



# Reticulocyte Analysis Provided by the Coulter GEN.S: Significance and Interpretation in Regenerative and Nonregenerative Hematologic Conditions

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## ABSTRACT

Automatic reticulocyte counting provides a new approach to reticulocyte analysis, with maturation indices and volume measurements. The Coulter GEN.S provides such an analysis that includes 7 parameters. After having calculated the normal range values from 1 group of normal donors, we studied the sensitivity and specificity of the various indices in diagnosing regenerative conditions, using 2 groups comprising hemolytic and non-hemolytic samples. With the exception of the immature reticulocyte fraction (IRF), the sensitivities of the maturation parameters were found to be greater than the sensitivity of reticulocyte count (91.1% and 88.6%, respectively), generally considered as the marker of regenerative anemia. The specificity of the results ranged from 79.4% (IRF) to 95.7% (reticulocyte count). However, the IRF appeared more independent of the reticulocyte count than the other maturation parameters, with a stronger relation to hemoglobin values. This finding suggested that the IRF could be an index of erythropoietic activity. The volume parameters (mean reticulocyte volume and mean sphered cell volume) were found to be closely related to the mean cell volume and should be interpreted only in relation to the mean cell volume. *Lab Hematol.* 1999;5:153-158.

**KEY WORDS:** Hematology automation · Coulter GEN.S · Reticulocyte count · Immaturity index

## INTRODUCTION

The Coulter GEN.S (Coulter Electronics, Hialeah, FL) is an automatic analyzer that is able to provide a complete hematologic profile including complete blood cell (CBC) count, white blood

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cell (WBC) differential count, and reticulocyte analysis. The ribosomal RNA of reticulocytes is first precipitated by a compound derived from methylene blue; then the red blood cells (RBCs) are sphered using an acidic reagent. This process is entirely automatic.

The treated RBCs are analyzed according to a flow cytometry procedure [1], which measures each cell for volume, light scatter of a laser beam, and cell opacity (radiofrequency). This technologic procedure is a new application of the procedure already in use in the Coulter instruments for the WBC differential [2-6]. RBCs are classified into mature cells or reticulocytes according to the extent of the scattering of the laser beam: Reticulocytes are RBCs with a higher scatter than mature RBCs. Furthermore, the level of immaturity of reticulocytes is correlated to the quantity of ribosomal RNA in their cytoplasmic structure, that is, the quantity of scatter light [1].

On the basis of this analysis, a 7-parameter reticulocyte profile is produced. The parameters may be classified into the following 3 categories:

1. Classic parameters are reported as reticulocyte percentage of RBCs (Ret%) and reticulocyte count per unit of peripheral blood (Ret#). The Ret# is expressed in  $10^9/L$ .

2. Maturation parameters are reported as immature reticulocyte fraction (IRF), percentage of high light-scatter reticulocytes (HLR%), and high light-scatter reticulocyte count per unit of peripheral blood (HLR#). By means of a 10-channel analysis of the laser light scatter, reticulocytes are classified as mature or immature; cells classified in the 8 upper channels are considered immature. The IRF is then calculated as the ratio of the immature Ret# to the total Ret#. The HLR% is calculated from the Ret% according to the formula  $HLR\% = Ret\% \times IRF$ . The HLR# is calculated according to the formula  $HLR\# = Ret\# \times IRF$  and is expressed as  $10^9/L$ .

3. Volumetric parameters, measured after the RBCs have been sphered, are reported as mean volume of the reticulocyte population (MRV), and the mean volume of the whole RBC population (MSCV).

The aim of this study was to gather any information that may be useful to the clinical interpretation of these new parameters. The

TABLE 1. Reference Values in 66 Healthy Donors\*

Classic Parameters	Maturation Parameters	Volume Parameters
Ret% 0.5-2	IRF (ratio) 0.2-0.4	MRV (fL) 100-125
Ret# (10 <sup>9</sup> /L) 22-98	HLR% 0.07-0.71	MSCV (fL) 84-104
	HLR# (10 <sup>9</sup> /L) 3-35	

The range values were calculated as the limits of the 95% confidence intervals.

