Cytokine modulation by PX differently affects specific acute phase proteins during sepsis in rats

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Voisin, Laure, Denis Breuillé, Benoît Ruot, Cécile Rallière, Fabienne Rambourdin, Michel Dalle, and Christiane Obléd. Cytokine modulation by PX differently affects specific acute phase proteins during sepsis in rats. Am. J. Physiol. 275 (Regulatory Integrative Comp. Physiol. 44): R1412–R1419, 1998.—To explore the regulation of the acute phase response in vivo, the effects of pentoxifylline (PX) treatment (100 mg/kg ip 1 h before infection) were investigated in infected and pair-fed rats 2 and 6 days after an intravenous injection of live bacteria (Escherichia coli). PX treatment prevented the increase in plasma tumor necrosis factor (TNF-α) (peak 1.5 h after the infection) and resulted in an 84 and 61% inhibition of plasma interleukin (IL)-1β and IL-6, respectively (peaks at 3 h). Plasma corticosterone kinetics were not modified by the treatment. Infection increased α1-acid glycoprotein (AGP), α2-macroglobulin (A2M), and fibrinogen plasma concentrations and decreased albumin levels. PX significantly reduced AGP plasma concentration as early as day 2 in infected animals but reduced A2M and fibrinogen plasma levels only at day 6. The treatment had no effect on the albumin plasma concentration. Hepatic AGP and fibrinogen mRNA levels increased in infected rats, whereas those of A2M were unchanged and those of albumin were decreased. Two days after infection, AGP and fibrinogen mRNA levels were reduced in treated infected animals. PX was ineffective in modifying those of A2M and albumin. These data demonstrate, in vivo, that different acute phase proteins are individually regulated in sepsis. The in vivo effects of PX treatment support the hypothesis that TNF-α plays an important role in the regulation of AGP production, whereas other factors seem to be involved in the regulation of A2M, fibrinogen, and albumin expression.

Tumor necrosis factor; interleukin-1; interleukin-6

Among other metabolic disturbances, sepsis causes a marked loss of weight and body proteins, muscle wasting, and a hepatic acute phase response. Total liver protein synthesis was markedly enhanced (11, 44), mainly due to an increased synthesis of exported proteins (acute phase proteins; see Ref. 44). Thus the liver response results in a concomitant increase in the circulating levels of these proteins. However, the levels of some plasma proteins such as albumin, named negative acute phase proteins, decrease (33, 42).

Cytokines, and especially tumor necrosis factor (TNF)–α (43) and interleukins-1 (IL-1; see Ref. 35) and 6 (IL-6; see Ref. 6), are considered to play key roles in the pathogenesis of sepsis. With respect to liver, administration of cytokines to healthy animals can reproduce the stimulation of protein synthesis (5, 13). The roles of cytokines in inducing individual acute phase protein changes have been studied extensively in various in vitro systems as hepatocyte primary cultures or hepato-cell lines. Based on these studies, IL-6 appears to be the main cytokine regulating the expression of the majority of the acute phase protein genes, whereas IL-1β and TNF-α regulate a different set of genes (17, 29, 33). Maximal expression of several acute phase protein genes is dependent on the presence of glucocorticoids (29). However, for a particular protein, different responses can be obtained in various cell systems (29), and the regulatory processes involved in the in vivo acute phase response might be much more complex. Some studies have been reported in which the role of IL-1 (28, 37) and IL-6 (20, 31) on the acute phase induction was studied in vivo. However, a major difficulty in defining the roles of various cytokines in vivo is their ability to induce each other (35, 43). Another approach, poorly documented in sepsis, consists of inhibiting the production or action of individual cytokines (21, 32, 42).

TNF-α is the first cytokine to appear in the circulation after administration of endotoxin or living bacteria in various species (43). Thus TNF-α is thought to be a proximal mediator of the inflammatory response and most likely triggers the release of other secondary mediators, including other cytokines. Pentoxifylline (PX), a methylxanthine derivative, has been demonstrated both in vitro and in vivo to suppress lipopolysaccharide (LPS)-induced TNF-α secretion (12, 30, 39, 41). PX may also modulate other cytokines, but this effect is more controversial (30, 39, 41), and its effect on glucocorticoid level is unknown. Thus, in this study, we explored in vivo the effect of PX treatment on TNF-α, IL-1, IL-6, and corticosterone levels in a rat model of gram-negative sepsis. Furthermore, we examined whether inhibition of TNF-α production could modulate the expression and plasma appearance of individual acute phase proteins during the acute septic phase (2 days postinfection) and the chronic septic phase (6 days postinfection).

