Anemia of the Belgrade Rat: Evidence for Defective Membrane Transport of Iron

By Barbara J. Bowen and Evan H. Morgan

The mechanisms underlying the impaired utilization of transferrin-bound iron by erythroid cells in the anemia of the Belgrade laboratory rat were investigated using reticulocytes from homozygous anemic animals and transferrin labeled with \(^{59}\)Fe and \(^{125}\)I. The results were compared with those obtained using reticulocytes from phenylhydrazine-treated rats and iron-deficient rats. Each step in the iron uptake mechanism was investigated, ie, transferrin–receptor interaction, transferrin endocytosis, iron release from transferrin, and transferrin exocytosis. Although there were quantitative differences, no fundamental difference was found in any of the abovementioned aspects of cellular function when the reticulocytes from Belgrade rats were compared with those from iron-deficient animals. The basic defect in the Belgrade reticulocytes must therefore reside in subsequent steps in iron uptake, after it is released from transferrin within endocytotic vesicles, ie, in the mechanism by which it is transferred across the lining membrane of the vesicles into the cell cytosol. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of reticulocyte ghosts extracts demonstrated a prominent protein band of mol wt 69,000 that was absent or present only in low concentration extracts from the other two types of reticulocytes. This may be a result of the genetic defect.

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MATERIALS AND METHODS

Animals. Heterozygous (b/?) male and female Belgrade rats were very kindly provided by Dr John A Edwards, Department of Medicine, State University of New York at Buffalo. These animals were used to produce a colony of anemic (b/b) and nonanemic (b/?) rats. Animals were fed a standard laboratory rat diet. Iron deficiency was produced in 3-month-old male nonanemic Belgrade rats by the removal of 5 mL of blood by heart puncture on four occasions at 1-week intervals while maintaining the rats on an iron-deficient diet. The animals were then kept on this diet for a further 2 months, being bled only as required for the studies described below.

Reticulocyte uptake was also induced in nonanemic (b/?) and ?/? rats by a single IP injection of neutralized phenylhydrazine HCl (10 mg/100 g of body weight). The animals were bled by heart puncture (2 mL/100 g of body weight) 2 days after the first bleed. This second sample of blood was used for the experiments described in this article. In one experiment, reticulocyte uptake was induced solely by bleeding by heart puncture (2 mL/100 g of body weight) three times in 1 week of (?/?) rats that had been injected intramuscularly (IM) with 50 mg iron as iron-dextran 1 week before the first bleed. Blood was obtained 3 days after the last bleeding for the iron uptake studies.

For brevity, the reticulocyte-rich blood cells from homozygous Belgrade (b/b) rats, heterozygous (b/?) rats, iron-deficient (?/?)
rats, and phenylhydrazine-treated (7/-) rats are referred to as Belgrade, heterozygous, iron-deficient, and control reticulocytes, respectively.

Purifications and labeling of transferrin. Rat and rabbit transferrins were isolated from plasma by ion-exchange chromatography on diethylaminoethyl (DEAE)-cellulose (DE52, Whatman) and gel filtration with Ultrogel ACA34 (LKB). The rat transferrin was labeled with 59Fe and 125I as described previously. It was used in the dextrif form in the experiments described below. The rabbit transferrin was used in cold-chase experiments and in the preparation of antisera in goats by three intramuscular injections of 5-mg quantities of the protein in Freund’s complete adjuvant at 2-week intervals.

Isolation of transferrin receptor. The transferrin receptor was isolated from the placenta of control rats on the nineteenth day of gestation. The placentas from four pregnant rats were washed with several changes of phosphate-buffered saline (PBS) and then homogenized with 3 vol of PBS using a Potter-Elvehjem homogenizer. The insoluble components of the placentas were sedimented by centrifugation for 30 minutes at 40,000 g (average) and washed three times by resuspension in PBS and centrifugation as before. They were then treated with 2% Triton X-100 (Sigma, St Louis), 1 mM of iodoacetic acid, 1.0 mM of phenylmethylsulphonyl fluoride in PBS for 1 hour, followed by centrifugation at 40,000 g (average) for 1 hour. The transferrin receptor was isolated from the supernatant solution by affinity chromatography using rat transferrin coupled to Sepharose 4B (Pharmacia, Piscataway, NJ) following the procedure described by van Driel and colleagues. The isolated receptor was contaminated with rat transferrin, which was largely removed by passage through a Sepharose 4B-anti-rat transferrin column equilibrated and washed with PBS, pH 7.4-0.2% Triton X-100. All procedures were performed at 4°C.

