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Mammalian reticulocytes lose adhesion to fibronectin during maturation to erythrocytes

(red blood cells/differentiation/extracellular matrix/spleen/anemia)

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ABSTRACT **We describe three situations in which a large fraction of circulating red blood cells attach tightly and specifically to fibronectin: (*i*) rabbits made anemic by repeated bleeding, (*ii*) patients with hemolytic anemia and functional asplenia and splenectomized normal humans, and (*iii*) splenectomized mice. Upon induction of anemia in rabbits, the proportion of circulating red blood cells capable of specifically attaching to fibronectin-coated plastic increased in parallel with the number of reticulocytes. Fibronectin-adherent red cells were barely detectable when the rabbit had recovered from the anemia. Attachment of reticulocytes to fibronectin was specific; cells did not attach to dishes coated with albumin, laminin, or collagen. None of these proteins promoted the attachment of normal erythrocytes. About 75% of the erythrocytes from splenectomized mice (but not control mice) also attached specifically to fibronectin 40 days after surgery. The effect of splenectomy was incomplete and transient; adherent cells were not detectable 8 weeks after splenectomy. As judged by labeling studies with [^{35}S]methionine, newly emergent reticulocytes preferentially attached to fibronectin. We suggest that about half of the reticulocytes in erythropoietically unstressed mice lose their ability to attach to fibronectin, possibly due to loss of fibronectin-adhesive components, during passage through the spleen. The others lose their ability to interact with fibronectin before release, in the bone marrow, or in some extrasplenic site.**

Erythrocyte production in adult mammals begins in the bone marrow; nucleated erythroid stem cells undergo proliferation and terminal differentiation into nonnucleated reticulocytes, the immediate precursor of mature erythrocytes. Before the final stages of maturation, reticulocytes are released from the bone marrow into the circulation. Reticulocyte maturation is completed within 1–3 days; during this period remnants of mitochondria, Golgi complex, and polyribosomes are eliminated. Reticulocyte maturation also involves the remodeling of the plasma membrane, resulting in loss of the ability to exhibit endocytosis (1–3).

Undifferentiated murine erythroleukemia (MEL) cells growing in culture adhere tightly to fibronectin, and erythroid differentiation is accompanied by the loss of adhesion fibronectin (4). Fibronectin is a glycoprotein associated with extracellular matrices of many tissues; it is tightly bound to the surface of many cells, and is also found in the blood plasma (5, 6). Fibronectin is a major component of the interstitial matrix in the bone marrow (7). We speculated that the bone marrow precursors of erythrocytes interact with fibronectin and that loss of fibronectin adhesion is associated with release of the cell into the circulation (4). But the function of such interactions during erythroid differentiation remains to be investigated.

We show here that human, rabbit, and mouse reticulocytes specifically attach to fibronectin and that the remodeling of the reticulocyte plasma membrane during maturation *in vivo* is indeed accompanied by the loss of adhesion to fibronectin. We also show that the spleen is one of the sites where reticulocytes lose their ability to attach to fibronectin.

MATERIALS AND METHODS

Rabbit Reticulocytes. From New Zealand White rabbits (Charles River Breeding Laboratories), weighing 2.5 to 3.5 kg, a total of 150 ml of blood was taken from the ear or by cardiac puncture over 5 days as indicated in Fig. 2. Lost body fluid was replaced immediately by subcutaneous injection of 40 ml of sterile saline solution containing 10% (wt/vol) glucose. After the last bleeding (day 5) animals were allowed to recover for 8 days. During this period blood samples (1 ml) for reticulocyte counts and cell adhesion assays were collected in 4 ml of Hanks' buffered salt solution that was supplemented with ACD (citric acid at 1 mg/ml, sodium citrate at 2.8 mg/ml, and glucose at 15 mg/ml) and 2 mM EDTA as anticoagulants. Blood hematocrit was measured by using a microhematocrit centrifuge. For reticulocyte counts, 100 μl of blood was mixed with an equal volume of 2% (wt/vol) new methylene blue solution and incubated for 10 min at 37°C (8). Each slide used 10 μl of suspension spread under a 22-mm^2 coverslip. A minimum of 200 cells were counted. Only large spherical cells containing an intensely stained reticulum were scored as reticulocytes.