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (Iffa Credo, Saint-Germain sur l’Arbresle, France) were individually housed in...
production in plasma from 1 to 24 h after the infection showed assays measured by using ELISA kits, according to the manufacturer.

Thus blood samples were collected in a lateral tail vein 1.5 h for IL-1β and IL-6 after bacteria administration (Fig. 1).

Acute phase protein concentration. Fibrinogen, α1-acid glycoprotein (AGP), α2-macroglobulin (A2M), and albumin plasma levels were measured by single radial diffusion, using anti-rat fibrinogen and albumin from Cappel and anti-rat AGP and A2M produced in the laboratory.

Northern blot analysis. Total RNA was extracted from 0.2 g of liver by the method of Chomczynsky and Sacchi (14). Twenty micrograms of RNA were electrophoresed in formaldehyde agarose gels (1%) and transferred electrophoretically to nylon membranes (GeneScreen; NEN Research Products, Boston, MA). RNA was covalently bound to the membrane by ultraviolet cross-linking. Membranes were hybridized with cDNA probes AGP (36), A2M (no. 63099; American Type Culture Collection, Rockville, MD; see Ref. 19), α1-fibrinogen (7), and albumin (25). Hybridizations were conducted overnight at 65°C with [32P]cDNA fragments labeled by random priming. After washing at the same temperature, filters were autoradiographed at −80°C with intensifying screens on Hyperfilm-MP (Amersham). After stripping of the different probes, the filters were reprobed with a mouse 18S ribosomal probe (no. 63178; American Type Culture Collection). Autoradiographic signals were quantified by digital image processing and analysis (NIH Image 1.54) and normalized using the corresponding 18S rRNA signals to correct for uneven loading.

Statistics. All data are expressed as means ± SE. The significance of differences was analyzed by one-way ANOVA and by Student’s t-test where appropriate. Differences among means were considered significant at P < 0.05.

RESULTS

Food intake, body weight, and muscle weight. Results presented in Figs. 2 and 3 show values obtained on rats studied for 6 days after the infection. Similar results were observed during the acute period in the group killed at day 2. Infection decreased food intake, especially during the acute phase period since rats ate only 5–15% of the preinfection intake (20–25 g). Thereafter, food intake of infected animals gradually increased to reach 75% of preinfection food consumption at the end of the study. Pretreatment of animals with PX before infection reduced anorexia, mainly at days 2 to 4 after infection compared with untreated rats (Fig. 2). On day 2 postinfection, the decrease of body weight observed in infected rats (INF) was significantly higher (27.5%) than in pair-fed animals (PF; Fig. 3), and the difference between these two groups strongly increased until 6 days after infection. By contrast, the body weight loss of
and abolished the atrophy of soleus muscle. The atrophy of gastrocnemius (12% vs. respective pair-fed rats) (Table 1). By contrast, PX treatment reduced the atrophy of INF rats was more severe (30 and 17%, respectively; Table 1). Infection significantly increased hepatic mRNA concentrations for AGP (19- and 8-fold, at days 2 and 6, respectively; Fig. 5) and fibrinogen (57 and 37% at days 2 and 6, respectively; Fig. 6). A2M mRNA levels increased significantly only on day 6 postinfection but more moderately than those of AGP (55% Fig. 5). By contrast, albumin mRNA levels were decreased by infection (50 and 57% at days 2 and 6, respectively; Fig. 6).

Administration of PX significantly decreased by 29% the rise in plasma AGP concentration in INF rats as early as 2 days after bacteria injection but did not significantly affect the increase in A2M and fibrinogen levels (Table 3). However, 6 days postinfection, the concentrations of these three positive acute phase proteins were significantly decreased in PX-INF rats compared with INF rats (65, 50, and 21%). No modification of the decrease of albumin concentration was observed with PX administration (Table 3). PX treatment reduced the increase in AGP and fibrinogen mRNA levels in PX-INF rats compared with nontreated infected rats 2 days after infection (Figs. 5 and 6), although they were not significantly different in PX-INF and nontreated infected rats on day 6 postinfection (Fig. 6).