Measurement of transferrin and iron uptake. Blood was obtained from the anemic rats by heart puncture, using heparin as an anticoagulant. The cells were washed three times with ice-cold PBS and were then suspended in Hanks’ and Wallace balanced salt solution. Theuffy coat was removed from the RBC layer at the end of each wash. After the third wash, the cells were centrifuged at 2,000 g for 30 minutes at 4°C and the top one-quarter of the RBC layer was removed to obtain a reticulocyte-enriched cell suspension. These cells were suspended in Hanks’ solution. The reticulocyte counts of these suspensions varied from 20% to 38% for the Belgrade reticulocytes, 21% to 49% for heterozygous reticulocytes, 22% to 59% for control reticulocytes and 18% to 37% for iron-deficient reticulocytes.

Aliquots of the reticulocyte suspensions were then incubated with 125I-Fe-labeled transferrin, washed with ice-cold PBS and treated with Pronase (Calbiochem-Boehringer, San Diego) at 4°C to measure endocytosed transferrin as previously described. Transferrin and iron exocytosis were measured by a cold-chase procedure, again using incubation with Pronase at 4°C to distinguish between membrane-bound and intracellular transferrin. The validity of this method for the measurement of transferrin endocytosis has been demonstrated by quantitative electron microscope autoradiography. To measure the number and affinity of transferrin receptors on the cell surface, samples of the cells were incubated for 30 minutes at 4°C with varying concentrations of radiolabeled transferrin in the presence of 10 mg/mL of bovine serum albumin (BSA) (Sigma, St Louis, Type A7030). Total cell-associated transferrin was then determined by centrifuging 150-μL aliquots of the cell suspension through a mixture of di-n-butyl phthalate and dioinyl phthalate (2:1; vol:vol) in a Beckman Microfuge for 15 seconds, and counting the resultant cell pellet for radioactivity. Nonspecific binding of transferrin was measured in duplicate incubations containing a 50-fold molar excess of unlabeled diferric rat transferrin. Specific binding was calculated as the difference between total and nonspecific binding, and was used to estimate the numbers of specific receptors on the cell membrane ($B_0$) and the association constant of the transferrin–receptor reaction ($K_a$) by Scatchard method. The total number of cellular receptors was measured in a similar manner except that the incubations were performed at 37°C. The percentage of total receptors situated on the cell membrane was calculated from these values.

Analytical procedures. Reticulocytes were determined by staining with new methylene blue, packed cell volume by the microhematocrit procedure and mean corpuscular volume (MCV) by the use of an automated cell counter (Model H-1; Technicon, Tarrytown, NY). Heme was extracted from washed cells by the method of Thurnell. Radioactivity was counted in a three-channel y-scintillation counter. Polyclarylamide gel electrophoresis (PAGE) was performed as described by Laemmli, using 10% polyacrylamide in the resolving gel, and reducing the samples by heating to 95°C in sample buffer containing 4% sodium dodecyl sulfate (SDS) and 10% mercaptoethanol. The samples were run in duplicate. One set of the duplicates was stained for protein using Coomassie brilliant blue R250. The transferrin receptor was identified in the other set of the duplicates by Western blot transfer of the proteins from the polyacrylamide gel to nitrocellulose membrane and by staining with anti-rat transferrin receptor serum, followed by reaction with goat anti-rabbit IgG coupled to horseradish peroxidase (Bio-Rad, Richmond, CA). The anti-rat transferrin receptor serum was produced in rabbits by injection of three doses of 100 μg of the receptor in Freund’s complete adjuvant. The antiserum was cleared of contaminating anti-rat transferrin by passage through a column of transferrin coupled to Sepharose 4B.