\*HLR% indicates percentage of high light-scatter reticulocytes; HLR# high light-scatter reticulocyte count; IRF, immature reticulocyte fraction; MRV, mean reticulocyte volume; MSCV, mean spheroid cell volume; Ret#, reticulocyte count; Ret%, reticulocyte percentage.

values in normal blood donors were established, followed by study of the values of the reticulocyte parameters in several types of pathologic conditions including anemias, both nonregenerative and regenerative. To interpret the parameters according to each other, it is necessary to know whether and how they are correlated. Furthermore, we investigated how the various parameters correlate with the peripheral oxygenation status.

## MATERIAL AND METHODS

### *Values in Normal Blood Donors*

From adult blood donors, 66 blood samples were collected in K<sub>3</sub>-ethylene diamine tetraacetic acid tubes (Hemoguard [5 mL], Becton-Dickinson, Meylan, France) and analyzed on the Coulter GEN.S for both CBC and reticulocytes. Samples with abnormal CBC values were excluded from the group. The normal reference ranges for each reticulocyte parameter were calculated from the mean (m) and SD as the 95% confidence intervals ( $m \pm 2$  SD), as reported in Table 1.

### *Values in Regenerative and Nonregenerative Samples*

A first group of 79 regenerative patients included patients suffering from sickle cell disease (42), malaria with anemia (20), major hemoglobin abnormalities (3 with homozygous thalassemias, 3 with hemoglobin S- $\beta$ -thalassemias, 3 with SC hemoglobinoses), and autoimmune hemolysis (8).

A second group of 209 nonregenerative patients included the 66 normal donors; the remaining 143 were patients suffering from malaria without anemia (22), lymphocyte blood diseases controlled

Coombs'-negative (47), acute leukemia (37), vitamin B<sub>12</sub> deficiency (7), chemotherapy (25), and polycythemia vera (5).

All blood samples were analyzed on the Coulter GEN.S for both CBC and reticulocytes. Studies of sensitivity (Table 2) and specificity (Table 3) were performed for each classic parameter and each maturation parameter, according to the criteria derived from the normal ranges: each value higher than the upper limit of the normal range was considered as a "flag" for detecting a hemolytic condition. In this view, the sensitivity of each flag was calculated as the percentage of hemolytic patients (first group) marked by a value of the corresponding parameter higher than the normal range. The specificity of each flag was calculated as the percentage of non-hemolytic patients (second group) marked by a normal or low value of the corresponding parameter.

### *Relation Between Classic Parameters and Maturation Parameters*

From the 2 groups just described, the 167 patients without impairment of the bone marrow were selected for the following study (ie, all hemolytic patients, 66 donors, and 22 patients without anemia). The IRF and HLR# were correlated to the reticulocyte number to evaluate whether these maturation parameters depend on the regenerative status measured according to the classic approach. The results of these correlation analyses are given in Figure 1.

### *Relation Between Mean Cell Volume and Volumetric Reticulocyte Parameters*

To know whether the volumetric parameters depend on the mean cell volume (MCV), a regression analysis was performed: MCV was taken as the independent variable and correlated to

TABLE 2. Sensitivity of Reticulocyte Parameters in Detecting Hemolytic Conditions

	Ret% >2	Ret# >100	HLR% >1	HLR# >35	IRF >0.4
Malaria with anemia (n = 20)	13	12	14	15	12
Hemoglobin abnormalities (n = 9)	9	9	8	8	5
Sickle cell diseases (n = 42)	42	42	42	42	40
Immune hemolysis (n = 8)	8	7	8	7	6
Total (n = 79)	72	70	72	72	63
Sensitivity (%)	91.1	88.6	91.1	91.1	79.8

The criteria for detection are given on the first line, as well as the number of patients meeting the respective criteria. The sensitivity for each parameter is given in the last line, as the total percentage of cases fulfilling the criteria. See Table 1 for explanation of abbreviations.

TABLE 3. Specificity of Reticulocyte Parameters Tested in Nonhemolytic Conditions\*

	Ret% <2	Ret# <100	HLR% <1	HLR# <35	IRF <0.4
Donors (n = 66)	64	64	65	64	65
Malaria without anemia (n = 22)	21	21	21	21	19
CLL Coombs'- (n = 47)	40	46	42	40	33
Acute leukemias (n = 37)	21	33	28	31	23
B <sub>12</sub> deficiencies (n = 7)	4	7	4	7	0
Chemotherapies (n = 25)	24	25	25	25	21
Polycythemia vera (n = 5)	5	4	5	4	5
Total (n = 209)	179	200	190	192	166
Specificity (%)	85.7	95.7	90.9	91.9	79.4

\*CLL indicates chronic lymphocytic leukemia. See Table 1 for explanation of other abbreviations.