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**DISCUSSION**

PX has been shown to increase animal survival in lethal models of infection (38) and to block LPS fever (30). The beneficial effects of PX in sepsis have been attributed to its ability to inhibit the production of TNF-α in vitro (39, 41) and in vivo (12, 30, 38). TNF-α is the first cytokine to appear in the plasma after endotoxemia or infection, and it induces the synthesis and release of other mediators, such as IL-1 and IL-6, generating a cytokine cascade (43). The neutralization of endogenous TNF-α with anti-TNF-α antibodies greatly diminishes the increases in IL-1β and IL-6-like bioactivity and glucocorticoid plasma levels. Administration of PX 1 h before infection suppressed the rise of the plasma TNF-α level (by 99%; Table 2). PX treatment induced a reduction of 84 and 61% of plasma IL-1β- and IL-6-like bioactivity concentrations, respectively, 3 h after infection (Table 2). In INF rats, plasma corticosterone levels were significantly higher 4 h after bacteria injection and were similar to normal values 24 and 48 h after the infectious stress (Fig. 4). PX treatment did not prevent the sepsis-induced increase in plasma corticosterone levels (Fig. 4).

Acute phase proteins. A2M, AGP, and fibrinogen concentrations were significantly increased 2 days after the infection (29-, 59-, and 2.3-fold, respectively) and 6 days after the infection (18-, 14-, and 2-fold, respectively) in INF rats compared with PF controls (Table 3). By contrast, albumin concentration was significantly reduced 2 and 6 days postinfection (45 and 54%, respectively). Infection significantly increased hepatic mRNA concentrations for AGP (19- and 8-fold, at days 2 and 6, respectively; Fig. 5) and fibrinogen (57 and 37% at days 2 and 6, respectively; Fig. 6). A2M mRNA levels increased significantly only on day 6 postinfection but more moderately than those of AGP (55% Fig. 5). By contrast, albumin mRNA levels were decreased by infection (50 and 57% at days 2 and 6, respectively; Fig. 6).

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IL-6-like bioactivity (measured 3 h after infection) may be the result of remaining IL-1β effect of PX treatment on corticosterone plasma level of the hypothalamic-pituitary axis (29). The lack of corticosterone (21). Nevertheless, TNF-α antagonist (ra) did not affect the increase in circulating of turpentine-injected mice with anti-IL-1 receptor presence of other factors sufficient to induce a maximal response to LPS (22, 26), after cecum ligature and puncture in rats (23), or after bacteria injection as shown in our study. A novel result after cecum ligature and puncture in rats (23), or after administration of IL-1β, IL-6, and glucocorticoids (17, 29, 33). Two types of acute phase genes with different levels, including various steps in the transcriptional and translational events. The analysis of mRNA levels and the measurement of transcription activity have provided evidence that transcriptional mechanisms play a central role in the regulation of expression of many acute phase proteins (9, 31, 36). Our results are in agreement with this view for AGP and fibrinogen, since mRNA levels were increased 2 and 6 days after the infection. By contrast, we found no coordinated changes between mRNA levels and plasma concentration of A2M, suggesting that the genes of these three proteins may be partially regulated by different mechanisms during inflammation. However, the increase in A2M mRNA level could be achieved before 48 h (19). Moreover, hepatic mRNA levels for A2M and AGP were found to increase to a maximum 24 h after LPS injection, with normal levels at 48 h for A2M but 10-fold over control values for AGP (42). Regulation of A2M may also occur on posttranscriptional levels, since Andus et al. (4) demonstrated that IL-6 markedly accelerated the secretion of A2M in hepatocyte primary cultures. For albumin, hepatic mRNA level and plasma concentration were diminished by 10.2±0.3% on May 20, 2017 http://ajpregu.physiology.org/ Downloaded from by 10.220.33.3 on May 20, 2017