Splenectomy of Mice. Six- to eight-week-old CD-1 female mice (Charles River Breeding Laboratories) were used. Mice were anesthetized with diethyl ether and a 2-cm-long incision was made on the abdomen. Excessive blood loss was prevented by ligating the splenic artery and vein with an absorbable surgical suture immediately before or after the removal of the spleen. The viscera were then tucked back into the abdomen and the incision was closed by suture. Sham-operated controls were treated exactly as above, except that the spleen, splenic artery, and splenic vein were left intact. For cell adhesion assays, about 50 μl of blood was collected from the tail into the Hanks' buffer containing anticoagulants, as above.

Cell Adhesion Assay. Blood samples from rabbits, mice, and human subjects, collected as above, were centrifuged at 1000 × *g* (maximum radius) for 12 min at 0–4°C to remove plasma. After four washings of the cells with 20 vol of Hanks' buffered salt solution containing 2 mM EDTA (HBS-EDTA) and one wash with 10 vol of serum-free Dulbecco's modified eagle's medium (DME medium), the cells were suspended at 1 × 10^6 per ml of DME medium. Attachment of cells to fibronectin was measured in 35-mm bacteriological plastic Petri dishes coated with 2 ml of fibronectin at 7.5 μg/ml in phosphate-buffered saline (4, 9, 10). Both fibronectin-coated and uncoated (control) dishes were coated with 1 ml of bovine serum albumin at 20 mg/ml prior to the assay because both erythrocytes and reticulocytes nonspecifically

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adhered to the dishes in the absence of the albumin. Two replicate dishes containing 2×10^6 cells in 2.0 ml were incubated at 37°C in a CO_2 incubator. After 2 hr, the medium was removed and the cells were washed twice with 1 ml of DME medium. These washings were combined with the medium; unattached cells in this suspension were counted in a Coulter Counter. Attached cells were detached (except where indicated otherwise) with a rubber policeman and counted as above. To determine the relative distribution of reticulocytes between the unattached and the attached fractions, cells concentrated by centrifugation were stained with new methylene blue and counted as above.

RESULTS

Human Reticulocytes Attach to Fibronectin. In our initial studies we utilized reticulocytes from patients with hemolytic anemia, who have higher proportions of reticulocytes in their circulating blood (15–30%) than do hematologically normal individuals (1–2%). About half of the erythrocytes from five patients with sickle cell anemia and one with sickle β-thalassemia attached to fibronectin-coated Petri dishes, but not to albumin-coated dishes (Table 1). Neither fibronectin nor bovine serum albumin promoted attachment of normal human erythrocytes. Cell attachement was dependent on the amount of fibronectin applied to the dishes: 25% adhesion was observed when dishes were coated with 2 ml of fibronectin at 3.5 μg/ml and 50% with 2 ml of fibronectin at 7.5 μg/ml. No significant increase in cell adhesion was observed when the amount of fibronectin was increased to 30 μg of fibronectin per dish (data not shown).

To determine whether the fibronectin-adherent population represented immature cells, blood from one of the sickle cell patients was centrifuged at $600 \times g$ (maximum radius) for 20 min at 0–4°C (12). Erythrocytes from the top 5% (immature cells) and bottom 5% (mature cells) of the cell column were collected, washed three times in HBS-EDTA, and examined for fibronectin adhesion. Almost all of the immature (less dense) cells, consisting of 35% reticulocytes, attached to fibronectin, but the mature (more dense) cell fraction, containing 4% reticulocytes, did not attach at all (Table 1). Since these anemic individuals were all functionally asplenic, we determined whether erythrocytes from individuals splenectomized for nonhematologic reasons also attached to fibronectin. Indeed, the two individuals examined had normal concentrations of reticulocytes in their blood but about 50% of their circulating erythrocytes attached to fibronectin (Table 1).