RESULTS

Transferrin receptors. The affinities and numbers of transferrin receptors on Belgrade, iron-deficient, and control reticulocytes are given in Table 1. There were no significant differences ($P > .05$) between the $K_a$ values for any of the three types of cells. Although the mean number of total sites per cell was significantly higher for the control reticulocytes than for the other two types ($P < .001$), the numbers on the outer cell membrane showed no significant differences ($P > .05$). Belgrade and iron-deficient reticulocytes had higher percentages of their total receptors on the cell membrane (57% and 69%), respectively, than did the control reticulocytes (30%). This difference was confirmed in experiments in which measurements were made before and after

| Table 1. Transferrin Receptor Affinities ($K_a$) and Numbers on Outer Cell Membrane, and Total Reticulocyte Numbers for Belgrade, Iron-Deficient, and Control Reticulocytes |
|---------------------------------|-------------------------------|-----------------|-----------------
|                                    | $K_a$ (M$^{-1}$) (x 10$^{-4}$) | Numbers (Molecules/Cell x 10$^{-4}$) | Outer Cell Membrane | Total |
| Reticulocytes                     |                               |                             |                  |
| Belgrade                         | 8.10 ± 1.25(3)                | 2.60 ± 0.23(7)             | 3.81 ± 0.42(7)   |
| Iron-deficient                   | 7.13 ± 0.80(3)                | 2.86 ± 0.33(5)             | 5.00 ± 0.46(5)   |
| Control                          | 7.87 ± 1.32(3)                | 2.85 ± 0.28(7)             | 9.66 ± 0.61(7)   |

Each value is the mean (± SEM) of the number of measurements shown in parentheses. The $K_a$ values and outer membrane receptor numbers were obtained by incubation of cells with 125I-labeled transferrin at 4°C, and the total receptor numbers were obtained by incubation at 37°C (described in text).

*Significant difference from control reticulocytes, $P < .01$.
incubating reticulocytes with Pronase at 4°C to remove surface receptors. This produced only a 22% reduction in total receptor numbers on the control reticulocytes but 55% and 71% reductions for the iron-deficient and Belgrade reticulocytes, respectively (means of two experiments). The total number of receptors in the three types of reticulocytes correlated to some extent with the mean MCV values, which were 21, 27, and 85 fl for the Belgrade, iron-deficient, and control reticulocytes, respectively. The MCV of mature erythrocytes from untreated nonanemic rats was 59 fl.

In the one experiment in which reticulocytes from the rats that had been bled >1 week after receiving an iron-dextran injection were used, the K_0 and receptor number values and the rates of iron and transferrin uptake were similar to those from the control reticulocytes.

The transferrin receptors were also examined by SDS-PAGE (Fig 1). The receptors from all cell types, including known heterozygous (B/-) rats, were identified as two closely opposed bands of similar mol wt, ~92,000. Although not evident in Fig 1, in some electrophoretic runs the Belgrade (B/B) receptor bands were of slightly faster mobility than the receptor from control reticulocytes. However, in these cases the receptor from the iron-deficient reticulocytes had mobility identical to that from the Belgrade cells. Hence, the change observed with the Belgrade reticulocytes appeared to be a consequence of iron-deficient erythropoiesis rather than of a genetic defect.

Several other differences were noted between the electrophoretic patterns of extracts from the ghosts of Belgrade and control reticulocytes. However, the same differences, with one exception, were noted when extracts of iron-deficient ghosts were compared with controls. The exception was the appearance of a strong protein band of approximate mol wt 69,000 in the Belgrade extracts as compared with its absence or near absence in the extracts from the other two types of reticulocytes including those from heterozygous (+/-) Belgrade rats (Fig 1).

Transferrin endocytosis and iron uptake. The rates of transferrin endocytosis and iron uptake were measured in eight separate experiments comparable to the one shown in Fig 2. As noted by earlier investigators, the outstanding feature was the relatively slow rate of iron uptake by Belgrade reticulocytes, even when compared with iron-deficient animals. Transferrin endocytosis was also slower in the Belgrade reticulocytes, but the relative decrease was not as great as that of iron uptake. This is illustrated very clearly by comparison of the mean rates of iron uptake and transferrin endocytosis in these experiments (Table 2). The mean rates of iron uptake obtained for the control and iron-deficient reticulocytes were almost twice those of transferrin endocytosis (Table 2), indicating that each molecule of diferric transferrin donated both its iron atoms to the cells during each intracellular cycle, as has been reported previously for rabbit reticulocytes.11 However, with Belgrade reticulocytes the mean rate of iron uptake was only 0.7 times that of transferrin. Hence, on the average, only ~35% of the iron carried into the cells by endocytosis of transferrin was being retained by the cells. Incorporation into heme of the iron taken up by the cells was also measured. The proportion of iron incorporated into heme during 30-minute incubation at 37°C was much lower for the Belgrade and Fe-deficient cells (9% to 30%) than for the controls (39% to 81%).

