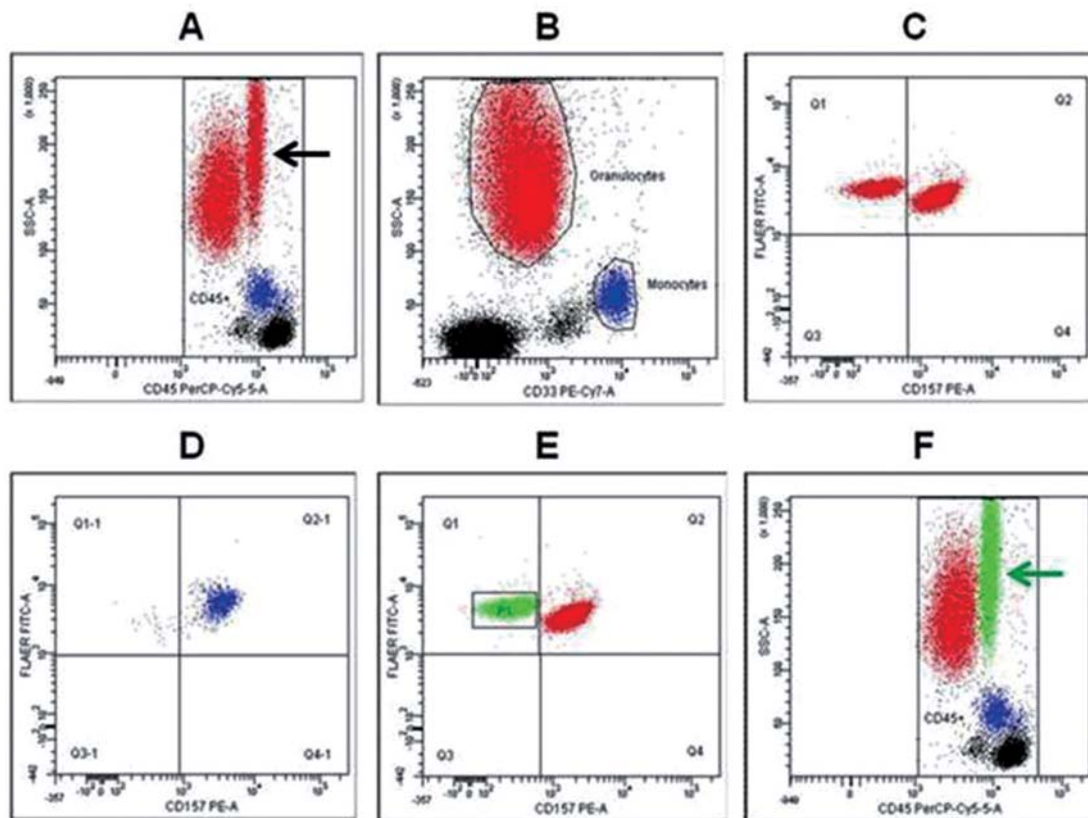

Fig. 2. Analysis of a sample from an allergic patient with eosinophilia (18% at automated counting). (*2A*) Gating of CD45$^+$ events. The *black arrow* shows a granulocytic population with higher SSC and CD45 properties. (*2B*) Gating strategy to select granulocytes (*red dots*) and monocytes (*blue dots*). (*2C*) A significant CD157$^-$/FLAER$^+$ granulocyte population (31.8% of granulocytes, 19% of CD45$^+$ events) are identified in Q1. (*2D*) Very few monocytes are CD157$^-$. (*2E*) Gate to select CD157$^-$/FLAER$^+$ granulocytes (P1). (*2F*) CD45/SSC backgating, which demonstrates that the P1 cell population coincides with the granulocytic population with higher SSC and CD45 (*green arrow*).

Flow cytometry

Flow cytometric assays were carried out by a FacsCanto II cytometer equipped with three lasers and assisted by the FacsDiva Software (both from Becton Dickinson). The diagnostic tube was organized with: CD45/PerCP-Cy5. 5; FLAER/Alexa Fluor 488; CD33/PE-Cy7; CD157/PE; CD14/APC-Cy7. An additional tube, with the association of CD45/PercCP-Cy5. 5; CD66b/FITC; CD33/PE-Cy7 and CD14/APC-Cy7 was added to complete the diagnostic panel. Both CD157 (Immunostep, Salamanca, Spain) and FLAER (Pinewood Scientific Services, Victoria, Canada) were pur-

chased from Lagitre.S.r.L (Milan, Italy). The other monoclonal antibodies were purchased from Becton and Dickinson. FLAER/Alexa Fluor 488 fluorescence was read in the FITC channel, because of similar excitation and emission spectra.