The numbers of samples with a normal value for each parameter are given in the respective columns. The specificity for each parameter is given in the last line as the total percentage of cases exhibiting normal values.

MRV and MSCV. To perform the analysis with both very low and very high values, 20 samples with macrocytosis and microcytosis were added, resulting in a total number of 187 samples. The results of these correlations are given in Figure 2.

#### Relation Between Anemia and Reticulocyte Parameters

In the group of patients without impairment of the bone marrow, 3 correlation analyses were performed between hemoglobin (taken as the independent variable) and reticulocyte number, IRF and HLR#, respectively. The results of these correlation analyses are given in Figure 3.

## RESULTS

The normal values for all reticulocyte parameters previously defined, calculated as the 95% confidence interval in a group of 66

blood donors, are given in Table 1. Most of these values are used as references in the following part of the study.

From the values obtained in regenerative and nonregenerative patients, we calculated the sensitivity for detection of hemolytic conditions and the parameter specificity of the criteria Ret% >2, Ret# >100, HLR% >1, HLR# >35, IRF >0.4.

The sensitivity results (Table 2) ranged from 79.8% (IRF) to 91.1% (Ret%, HLR%, HLR#). The sensitivity of the parameter Ret# (discriminating value:  $100 \times 10^9/L$ ), which is generally considered as the marker of regenerative anemia, was 88.6%. Furthermore, the score of each parameter for the separate pathologic conditions was given. It is worth noting that the highest proportion of complete regenerative pattern (high value for all parameters) was seen in patients with sickle cell disease (40 of 42).

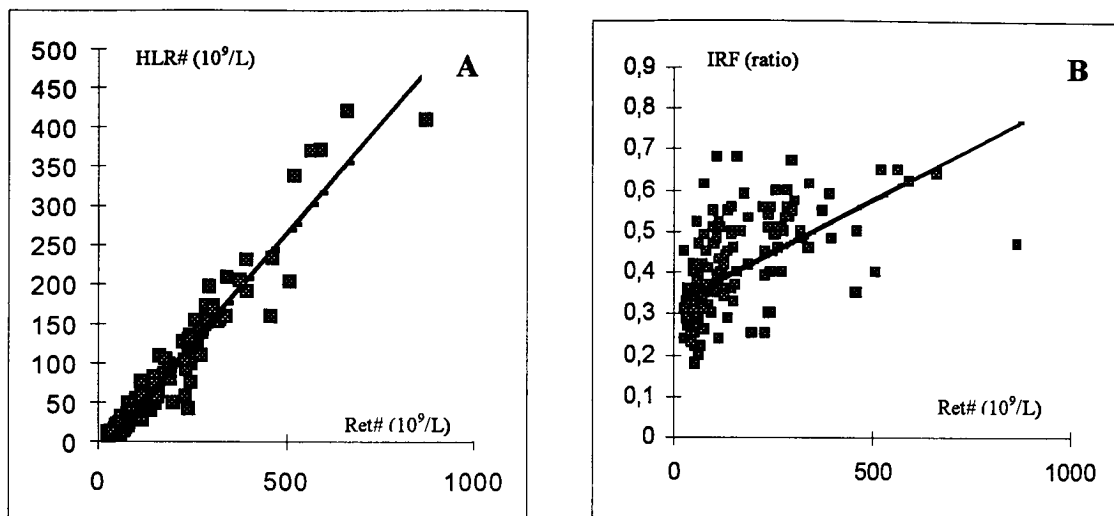


FIGURE 1. A. Relation between the reticulocyte number (Ret#; x axis) and the high light-scatter reticulocyte count (HLR#; y axis);  $r = 0.967$ . B. Relation between the Ret# (x axis) and the immature reticulocyte fraction (IRF; y axis);  $r = 0.637$ .