Two other experiments using agents known to affect the rates of transferrin endocytosis and iron uptake, phorbol 12-myristate 13-acetate (PMA) and hemin, were also performed. PMA produced increases in the rates of transferrin endocytosis by all three types of cells (Table 3). In control and iron-deficient reticulocytes, this was accompanied by an increase in the rate of iron uptake, but with Belgrade reticulocytes no such increase was observed. In contrast, hemin inhibited transferrin endocytosis, and the degree of inhibition was greater with the control reticulocytes than with the other two types. In iron-deficient cells, the rate of iron uptake was inhibited to almost the same extent. The transferrin receptor is the double band at the 92.5-mol wt position as shown by Western blot.
MEMBRANE IRON TRANSPORT IN BELGRADE ANEMIA

Table 2. Rates of Iron Uptake and Transferrin Endocytosis and the Ratio of Two Measurements for Belgrade, Iron-Deficient, and Control Reticulocytes

<table>
<thead>
<tr>
<th>Reticulocytes</th>
<th>Iron Uptake (Atoms/Cell/ min x 10^-14)</th>
<th>Transferrin Endocytosis (Molecules/Cell/ min x 10^-10)</th>
<th>Iron:Transferrin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belgrade</td>
<td>0.68 ± 0.082 *†</td>
<td>1.05 ± 0.21 †</td>
<td>0.65 ± 0.13 *†</td>
</tr>
<tr>
<td>Iron-deficient</td>
<td>2.33 ± 0.62 †</td>
<td>1.18 ± 0.31 †</td>
<td>1.97 ± 0.56 †</td>
</tr>
<tr>
<td>Control</td>
<td>4.05 ± 0.47</td>
<td>2.10 ± 0.25 †</td>
<td>1.93 ± 0.35 †</td>
</tr>
</tbody>
</table>

Each value is the mean (± SEM) of eight measurements with Belgrade and control reticulocytes and five measurements with iron-deficient reticulocytes.

*Significant difference from control reticulocytes, P < .01.
†Significant difference from iron-deficient reticulocytes, P < .01.
‡Significant difference from control reticulocytes, P < .05.

degree as that of transferrin endocytosis. However, with Belgrade reticulocytes there was little change in the rate of iron uptake. Hence, the rate of iron uptake relative to that of transferrin endocytosis increased considerably (Table 3).

The rates of transferrin endocytosis and iron uptake were also measured in two experiments in which heterozygous reticulocytes were compared with Belgrade and control cells. The results confirmed the abovementioned differences between Belgrade and control reticulocytes but showed no differences between control and heterozygous reticulocytes.

**Transferrin exocytosis and iron release from reticulocytes.** The rates of transferrin exocytosis and iron release from reticulocytes labeled with 59Fe and 125I-transferrin by prior incubation with doubly labeled transferrin were measured in four experiments similar to that shown in Fig 3. In all cases, the rate of transferrin exocytosis was as fast from the Belgrade and iron-deficient reticulocytes as from the control cells. However, in confirmation of earlier observations, the Belgrade reticulocytes released relatively more of their radioiron than did the control reticulocytes. This was not a simple consequence of iron deficiency since it was not observed with the iron-deficient cells.