Blood samples (100 μL/tube) were incubated with saturating amounts of reagents for 20 min and subjected to erythrocyte lysis by means of NH_4Cl. At least 100,000 $CD45^+$ events were acquired (Fig. 2A), and granulocytes and monocytes were selected by exploiting the different CD33 expression (Fig. 2B). The whole granulocyte population was analyzed in a CD157/FLAER cytogram and, as shown in Fig. 2C, the quadrant statistics was used to detect and enumerate: PNH clones, if any (Q3 quadrant); $CD157^+/FLAER^+$ normal granulocytes (Q2 quadrant); $CD157^-/FLAER^+$ granulocytes (Q1 quadrant). Granulocytes within the Q1 quadrant were quantified and results were expressed as: percentage of the whole granulocytic population; percentage of $CD45^+$ cells. This latter percentage was compared with the percentage of eosinophils enumerated by the automated ADVIA 2120 cell counter and with the percentage obtained with manual counting.

Sorting assays

To demonstrate that the $CD157^-/FLAER^+$ granulocytic population consisted of eosinophils, we performed sorting studies by means of a S3 Cell Sorter (Bio-Rad, Hercules, USA-CA) equipped with ProSort® Software (Bio-Rad) and applying "Purity" sort mode. 500 μL of peripheral blood, collected in K_3EDTA pre-treated tube, were stained with FLAER-Alexa Fluor 488 and CD157-PE reagents. After 30 sec at 4℃, erythrocytes were lysed applying Red Blood Lysis Solution from Miltenyi Biotec (Bergisch Gladbach, Germany) and washed with pre-refrigerated AutoMACS Running Buffer (Miltenyi Biotec). Gating granulocytic events as FSC^{high} SSC^{high}, sort regions were defined on PE *vs* FITC cytogram in order to collect $CD157^+/FLAER^+$ and $CD157^-/FLAER^-$ fractions. About 250,000 sorted cells were then cytocentrifuged on glass slides and processed for May Grünwald-Giemsa stain on a Wescor Aerospray® 7120 automatic slide stainer (ELITech Group, Puteaux, France). Pictures were taken using standard DM RB Leica microscope (Leica, Wetzlar, Germany) equipped with LAS image acquisition software (Leica).

Statistics

Results were analyzed by the Spearman linear correlation test.

RESULTS

Detection and enumeration of eosinophils by CD157/FLAER staining

Variable percentages of $CD157^-/FLAER^+$ events were detected in Q1 quadrants, with a very high correlation with the percentage of circulating eosinophils (Fig. 3). After setting a specific gate to include $CD157^-/FLAER^+$ events (Fig. 2E), and CD45/SSC back-gating, this granulocytic subpopulation was found to be characterized by higher CD45 expression and higher SSC properties, as expected in the case of eosinophils (Fig. 2F). Only very few monocytes did not express CD157 (Fig. 2D).

Manual counting confirmed the presence of eosinophils. The percentages of eosinophils registered by manual counting were compared with those obtained by flow cytometry, and a very significant correlation was obtained (Fig. 3).

Sorting assays

As shown in Fig. 4, the sorted $CD157^-/FLAER^+$ cell population consisted of virtually 100%, well-recognizable eosinophils, while the sorted $CD157^+/FLAER^+$ cell population was

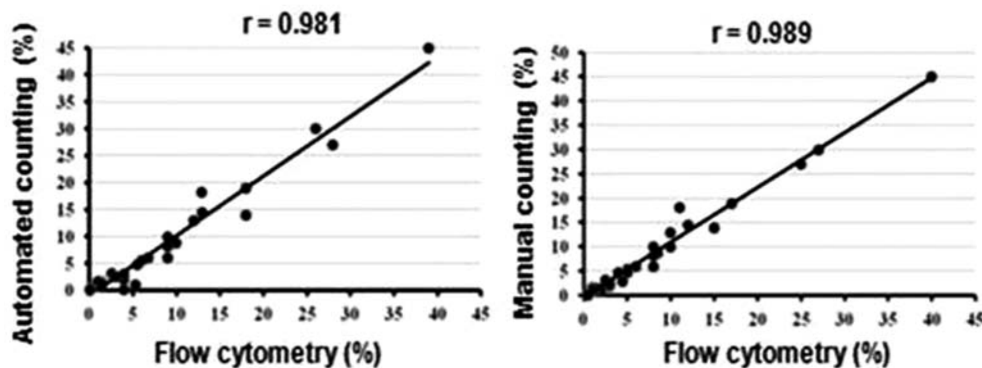

Fig. 3. Linear correlation between percentages of eosinophils detected by flow cytometry as $CD157^-/FLAER^+$ events and percentages of eosinophils enumerated by automated counting (*left*) and by manual counting (*right*). $p < 0.0001$.