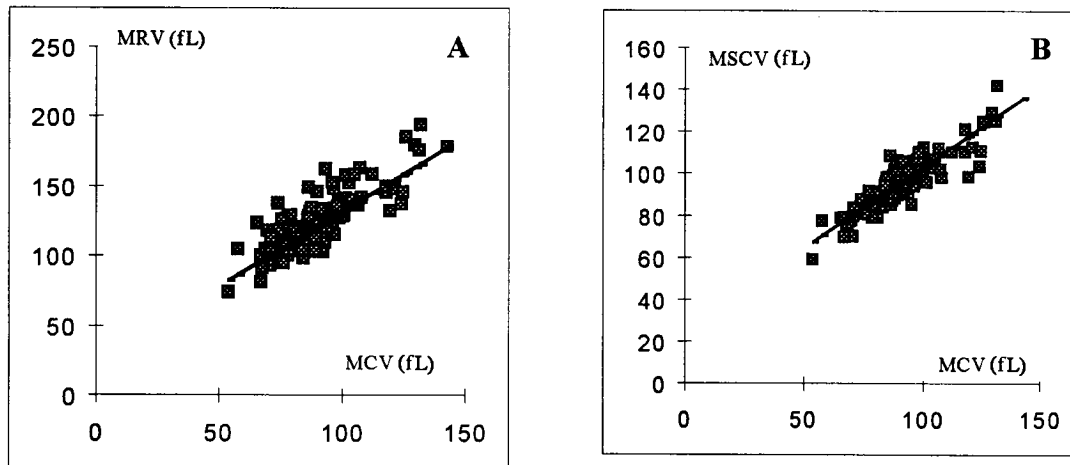


FIGURE 2. A. Relation between the mean cell volume (MCV; x axis) and the mean reticulocyte volume (MRV; y axis);  $r = 0.776$ . B. Relation between the mean cell volume (x axis) and the mean spherical cell volume (MSCV; y axis);  $r = 0.892$ .

The specificity results (Table 3) ranged from 79.4% (IRF) to 95.7% (Ret#). Thus, the IRF appeared to be the less specific parameter for detection of hemolytic conditions, especially in bone marrow invasion due to acute leukemias and chronic lymphoid leukemias. In the 7 cases of vitamin B<sub>12</sub> deficiency, the IRF appeared constantly elevated. On the other hand, the 5 cases of polycythemia vera were associated with a normal IRF.

The global efficiency of each parameter was calculated from the results given in Tables 2 and 3, as the percentage of the total population (288 patients from groups 1 and 2) correctly classified as hemolytic or nonhemolytic conditions by means of the various criteria. These were the results: Ret% >2% (87.2%); Ret# >100 × 10<sup>9</sup>/L (93.8%); HLR% >1% (91%); HLR# >35 × 10<sup>9</sup>/L (91.7%); IRF >0.4 (79.4%).

The correlation analysis of the maturation parameters versus the reticulocyte number, performed in patients without bone marrow

impairment, showed a strong relation between the HLR# and the Ret# ( $r = 0.967$ , Figure 1A) and a weaker one (although statistically significant) between the IRF and the Ret# ( $r = 0.637$ , Figure 1B).

From the results given in Figure 2, a significant relation is seen between MCV and both MRV ( $r = 0.776$ ) and MSCV ( $r = 0.892$ ). The MCV values ranged from 60 to 150. Thus, the volumetric parameters cannot be interpreted without reference to the cell volume.

Significant negative relations were found between the hemoglobin value (taken as independent variable) and the reticulocyte parameters Ret# ( $r = 0.567$ ), HLR# ( $r = -0.589$ ), and IRF ( $r = -0.703$ ). The strongest relation is between hemoglobin and IRF (Figure 3).

## DISCUSSION

The Ret# has long been used as a noninvasive method to evaluate the status of bone marrow production and as a means of