**Iron release from transferrin.** The above experiments showed that the rate of iron accumulation by Belgrade reticulocytes is less than one-half that which would be expected from the rate of endocytosis of transferrin and that a significant proportion of the iron taken up by these cells is later returned to the extracellular medium. These results suggested that the Belgrade reticulocytes were defective in releasing iron from transferrin within the endocytic vesicles or that the iron after its release was unable to pass through the lining membranes of the vesicles into the cell cytosol. Two types of experiments were used to distinguish these possibilities. The first was based on the observation that certain ferrous iron chelators such as 2,2'-bipyridine are able to chelate iron after it has been released from transferrin by the action of reticulocytes and to transport it from the cells, thus preventing its accumulation by the cells. The chelator-bound and transferrin-bound iron can be distinguished by treatment with ice-cold ethanol, which precipitates transferrin-iron quantitatively but does not precipitate bipyridine-iron. The second approach was to perform cold-chase experiments with rabbit rather than rat transferrin so that radioiron that has been released from 59Fe-labeled rat transferrin and bound by the rabbit protein can be distinguished by precipitation of the rabbit protein with a specific anti-rabbit transferrin antiserum. A similar procedure has been used for other purposes with human and rabbit transferrin and rabbit reticulocytes.

When control and iron-deficient reticulocytes were incubated with 59Fe-125I-rat transferrin in the presence of 2,2'-bipyridine, the radioactive iron was transferred to bipyridine at almost the same rate as it was accumulated by reticulocytes in the absence of bipyridine (Fig 4). However, with Belgrade reticulocytes, the rate of transfer of 59Fe to bipyridine was 2.7 ± 0.25 times as fast as iron accumulation by control cells (mean ± SEM of eight measurements). No transfer to bipyridine occurred in the absence of cells, and with all cell types the transfer was inhibited by NH4Cl (15

Table 3. Effects of PMA and Hemin on Iron Uptake and Transferrin Endocytosis by Belgrade, Iron-Deficient, and Control Reticulocytes

<table>
<thead>
<tr>
<th>Reticulocytes</th>
<th>Iron Uptake (Atoms/Cell/ min x 10^-14)</th>
<th>Transferrin Endocytosis (Molecules/Cell/ min x 10^-10)</th>
<th>Iron:Transferrin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belgrade</td>
<td>0.60</td>
<td>1.05</td>
<td>0.57</td>
</tr>
<tr>
<td>Control</td>
<td>0.60</td>
<td>1.25</td>
<td>0.48</td>
</tr>
<tr>
<td>Iron-deficient</td>
<td>2.12</td>
<td>1.14</td>
<td>1.86</td>
</tr>
<tr>
<td>Control</td>
<td>2.50</td>
<td>1.38</td>
<td>1.81</td>
</tr>
<tr>
<td>PMA</td>
<td>5.69</td>
<td>2.97</td>
<td>1.92</td>
</tr>
</tbody>
</table>

Reticulocytes were preincubated with PMA (10^-6 mol/L) or hemin (4 x 10^-9 mol/L) for 15 minutes at 37°C prior to the addition of labeled transferrin. Results are from two separate experiments.
mmol/L), nigericin (10 μmol/L), monensin (10 μmol/L) and N-ethylmaleimide (2 mmol/L), which also inhibited iron uptake by cells incubated without bipyridine.

The results of one cold-chase experiment with rabbit transferrin are shown in Fig 5. Similar results were obtained in a second such experiment. The fraction of intracellular 59Fe released from the cells during 5-minute cold chase with rabbit transferrin varied with the duration of the period used to label the cells with radiolabeled transferrin. It was low with both control and iron-deficient reticulocytes but high with Belgrade reticulocytes. The values obtained with the first two types of cells changed from 8% to 1% as the uptake time was increased from 1 to 16 minutes. However, with Belgrade reticulocytes, 50% to 54% of the radioiron taken up during 1 to 4 minutes was released and 24% of that was taken up during 16 minutes. Of this 59Fe, 74% to 77% was present on the chase rabbit transferrin, indicating that it had been released from its initial complex with rat transferrin during the intracellular cycle. A control experiment was performed by incubating 1, 2, 5 and 10 μg 59Fe-151-labeled rat transferrin with 1 mL of 2.5 mmol/L of 50% iron-saturated rabbit transferrin for 10 minutes at 37°C followed by precipitation of the rabbit transferrin with specific antiserum under the conditions used in the above experiments. Only 0.8% to 1.6% of the 59Fe was precipitated by the antiserum. Hence, the results of these experiments confirm the conclusions from the bipyridine experiments that Belgrade reticulocytes are unimpaired in their ability to release iron from transferrin.