Effect of Splenectomy. The role of the spleen in the loss of adhesion to fibronectin during maturation of reticulocytes to erythrocytes was also examined in mice. Seven mice were splenectomized, and two were sham-operated. Blood was tested for fibronectin-adherent erythrocytes once each week for 9 weeks after surgery. Data from one of two such experiments are illustrated in Fig. 1. The concentration of fibronectin-adherent erythrocytes in the circulation progressively increased, reaching a maximum of 75% after approximately 6 weeks. The adherent fraction rapidly declined to less than 5% by 8 weeks after splenectomy. Erythrocytes from unoperated and sham-operated mice, assayed in parallel, did not attach to fibronectin. At the peak of the response, blood from splenectomized mice contained less than 1% reticulocytes. Moreover, erythrocytes from splenectomized mice attached specifically to fibronectin, but not to dishes coated with bovine serum albumin, laminin, or type I collagen (Table 2). Under these assay conditions, laminin promoted the attachment of 90% of added human hepatoma cells, and type I collagen promoted attachment of over 85% of added Chinese hamster ovary cells (4).

Since mouse erythrocytes have a normal lifetime of 44 days, the above results suggested that, after splenectomy, aged, nonadherent, cells were gradually replaced with new, adherent, ones. To establish directly that newly released reticulocytes are adherent, we administered 2.5 mCi (1 Ci = 37 GBq) of [^{35}S]methionine to a mouse 3 days after splenectomy. Four days later, we determined the ability of both the labeled newly emergent cells and the total population of cells to attach to fibronectin. While only 10% of the total erythrocytes attached to fibronectin, 50% of the labeled ones attached (Table 3). Controls, labeled erythrocytes from normal mice that also received [^{35}S]methionine, did not attach to

Table 1. Attachment of human erythrocytes to fibronectin-coated plastic dishes

Condition of donor	Age, yr	Blood reticulocytes, %	% attachment: Without fibronectin	% attachment: With fibronectin
Normal	17	<1	<5.0	<5.0
Sickle cell anemia				
Patient 1	18	33	<5.0	63 ± 6
Patient 2	11	23	<5.0	50 ± 5
Patient 3	19	26	<5.0	45 ± 4
Patient 4	4	28	<5.0	47 ± 3
Patient 5	9			
Total cells		22	<5.0	46 ± 3
Immature cells		35	<5.0	78 ± 6
Mature cells		4	<5.0	5 ± 3
Sickle β-thalassemia	21	19	<5.0	46 ± 4
Idiopathic thrombocytopenic purpura*	10	<1	<5.0	47 ± 5
Splenectomized†	22	<1	<5.0	53 ± 4

Attachment of cells to Petri dishes coated with 2 ml of fibronectin at 7.5 μg/ml was measured; fibronectin-coated dishes and controls without fibronectin were coated with 1 ml of bovine serum albumin at 20 mg/ml. The attached cells in this experiment were detached by 5-min incubation in 0.2% trypsin/0.02% EDTA at 37°C. The detached cells were centrifuged at $1000 \times g$ for 12 min and the cell pellet was resuspended in DME medium containing soybean trypsin inhibitor (25 μg/ml) for counting. Data reported are mean ± SEM for two replicate dishes. Howell–Jolly bodies were detected in the blood of all hemolytic anemia patients, suggesting that the patients' spleens were not functional (11).

*Patient splenectomized to prevent platelet destruction approximately 3 months before the day of experiment.

†Adult human splenectomized after auto accident approximately 6 months before the day of experiment.

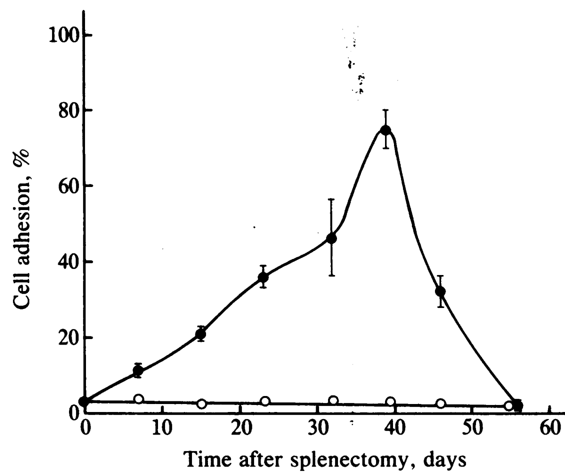

FIG. 1. Effect of splenectomy on the kinetics of appearance of circulating erythrocytes that attach to fibronectin. The adhesive capacity of erythrocytes obtained on the indicated days from splenectomized (●) and sham-operated and unoperated (○) mice is shown as the percentage of total cells that attached to fibronectin. Data presented are mean ± SEM for seven splenectomized mice. Erythrocytes from two sham-operated and two unoperated mice exhibited less than 5% attachment to fibronectin. None of the cells attached to dishes coated with bovine serum albumin.

