Vol. 180, No. 1, 1991
October 15, 1991

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 23-32

FORMALDEHYDE TREATED ALBUMIN CONTAINS MONOMERIC AND POLYMERIC FORMS THAT ARE DIFFERENTLY CLEARED BY ENDOTHELIAL AND KUPFFER CELLS OF THE LIVER: EVIDENCE FOR SCAVENGER RECEPTOR HETEROGENEITY

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Received June 13, 1991

SUMMARY: Formaldehyde treated albumin (F-HSA) was found to consist of a monomeric and a polymeric fraction. Both fractions were primarily endocytosed by rat liver sinusoidal cells. However, immunohistochemical staining of endocytosed material showed that the relative contribution of the endothelial and Kupffer cells in uptake of the monomer and the polymer differed significantly, with the monomer mainly having an endothelial cell- and the polymer predominantly having a Kupffer cell pattern of distribution. To directly confirm these heterogeneous patterns, we injected in vivo the ^{125}I-labeled F-HSA fractions and isolated the endothelial and Kupffer cells by centrifugal elutriation. 73.7% of the monomeric F-HSA was found in endothelial cells and only 14.9% was found in Kupffer cells. In contrast, the polymeric F-HSA (1500 kD) was mainly endocytosed by Kupffer cells (71%), whereas the endothelial cells contributed only for 24% in hepatic uptake. In vivo studies and isolated perfused rat liver experiments showed that endocytosis of both monomer and polymer was inhibited by co-administration of polyinosinic acid, a well known inhibitor for scavenger receptors, indicating that these receptors on endothelial and Kupffer cells are mainly involved in this uptake process.

Albumin is subject to a constant turnover, it is synthesized by the hepatocytes at the same rate as it is removed from the circulation and degraded (1). A potential mechanism for

ABBREVIATIONS
HSA, Human Serum Albumin; F-HSA, formaldehyde denatured HSA; FPLC, Fast Protein Liquid Chromatography.

0006-291X/91 $1.50

the removal of senescent albumin is a high affinity receptor on macrophages, recognizing plasma proteins that have been modified by long term exposure to glucose (2). In this respect it was found that about 10% of the circulating albumin is glucosylated (3), and upon further aging forms advanced glycosylation end products (AGE). Denatured proteins and chemically modified lipoproteins are rapidly cleared from the circulation by a scavenger system on liver non-parenchymal cells (4,5). It has now been well established that acetyl LDL is endocytosed by a scavenger receptor on endothelial cells in the liver (6-8). This receptor is supposed to be distinct from the scavenger system recognizing F-HSA (9). Although the F-HSA receptor was characterized (10), and isolated from sinusoidal cells (11), conflicting data have been reported regarding the liver cell type responsible for uptake of F-HSA. Blomhof et al. (12) and Eskild et al. (13) provided evidence that F-HSA is mainly taken up by endothelial cells, whereas Buys et al. (14) presented data suggesting a specific Kupffer cell uptake. Yokota et al. (15) described uptake in both endothelial and Kupffer cells.

In this study we show that F-HSA prepared according to the general applied procedures (16), consists of a polymeric and a monomeric fraction. We purified both fractions and show, both by cell separation techniques and immunohistochemistry, that the monomer is predominantly endocytosed by endothelial cells whereas the polymer is mainly endocytosed by Kupffer cells. The fact that denaturation of albumin with formaldehyde results in a mixture of monomers and polymers, may very well explain the reported conflicting literature data concerning the cellular distribution of F-HSA. The results also indicate that the scavenger systems on endothelial and Kupffer cells display different substrate specificities towards the negati-

vely charged denatured proteins, in which the Kupffer cells prefer to endocytose the large size fractions.

MATERIALS AND METHODS

Preparation of F-HSA. Formaldehyde treatment was performed according to Mego et al. (16). Briefly, HSA (500 mg) was dissolved in 50 ml 0.2 M Na_2CO_3 pH 10.0, formaldehyde was added to a final concentration of 20% and this solution was stirred for 72 hours in the dark at room temperature. The solution was filtered over a 0.2 µm filter to remove insoluble material, purified on a Sephadex G25 column, washed with distilled water on a PM10 membrane in an Amicon Stirred Cell Concentrator and finally lyophilized. F-HSA was labeled with ^{125}I to a specific activity of 2.5 µCi per µg by using a chloramine-T method (22). Immediately prior to experiments, labeled F-HSA was chromatographed on PD-10 columns, and radioactivity recovered in the void volume was >98% precipitable with trichloroacetic acid (TCA) at a final concentration of 20%.