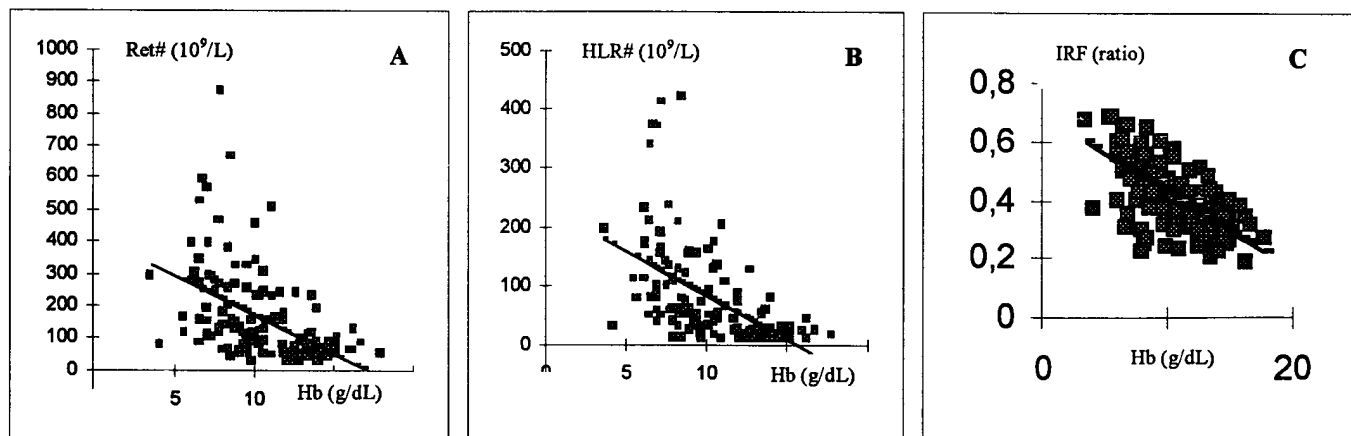


FIGURE 3. A. Relation between the hemoglobin (Hb; x axis) and the reticulocyte number (Ret#; y axis);  $r = -0.567$ . B. Relation between the hemoglobin (x axis) and the number of high light-scatter reticulocytes (HLR#; y axis);  $r = -0.589$ . C. Relation between the hemoglobin (x axis) and the immature reticulocyte fraction (IRF; y axis);  $r = -0.703$ .

knowing whether an anemia is related to a bone marrow insufficiency or to an excessive peripheral destruction (hemolysis). However, the method, performed microscopically, was limited by the lack of standardization [7,8] and the lack of reproducibility [9], owing to the small number of reticulocytes counted. The development of automated methods, using flow cytometry, made it possible to perform both a precise quantification of reticulocytes [9-12] and a further analysis of the reticulocyte population with the evaluation of the IRF [9,13-15].

The introduction of new indices for evaluating the maturation status of the reticulocyte population resulted in various studies [14-17]. The results suggest that the IRF, interpreted in association with the Ret#, may be a useful index of erythropoietic activity. The presence of both a low Ret# and a low IRF suggests a low level of erythropoiesis. The data presently available led to a classification of anemias on the basis of both IRF and Ret# [9,17].

The GEN.S analysis of reticulocytes was first performed in 66 adult volunteers to establish the normal nonregenerative values. The ranges of normal values given in Table 1 are consistent with those previously calculated by Lahary et al [18]. It is worth noting that the values were calculated from the 95% confidence interval, and 2 individual donors had a value higher than normal for Ret% and Ret#, as well as for HLR#. One donor exhibited higher-than-normal values for HLR% and IRF. The normal values, as given in Table 1, thus define the normal nonregenerative pattern.

Among the regenerative samples studied by means of the Coulter GEN.S (Table 2), the sensitivity studies suggested that the maturation parameters HLR% and HLR# were the most sensitive (91.1%) in detecting the hemolytic diseases. The Ret# was higher than  $100 \times 10^9/L$  in 88.6% of cases. The regenerative status was accompanied by a high IRF in 79.8% of cases. Among the hemolytic subgroups studied, a pattern of chronic hemolysis could be defined in most samples of the sickle cell disease group (40/42); this group may be distinguished from the others by high Ret# and by higher-than-normal IRFs.

The nonregenerative samples exhibited more variable patterns, according to the subgroups. A constant pattern of nonregenerative anemia (Ret#  $<100 \times 10^9/L$ ) with very high IRFs ( $\sim 0.5$ ) was found in the 7 cases of vitamin B<sub>12</sub> deficiencies recorded, consistent with the data of Watanabe et al [17]. The nonanemic malaria subgroup looked like the donor group. All patients of the chemotherapy subgroup were aregenerative, with very low Ret# and HLR#; however, 4 of them were marked by an IRF higher than normal.