fibronectin. Radioactivity in the fibronectin-attached fraction cannot be attributed to labeled nonerythroid cells, such as lymphocytes, because nucleated cells were not detected when the attached fraction was concentrated, stained with hematoxylin, and examined under the microscope. Also, labeled nonerythroid cells did not contribute significantly to attached or unattached fraction, because analysis of both the attached and unattached fractions by sodium dodecyl sulfate/polyacrylamide gel electrophoresis revealed that ≈80% incorporation of [^{35}S]methionine had occurred into erythrocyte-specific proteins such as hemoglobin (data not shown).

Rabbit Reticulocytes Attach to Fibronectin. To determine whether functional asplenia *per se* affected the adhesion capacities of erythrocytes, anemia was induced in rabbits by repeated bleeding over 5 days. The animals were allowed to recover for 8 days. The number of reticulocytes and fibronectin-adherent cells in the circulation was monitored during the entire 13-day period. The circulating reticulocytes resembled stress reticulocytes‡ and were large, spherical, and intensely stained with new methylene blue. The proportion of reticulocytes increased progressively in the circulation, reaching a peak (50%) at day 5, and decreased rapidly to about 4% by day 11 (Fig. 2). The number of circulating cells capable of attaching to fibronectin increased in parallel with the number of reticulocytes and also peaked by day 5. However, the fibronectin-adherent cells disappeared from the circulation more slowly than reticulocytes. Blood samples from animals at 7 to 9 days after bleeding contained as many as 30% cells that were slightly discocytic, large, and lightly stained with new methylene blue. Such cells were not counted as reticulocytes but they were invariably recovered in the attached fraction, thus contributing to the apparently rapid disappearance of reticulocytes.

On day 5 the fibronectin-attached cells resembled large flat spheres with granular cytoplasm. Staining the attached and the unattached populations of cells revealed that 80–90% of the attached cells were reticulocytes (data not shown). As

‡Rabbit reticulocytes produced by phenylhydrazine treatment or by repeated bleeding are larger than those in normal blood. To make this distinction, such reticulocytes are often called "stress reticulocytes" (13).

Table 2. Erythrocytes from splenectomized mice and an anemic rabbit attach specifically to fibronectin

Donor	% of total attachment of cells to dishes coated with				
	Albumin	Fibronectin	Laminin	Collagen Type I	Collagen Type IV
Untreated mice	2	3	3	3	4
Sham-operated mice	1	1	3	4	2
Splenectomized mice	3	31	2	5	3
Untreated rabbit	2	3	2	2	ND
Anemic rabbit*	2	52	4	2	ND

Falcon Petri dishes (35-mm diameter) were coated with 2 ml of bovine serum albumin at 10 mg/ml, fibronectin at 6 μg/ml, laminin at 10 μg/ml, type I collagen at 100 μg/ml, or type IV collagen at 50 μg/ml as described elsewhere (4). Erythrocytes from sham-operated and splenectomized mice were tested 25 days after the operation. ND, not done.

*Cells obtained from anemic rabbit on day [illegible] of the experiment shown in Fig. 2.

is the case for undifferentiated MEL cells (4), reticulocytes attached specifically to fibronectin but not to dishes coated with bovine serum albumin, laminin, or collagen (Table 2). None of these protein substrates promoted attachment of mature erythrocytes obtained from the control rabbit (Table 2). Thus, reticulocytes in anemic rabbits with a functional spleen do attach tightly and specifically to fibronectin. Maturation of reticulocytes to erythrocytes was correlated with the loss of adhesion to fibronectin.