FPLC characterization of F-HSA.
Molecular weight: We injected 100 µl (1 mg/ml) F-HSA into the FPLC system (Pharmacia) equiped with a Superose-12 column (Pharmacia). Elution was performed with PBS pH 7.4 at a flow rate of 0.5 ml/min.
Charge: The relative net negative charge of the F-HSA fractions was determined on the FPLC system equipped with a Mono-Q anion exchange column (Pharmacia). Buffer A was a Tris HCl buffer (0.02M) pH 7.4 and buffer B was buffer A plus 1M NaCl. Elution was performed at a flow rate of 0.25 ml/min with a gradient from 100% A to 100% B in 30 minutes. Samples were dissolved to 1 mg/ml in buffer A and 100 µl was injected into the FPLC system.

Isolated perfused rat liver experiments. Experiments with isolated perfused livers were performed as previously described (21). Briefly, rats were anesthetized with pentobarbital (Nembutal® 60mg per kg i.p.). The bile duct, the portal vein and the superior vena cava were cannulated. The liver was excised and placed in the perfusion apparatus. Temperature was kept at 37°C, perfusate flow was maintained at 35 ml/min at a hydrostatic pressure of 10-12 cm. The recirculating perfusion medium (100 ml) consisted of a Krebs-bicarbonate buffer supplemented with 0.1% glucose and 1% BSA and was constantly gassed with 95% oxygen and 5% carbon dioxide. To replace bile salts, an infusion of sodium taurocholate was given (15 µmol/-hr). After a stabilization period of 20 min, 100 ng ^{125}I-labeled F-HSA was injected in the mixing chamber and at the indicated times, perfusate samples of 300 µl were taken and mixed with 300 µl icecold TCA 20% and centrifuged at 2500 rpm for 10 minutes. The pellet was washed with 600 µl TCA 20%. The radioactivity of the combined supernatants and the pellet was counted in a LKB-Multichannel γ counter. In the competition experiments, 5 mg polyinosinic acid was given 5 minutes prior to addition of ^{125}I-labeled F-HSA and again at t=20 min.

In vivo serum clearance and liver association. Rats were anesthetized by intraperitoneal injection of 20 mg Nembutal®. The abdomen was opened, and radiolabeled compounds were injected into the inferior vena cava at the level of the renal veins. The body temperature was maintained at 37°C by an

infrared heating lamp. At the indicated times, 0.2 ml of blood was taken from the inferior vena cava at least 2 cm distal of the injection point. The samples were centrifuged for 2 min at 20,000 g, and the radioactivity in the supernatants was counted. Liver lobules were tied off and excised at the indicated times. After weighing the lobule and counting its radioactivity, the total liver uptake was calculated using the assumption that 3.75% of the total body weight is contributed by the liver. The amount of liver that was tied off was 2-3% at each time point, so that at the longest circulation time still less than 15% of the total liver weight was removed.

Immunohistochemical staining of endocytosed material. 1 mg of the particular F-HSA fraction was injected in the vena penis dorsalis and after 10 minutes the animals were killed. Pieces of liver were immediately frozen in isopentane (-80°C) and stored at -80°C until sectioning. Sections of 4 μm were cut in a cryostat (-20°C). Simultaneously staining of the endocytosed material and Kupffer cells was performed as described previously (17) Briefly: sections were fixed in acetone for 10 min, incubated with a Rabbit anti-Human Serum Albumin antibody (1:250 diluted in a buffer containing 0.02M Tris, 0.15M NaCL, 0.5M $CaCL_2$ pH 7.8) for 30 min and subsequently the sections were incubated with a peroxidase conjugated Swine anti-Rabbit antibody (1:20 diluted in the same buffer) for 15 min. The sections were then incubated with the monoclonal antibody ED2 (directed against Kupffer cells) for 30 min and next incubated with an alkaline phosphatase conjugated Rabbit anti-Mouse antibody for 15 min. Finally the alkaline phosphatase was visualized with naphthol AS-MX/Fast Blue BB reaction for 30 min and the peroxidase activity was visualized with the amino-ethyl-carbazole (AEC) reaction for 10 min.