**DISCUSSION**

The results of these experiments demonstrate that the basic defect of iron metabolism inherent in the Belgrade reticulocytes is an impairment of iron transfer into the cells after it has been released from transferrin. Iron release from transferrin occurs within endocytotic vesicles.5 Hence, the site of impaired transfer can be concluded to be the lining membrane of these vesicles. The membrane may contain a carrier for iron that is defective as a consequence of the genetic abnormality expressed in homozygous (b/b) Belgrade rats. Because evidence shows that the iron of transferrin is reduced to the ferrous state within the endocytotic membrane of these vesicles.7 Hence, the results from the experiments with 2,2'-bipyridine and the cold chase with rabbit transferrin clearly show that the amount of iron released from transferrin during its endocytotic cycle in Belgrade reticulocytes is far in excess of that which becomes fixed by the cells. Indeed, the amount released is equivalent to that which can be accounted for by the measured rate of transferrin endocytosis. Also, this release is inhibited by the lysosomotropic agent NH₄Cl and the proton ionophores nigericin and monensin, indicating that the release process is dependent on acidification of the endocytotic vesicles as it is in normal reticulocytes.5 Hence, there is no evidence for a
decreased capacity of Belgrade reticulocytes to release iron from transferrin.

The results of the experiments with PMA and hemin are compatible with the above conclusions and explain why hemin has little effect on iron uptake by Belgrade reticulocytes.23 As previously reported,16 PMA stimulated transferrin endocytosis and iron uptake by control and iron-deficient reticulocytes but had no effect on iron uptake by Belgrade reticulocytes even though the rate of endocytosis was accelerated in these cells. Presumably, the defect in membrane transport of iron prevented the cellular uptake of the increased amount of iron being endocytosed with the transferrin. In contrast, hemin inhibits the rate of transferrin endocytosis and recycling.17 Because these processes are the rate-limiting steps in iron uptake by normal and iron-deficient reticulocytes, there is a proportionate reduction in iron uptake. However, with the Belgrade reticulocytes, the rate-limiting step is the membrane transfer of iron. Therefore, reduced rate of delivery of iron to the site of this transfer by endocytosis would have little effect on the rate of iron uptake until the delivery became rate limiting.

This investigation confirms and extends an earlier report that iron-deficient reticulocytes show several alterations in iron metabolism when compared with iron-replete reticulocytes,24 and demonstrates that similar changes occur whether the iron-deficient cells are the result of a genetic defect or are due to iron depletion produced by dietary means combined with bleeding. Thus, total transferrin receptor numbers were lower, but a greater proportion of the receptors were on the outer cell membrane in iron-deficient than in normal reticulocytes. This was accompanied by a diminished rate of iron uptake and impaired incorporation of iron into heme.

Whether the control reticulocytes used in the present work were normal cells is debatable. Because they were collected 7 days after the injection of phenylhydrazine, it is unlikely that they were directly affected by the drug. This is supported by the observations that their transferrin receptor numbers, distribution, and function were similar to those of iron-replete reticulocytes produced solely by bleeding. However, the control reticulocytes were undoubtedly produced as a consequence of greater erythropoietic stimulation than that received by the iron-deficient and Belgrade cells and hence were probably more immature than the latter cells. This would account for their relatively large size when compared with mature rat erythrocytes and for some of the quantitative differences between the control and other types of reticulocytes. However, the changes in the efficiency of iron incorporation into heme, transferrin receptor distribution, and electrophoretic appearance of cell membrane extracts in the Belgrade and iron-deficient reticulocytes when compared with the control cells are likely to be a consequence of iron deficiency rather than of cell maturity. Such changes were not observed in any samples of control reticulocytes even though reticulocyte count and, presumably, cell maturity varied considerably from one sample to another. The mechanism by which iron deficiency produces the above changes is uncertain but is probably associated in part with the structural changes that lead to their microcytic appearance. The altered electrophoretic properties of the transferrin receptor observed in some electrophoretic runs may be a consequence of altered glycosylation.

The observation that Belgrade reticulocyte ghosts contain a protein absent, or present only in very low concentration, in the membrane of heterozygous, iron-deficient, and control reticulocytes is of particular interest. This may be a result of the genetic defect and directly responsible for the disorder of membrane transport of iron. Future work will be directed at studying the nature and function of this protein.

ACKNOWLEDGMENT

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