The subgroups of patients with invaded bone marrow (Coombs'-negative chronic lymphoid leukemias and acute leukemias) generally exhibited a nonregenerative pattern, with Ret#  $<100 \times 10^9/L$  in 79 cases of 84. However, the IRF was found higher than normal in about one third of these patients. The pattern of invaded bone marrow thus appears to include a variable IRF. This finding is in agreement with the results of Tsuda and Tatsumi [15], who found, by means of another automated method, in patients with acute leukemias as well as those with malignant lymphomas, a slightly increased IRF compared with that of patients with no known hematologic disease. Furthermore, Watanabe et al [17] found that the IRF might be increased in patients with acute leukemias.

A special mention must be made concerning the patients suffering from polycythemia vera: 4 of the 5 patients included in this subgroup

were nonregenerative; the fifth exhibited a Ret# slightly over the normal range. Furthermore, the IRF was in the normal range in all cases.

The knowledge of the degree of interdependence among the reticulocyte parameters may be useful in the clinical interpretation of the results. In this study, several correlation analyses were performed in patients with healthy bone marrow. It was first shown that the parameters Ret# and HLR# were closely related to each other. This finding, together with the results of sensitivity and specificity, suggests that these 2 parameters have the same significance; with a borderline reticulocyte value, a hemolytic condition may be detected by the presence of an elevated HLR#.

On the other hand, the relation between the Ret# and the IRF, although statistically significant, appears weaker, suggesting that the 2 parameters can be interpreted independently of each other. This result is similar to those obtained from the Sysmex R3000 (Sysmex Corporation of America, Long Grove, IL) by Chang and Kass [19], who found a weak but significant relation between the absolute Ret# and the IRF calculated from the R3000 results (sum of the high-fluorescence reticulocytes and the medium-fluorescence reticulocytes). From this point of view, the IRF could be more representative of the current status of the erythropoietic activity, as seen in some nonregenerative conditions, where it was shown that an increased IRF may be seen before the increase of the Ret# [20,21]. Accordingly, the IRF could be interpreted as an index reflecting the status of the tissue oxygenation. This point was further investigated by performing a correlation analysis between IRF and hemoglobin (considered as a value correlated to the oxygen tissue status) in patients (anemic and nonanemic) with functional bone marrow.

For comparison purposes, 2 other correlation analyses were performed on the same samples: Ret# versus hemoglobin and HLR# versus hemoglobin. Our results showed that, among the relations studied, the strongest one was between IRF and hemoglobin. A significant relation was also found by Davis et al [22] in anemic patients (hemoglobin  $<12$  g/dL), between hemoglobin and the reticulocyte maturity index (RMI, measured as the mean fluorescence of reticulocytes stained with thiazole orange), which depends on the RNA content of the reticulocytes. The selection of Davis's group (anemic patients without regard to the bone marrow status) may explain why the relation was weaker and that no relation was found between hemoglobin and Ret#.

These points suggest that the best index of the current erythropoietic activity is the IRF. A good demonstration of this statement should be the discovery of a strong relation between erythropoietin values and IRF in patients with healthy bone marrow. This relationship was found by Davis et al [22]. We had no erythropoietin data to enable us to study such a relationship, but 2 other points in our study seem to support this hypothesis: all patients with polycythemia vera (a condition classically associated with low erythropoietin levels) had normal IRF, and all but 2 patients suffering from sickle cell disease (a condition in which the low hemoglobin affinity for oxygen results in a decreased delivery to peripheral tissues) exhibited an increased IRF.

There was a strong relation between the reticulocyte volume parameters (MRV and MSCV) and the MCV in all patients, which means that these parameters cannot be interpreted independently of the MCV. The significance of MRV remains unclear. For the MSCV, a team working on hereditary spherocytosis recently found that the patients suffering from this disease had a MSCV lower

than the corresponding MCV [23], a feature that may be used as a screening tool for hereditary spherocytosis with a sensitivity of 100% and a specificity of 93%.

In conclusion, the reticulocyte profile measured by the GEN.S includes several useful and significant parameters, which can be helpful in diagnosing the hematologic diseases. Further studies are necessary to confirm their significance, especially with reference to the erythropoietic activity in regenerative conditions.

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