Isolation of endothelial and Kupffer cells by centrifugal elutriation. 100 ng of the particular ^{125}I labeled F-HSA was injected in the tail vein of the 3 months old male Wistar rats. After 10 minutes the endothelial cells (EC), the Kupffer cells (KC) and the parenchymal cells (PC) were isolated according to Nagelkerke et al. (7), with the modification that parenchymal cells were initially removed by centrifugation (30", 50 g). The viability of the cells was greater than 95% as determined by their ability to exclude trypan blue. Cell purity was checked by peroxidase staining with diamino benzidine (DAB).

RESULTS AND CONCLUSIONS

FPLC analysis of F-HSA (Figure 1) using a Mono Q anion exchange column showed a 1.9 ml increase in elution volume, compared with the parent albumin, indicating that the net negative charge has considerably increased. This enhanced negative charge is most likely due to formation of hydroxymethyl adducts and Schiff bases and consequently removal of the positive charge on the ϵ-NH_2 groups of lysine. Reaction of F-HSA with trinitrobenzene sulphonic acid according to Habeeb (18) indicated that 90% of the free ϵ-NH_2 groups in the albumin molecule were derivatized.

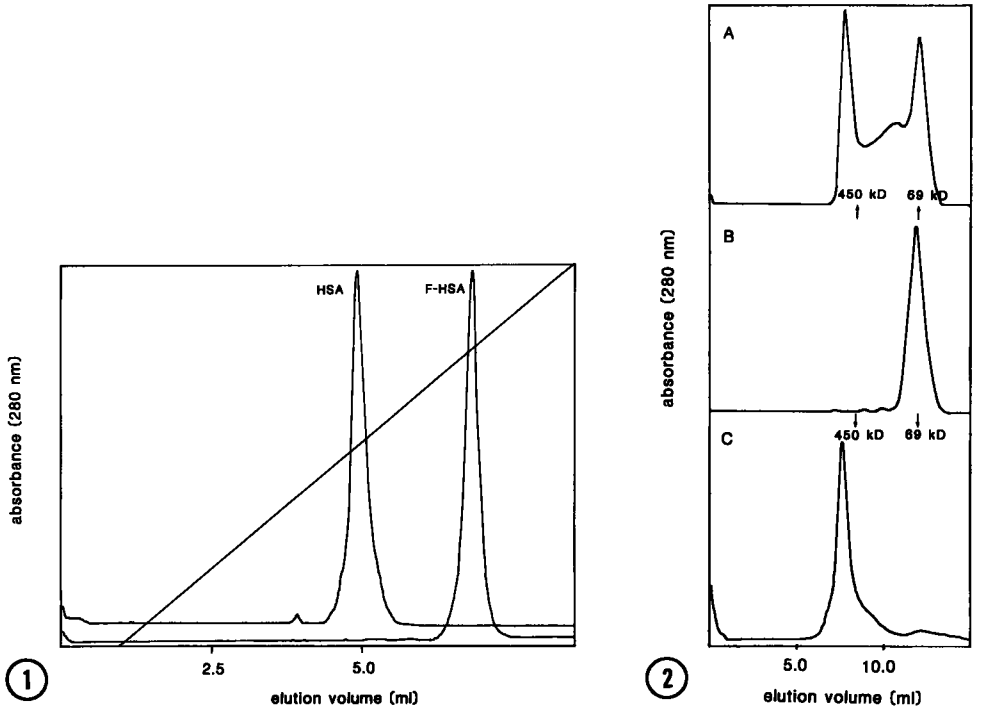
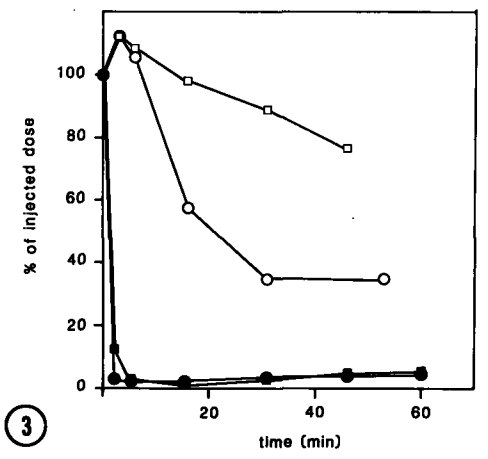


Figure 1. FPLC characteristics using a Mono-Q anion exchange column. The elution volume is related to the net negative charge.