DISCUSSION

We have described three situations in which a large fraction of the circulating red blood cells bind tightly and specifically to fibronectin: (*i*) rabbits made anemic by repeated bleeding, (*ii*) hemolytic anemia patients with functional asplenia and splenectomized normal humans, and (*iii*) splenectomized mice. It is unlikely that adhesion of reticulocytes to fibronec-

Table 3. Effect of splenectomy on adhesion of newly released (young) erythrocytes to fibronectin

Exp.	Mice	% cell adhesion	Acid-precipitable [^{35}S]methionine: Total cpm	Acid-precipitable [^{35}S]methionine: % in attached cells
1	Untreated	1	3,[illegible]98	2
	Sham-operated	2	[illegible]426	1
	Splenectomized	10	[illegible],498	51
	Splenectomized	11	[illegible],249	50
2	Untreated	2	3,565	3
	Sham-operated	2	8,380	2
	Splenectomized	9	14,748	51
	Splenectomized	10	5,409	44

Unoperated, sham-operated, and splenectomized mice were injected subcutaneously with 2.5 mCi of [^{35}S]methionine (1350 mCi/mmol; Amersham) 3 days after surgery. The capacity of the circulating erythrocytes to attach to fibronectin was determined 7 days after surgery. The numbers of cells in the unattached and the attached fractions were determined by counting 0.2-ml suspensions in a Coulter Counter. Proteins in the remaining attached and unattached fractions were precipitated with ice-cold trichloroacetic acid in the presence of bovine serum albumin (150 μg/ml) and hydrogen peroxide (3%, vol/vol) added as carrier and bleaching agent, respectively. The precipitated labeled proteins were washed once in 10% trichloroacetic acid, dissolved in 1 M NaOH, and neutralized with HCl, and radioactivities were measured in 20 ml of Aquasol (New England Nuclear) in a liquid scintillation spectrophotometer. Data presented are means for two replicate determinations.

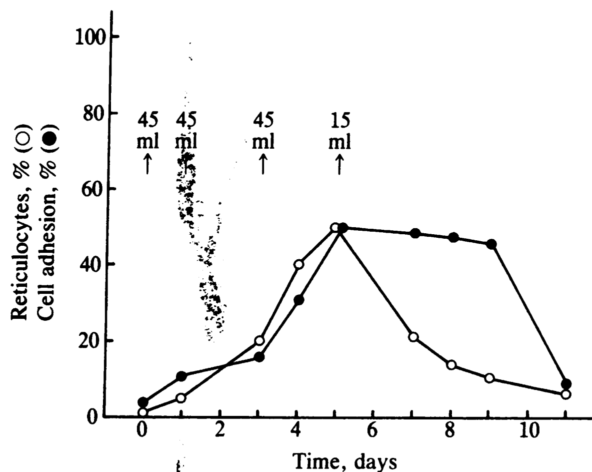

FIG. 2. Kinetics of appearance and loss of circulating reticulocytes and fibronectin-adhesive cells during induction of anemia and recovery in rabbits. Anemia was induced by withdrawing a total of 150 ml of blood in four installments as shown by the arrows. The proportion of reticulocytes (○) and the percentage of cells capable of attaching to fibronectin (●) in the peripheral blood of rabbit are shown. Erythrocytes from a hematologically normal rabbit assayed in parallel exhibited less than 5% attachment to fibronectin (not shown). Each point represents the mean of duplicate determinations. The data presented here were obtained from a single rabbit, although the results showed little variance among the eight rabbits examined.

tin is due to nonspecifically adsorbed plasma proteins, such as fibrinogen or complement factor $C1_q$, since the cells were washed three or four times with Hanks' buffered salt solution containing 2 mM EDTA prior to the cell adhesion assay. As with undifferentiated MEL cells, attachment is specific for fibronectin; cells do not adhere to laminin or type I or type IV collagen, other extracellular matrix proteins (4).

The loss of fibronectin-adherent cells from the circulation during recovery of rabbits from anemia could occur by two different mechanisms: selective destruction of reticulocytes or surface remodeling of the reticulocyte plasma membrane during maturation. While both mechanisms may be operating to some extent, loss of adherent cells cannot be attributed solely to the destruction of reticulocytes; the blood hematocrit actually increased from 30% on day 4 to about 46% on day 11 after bleeding (data not shown). The stress reticulocyte has a larger membrane surface area and volume than the mature erythrocyte (14). Reticulocyte maturation involves a disproportionate loss of plasma membrane compared to cell volume (13, 15). Reticulocyte maturation also involves the loss of cell surface glycoproteins, such as the transferrin receptor (16–18). We suggest that maturation of reticulocytes to erythrocytes is accompanied by the loss or modification of the cell surface fibronectin-binding sites.