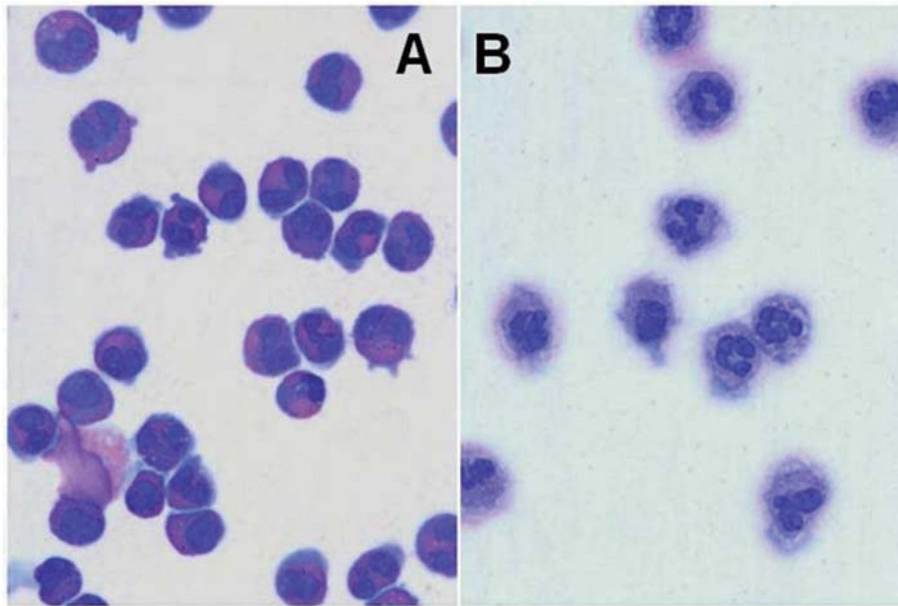

Fig. 4. Cytospins after sorting procedures. (***4A***) CD157^{-}/FLAER^{+} cells are recognizable as eosinophils. (***4B***) CD157^{+}/FLAER^{+} cells can be identified as neutrophil granulocytes. ×1,000.

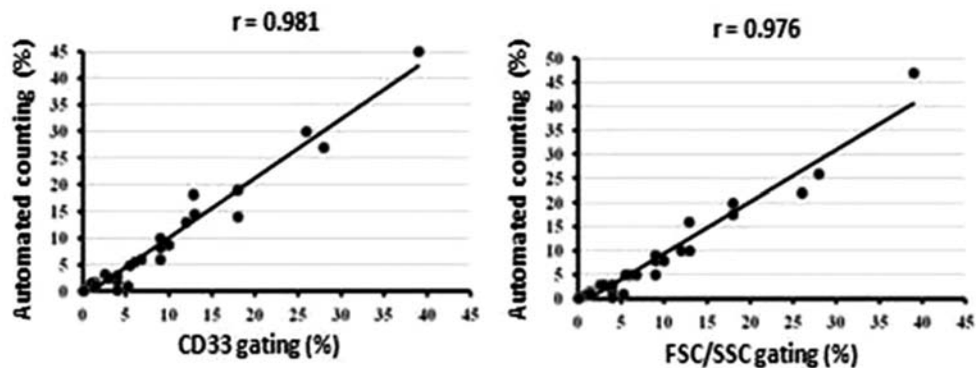

Fig. 5. Linear correlation between percentages of eosinophils detected by CD33 gating (*left*) and by FSC/SSC gating (*right*), compared with automated counting. $p < 0.0001$.

represented by neutrophil granulocytes (Fig. 4). Since the gating strategy used in sorting experiments was based on FSC/SSC properties of granulocytes, the percentages of eosinophils obtained with this procedure were compared with those registered with CD33 gating strategy. No differences were found in terms of correlation values (Fig. 5).

DISCUSSION

Our study shows that the differential expression of CD157 by eosinophils and neutrophils can be exploited to separate these two cell populations by flow cytometry.

We think that the simple application of CD157 plus FLAER may be useful to improve detection of circulating eosinophils in peripheral blood from both normal and patients with eosinophilia. Eosinophil quantification by flow cytometry showed a very significant correlation with both automated and manual counting.

Different gating strategies may be applied, since CD33 gating and FSC/SSC gating yielded highly comparable results.

We think that, by using this simple method, other antigens may be studied and sorting studies may be carried out in order to analyze eosinophils with very high degree of purification.

Since CD157 is gradually up-regulated throughout bone marrow neutrophil maturation,[21] our method cannot be ap-

plied to samples containing immature myeloid cells, such as myeloblasts, promyelocytes and myelocytes. Thus, clinical conditions such as, for example, chronic myeloid leukemia, are not suitable for applying a method based on CD157 expression.

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CONFLICT OF INTEREST: The authors declare no conflict of interest.

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