Figure 2. FPLC characteristics using a Superose-12 column, of total F-HSA (A), monomeric (B) and polymeric 750 Kd F-HSA (C). The elution volumes of the 750 Kd and 1500 Kd (not shown) polymer are 7.34 and 6.18 ml respectively.

Figure 2A shows that F-HSA consisted of a monomeric (70 kD) and a polymeric fraction (750 kD). This is in agreement with the results of Buys *et al*. (19). Polymers are probably formed due to the formation of methylene bridges between protein molecules (20). The length of the polymer was not the same in each batch of F-HSA synthesized, and varied from 750 kD to about 1500 kD. Monomer and polymers showed the same chromatographic behaviour on the Mono Q anion exchange column, indicating that their negative charge density does not differ significantly.

After purification of monomers and polymers (Figure 2B, 2C), we compared their hepatic disposition. The in vivo plasma disappearance curves (Fig. 3) combined with the in vivo liver uptake curves (Fig. 4) and the perfusate dissapearance profile in the isolated perfused rat livers (Fig. 5) showed that both monomeric- and polymeric F-HSA are rapidly removed from the



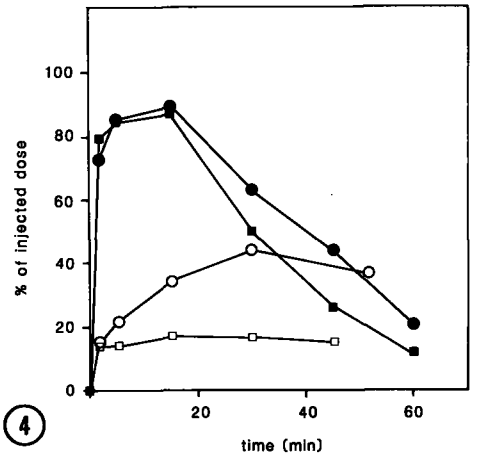


Figure 3. *In vivo* plasma disappearance curves of ^{125}I-labeled monomeric F-HSA (squares) and polymeric F-HSA (circles). Closed symbols represent controls without competitor and open symbols denote the plasma disappearance after pre-injection (1 min prior to the radiolabeled ligand) of 5 mg polyinosinic acid. Points are the mean of two separate experiments.

Figure 4. *In vivo* liver uptake curves of ^{125}I-labeled monomeric F-HSA (squares) and polymeric F-HSA (circles). Closed symbols represent controls without competitor and open symbols denote the liver uptake after pre-injection (1 min prior to the radiolabeled ligand) of 5 mg polyinosinic acid. Points are the mean of two separate experiments.

circulation by the liver. Competition experiments with the scavenger receptor competitor polyinosinic acid, clearly show that scavenger receptors are responsible for the uptake (Fig.

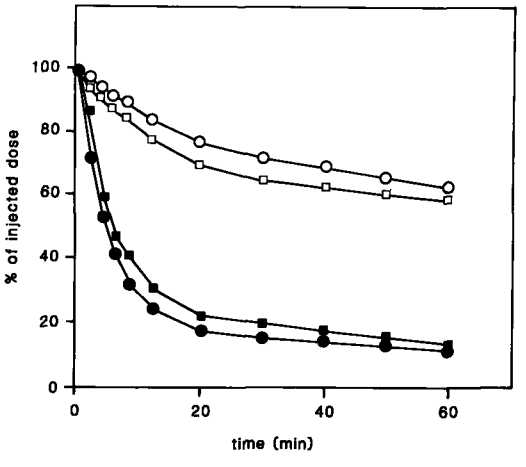


Figure 5. Isolated perfused rat liver perfusate disappearance curves of ^{125}I-labeled monomeric F-HSA (squares) and polymeric F-HSA (circles). Closed symbols represent controls without competitor and open symbols denote the perfusate disappearance after preadministration of 5 mg polyinosinic acid and again at 20 min. Points are the mean of two separate experiments.