Our studies with hemolytic anemia patients suggest that both functional asplenia and erythropoietic stress (high blood reticulocyte count) may have contributed to the increased number of fibronectin-adhering erythrocytes in the circulation. However, we emphasize that we have examined only a few patients (Table 1). It will be important to study many patients with different types of hemolytic diseases to establish a significant correlation between fibronectin-adherent erythrocytes and hematologically significant variables, such as the duration of disease, degree of anemia, and state of the spleen. It will also be important to determine whether splenectomy of hematologically normal human subjects exerts only short-term effects on the concentration of adherent cells in the circulating blood, as we observed with splenectomized mice.

After splenectomy of the mouse there was no increase in the blood reticulocyte count; thus the animals appeared to be in an erythropoietically unstressed condition. However, a significant proportion of erythrocytes from the splenectomized mice, but not control mice, attached specifically to fibronectin (Table 2). However, the effect of splenectomy was incomplete, since only 75% of the circulating cells were adherent at the peak of the response at 6 weeks. Adherent cells were not detectable 8 weeks after splenectomy (Fig. 1). This response is consistent with the notion that the spleen is one of the sites where newly emergent reticulocytes complete their maturation into erythrocytes. Since erythrocyte turnover is not significantly affected by splenectomy, we calculated, on the basis of the average lifespan of the mouse erythrocyte of 44 days, that 7 days after splenectomy 11% of the circulating cells would have been made since the operation. This agrees with the 10% fibronectin adhesion observed 7 days after splenectomy (Fig. 1 and Table 3). If all of the newly released cells were capable of attaching to fibronectin, 100% of the newly emerged $[^{35}S]$methionine-labeled erythrocytes would be adherent. However, only about 50% of the radioactive cells attached (Table 3). Assuming that all reticulocytes, at some stage of their maturation, are adherent to fibronectin, this study suggests that about half of the cells lose their adherent components during passage through the spleen. The others lose their fibronectin-adhesive molecules either in the bone marrow before release or in some extrasplenic site. Since 8 weeks after splenectomy no circulating cells adhere to fibronectin, some other organ takes over the function of the spleen in removing these molecules from reticulocytes and, presumably, in other aspects of remodeling of the reticulocyte membrane.

Thus, in erythropoietically unstressed mice reticulocytes capable of attaching to fibronectin are released from the bone marrow and lose this ability as they mature into erythrocytes. The spleen appears to be a significant mediator of this process, but it is not the only site where reticulocytes can complete their maturation process. It is significant that in both splenectomized humans (19) and rats (3, 13, 15) there is an increase in the phospholipid and cholesterol content of mature erythrocytes. Erythrocyte membranes from splenectomized humans also contain a high molecular weight protein aggregate not found normally (20). Moreover, splenectomy in rats decreases the rate and extent of membrane lipid loss during reticulocyte maturation, resulting in mature erythrocytes rich in lipid (3, 15).

The physiological significance of the reticulocyte–fibronectin interaction described here remains to be established. The mechanisms involved in the release of reticulocytes from the interstitial matrix of the bone marrow are complex. We have suggested that the loss or modification of the cell surface fibronectin binding sites may be involved in the release of reticulocytes from the bone marrow (4). Loss of fibronectin-adhesive molecules from the cell surface of circulating reticulocytes may occur by an endocytotic mechanism, since spectrin-free surface invaginations and intracellular vesicles have been observed in reticulocytes within normal rabbit spleen (21). Fibronectin or fibronectin-adhesive molecules on the surface of reticulocytes may also serve as an attachment site for splenic macrophages that participate in remodeling of the surface membrane, because macrophages are known to adhere and ingest fibronectin-coated particles (22–24). Interactions of cells with extracellular matrices are essential for many developmental processes (25, 26), and it will be important to determine the specificity of interactions among erythroid precursor cells, extracellular matrix proteins, and macrophages. As judged by iodination with extracellular lactoperoxidase and by selective detergent extraction of membranes, rabbit reticulocytes contain three major integral membrane proteins absent from

erythrocytes. It will be of interest to determine whether one or more of these is the cell surface fibronectin-binding sites.

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