3, 4 and 5). Uptake of the F-HSA fractions in rat livers after i.v. injection is visualized by immuno-histochemistry as shown in figure 6. It can be seen that the endothelial cells are mainly involved in uptake of the monomeric form, and Kupffer cells are predominantly responsible for uptake of polymeric F-HSA. Since monomeric and polymeric F-HSA exhibited the same plasma disappearance profile and were taken up in the isolated perfused rat liver and in the liver in vivo at the same rate (Fig. 3, 4 and 5), the higher staining intensity seen in figure 6 B for the polymeric F-HSA as compared with figure 6 A, is probably caused by a greater reactivity of the polymer with the polyclonal antibody against HSA.

The idea that the molecular weight of F-HSA is a crucial factor for uptake via scavenger receptors on the endothelial cells and the Kupffer cells, was strongly supported by the data obtained from the cell isolation experiments (Table 1). The endothelial cells contributed for at least 73% in the total hepatic uptake of the monomeric form of F-HSA. Whereas the Kupffer cells contributed only for 14%. In contrast, polymeric F-HSA (1500 kD) was predominantly endocytosed in Kupffer cells (71%), and to a much lesser extent in endothelial cells (24%). The smaller polymer of F-HSA (750 kD) resulted in an intermediate cellular distribution pattern (58% in Kupffer cells and 42% in endothelial cells).

The apparent K_s of removal of the different F-HSA fractions was determined through kinetic analysis of the nonlinear decay curves in the isolated perfused liver experiments. For the monomer and polymer (1500 kD) we calculated a K_s of $7.5 * 10^{-8}$ M and $4.4 * 10^{-9}$ M respectively, indicating that the affinity of the F-HSA monomer and polymer for their receptors differs.

In the past, several authors have reported a rapid uptake of formaldehyde denatured albumin via scavenger receptors in rat livers. There was no consenses however, whether the supposed receptor for F-HSA resides on the endothelial cells, the Kupffer cells, or on both of these major sinusoidal cell types. The results presented in this study show that both endothelial and Kupffer cells recognise and endocytose F-HSA, indicating that both cell types may express some kind of scavenger receptor. The monomeric form of F-HSA was preferably endocytosed by endothelial and the polymeric form of F-HSA was predominantly endocytosed by Kupffer cells. Recently, the existance of a scavenger receptor system on Kupffer cells,

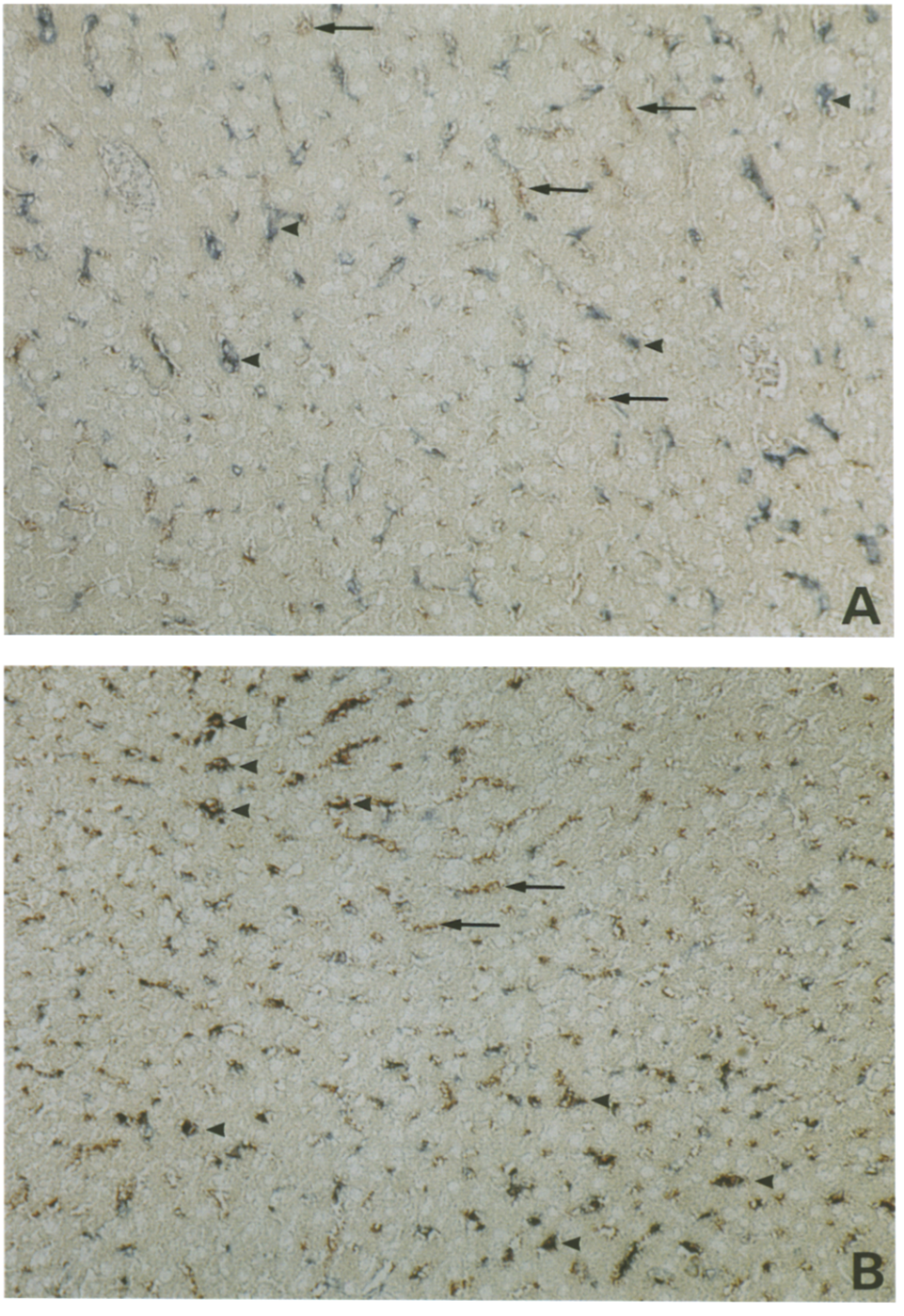


Figure 6. A. Cryostat sections of rat liver 10 min after i.v. injection of monomeric (A) and polymeric F-HSA (B). Endocytosed material is stained red/brown (arrows) and simultaneously, Kupffer cells are stained blue (arrow-heads). In panel A, the endocytosed material is separated from the Kupffer cells, indicating endothelial cell uptake. In panel B, most of the endocytosed material is associated with the blue spots, indicating Kupffer cell uptake. Magnification: 140.

TABLE 1. Uptake of ^{125}I-labeled F-HSA fractions in endothelial cells (EC), Kupffer cells (KC) and parenchymal cells (PC)

	EC	KC	PC
Monomer	74.9	14.5	10.6
Polymer (750 kD)	53.4	42.8	3.8
Polymer (1500 kD)	24.4	71.0	4.6

Values represent the percentage of total liver uptake and are the mean of two (polymers), and three (monomer) separate experiments.

distinct from the scavenger receptor on endothelial cells, has been described by Jansen et al. (23) and Van Berkel et al. (24).

The monomer to polymer ratio and the length of the polymer of different batches of F-HSA may vary significantly, probably due to differences in reaction conditions. If, in the previously reported studies, very different mixtures of polymeric and monomeric F-HSA were used, the reported dissimilar hepatic distribution can be explained. Our results indicate that Kupffer cells are better equipped to dispose relative large negatively charged molecules. The nature or the clustering of the particular type of scavenger receptor may in that respect differ from that on endothelial cells. This is in line with the general picture that the liver macrophage preferably recognizes and removes particulate material or molecules with a large size.

ACKNOWLEDGMENT

This study was supported by a grant from the Dutch Organisation for Scientific Research (NWO).

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