Table 1. Effect of PX treatment on muscle weights 2 and 6 days postinfection

<table>
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<th>Days Postinfection</th>
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<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>PF INF PX-PF PX-INF</td>
</tr>
<tr>
<td>Gastrocnemius, g</td>
<td>1.69 ± 0.02 1.44 ± 0.02* 1.81 ± 0.03† 1.56 ± 0.03†</td>
</tr>
<tr>
<td>Soleus, mg</td>
<td>125 ± 3 109 ± 2* 128 ± 6 112 ± 4*</td>
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Values are means ± SE for 5 or 6 animals. INF, infected rats; PF, pair-fed control of INF rats; PX-INF, infected rats treated with pentoxifylline (PX); PX-PF pair-fed control treated with PX of PX-INF. * P < 0.05, one-way ANOVA vs. respective pair-fed controls. † P < 0.05, one-way ANOVA vs. respective infected animals.

remaining IL-1β can be sufficient to produce a large increase of IL-6 plasma level in PX-treated animals.

Plasma corticosterone levels were increased soon after administration of IL-1β (47) and LPS (22, 26), after cecum ligation and puncture in rats (23), or after infection as shown in our study. A novel result of this study is the inability of PX administration to modify glucocorticoid levels. Proinflammatory cytokines, like TNF-α and IL-1β, were reported to increase glucocorticoid synthesis and release via the stimulation of the hypothalamic-pituitary axis (29). The lack of effect of PX treatment on corticosterone plasma level may be the result of remaining IL-1β and/or the presence of other factors sufficient to induce a maximum production of glucocorticoids, since pretreatment of turpentine-injected mice with anti-IL-1 receptor antagonist (ra) did not affect the increase in circulating corticosterone (21). Nevertheless, TNF-α did not appear to be the main mediator of corticosterone production in sepsis. Moreover, our results suggest that the mechanisms of PX action are independent of corticosterone levels or that corticosterone has only minor direct effects in sepsis.

PX administration reduced, but did not prevent, anorexia induced by infection, as previously observed in our laboratory (12) or by others using TNF-α monochlonal antibody pretreatment in endotoxemic rats (42). These data are in agreement with the view that TNF-α and IL-1β have anorexic effects (45). PX treatment suppressed body weight difference between INF rats and their PF control over the entire course of the study. Thus the effect of infection on body weight change completely disappeared in infected treated animals. Moreover, muscle atrophy linked to infection was significantly reduced by PX treatment and even abolished at day 6 in soleus muscle. This rapid recovery of muscle weight of treated animals is consistent with the inhibition of proteolysis (46, 48) reported previously with inhibitors of TNF-α secretion or action and IL-1ra.

The acute phase response associated with sepsis is accompanied by changes of the plasma concentration of a large number of proteins. Increased rates of incorporation of radiolabeled amino acids into proteins suggest, at least in part, that the increase of the plasma concentration of acute phase proteins during inflammation or sepsis is due to increased rates of their synthesis (40). The regulation of protein synthesis may occur at different levels, including various steps in the transcriptional and translational events. The analysis of mRNA levels and the measurement of transcription activity have provided evidence that transcriptional mechanisms play a central role in the regulation of expression of many acute phase proteins (9, 31, 36). Our results are in agreement with this view for AGP and fibrinogen, since mRNA levels were increased 2 and 6 days after the infection. By contrast, we found no coordinated changes between mRNA levels and plasma concentration of A2M, suggesting that the genes of these three proteins may be partially regulated by different mechanisms during inflammation. However, the increase in A2M mRNA level could be achieved before 48 h (19). Moreover, hepatic mRNA levels for A2M and AGP were found to increase to a maximum 24 h after LPS injection, with normal levels at 48 h for A2M but 10-fold over control values for AGP (42). Regulation of A2M may also occur on posttranscriptional levels, since Andus et al. (4) demonstrated that IL-6 markedly accelerated the secretion of A2M in hepatocyte primary cultures. For albumin, hepatic mRNA level and plasma concentration were diminished by ~50% 2 and also 6 days after infection. Such correlated decreases, suggesting that hypalbuminemia is due to a decreased synthesis of the protein, have been described 24–48 h after LPS and turpentine injections, but they were followed by recovery over the next 48–72 h (2, 42). These results underline the long-lasting perturbations associated with our model, as described previously (11).

Cytokines have been implicated in the induction of the acute phase response. Most studies devoted to the exploration of the role of cytokines and hormones in inducing acute phase proteins have been carried out in cell culture systems and have shown direct cytokine effects. Prominent stimulatory functions have been ascribed to TNF-α, IL-1β, IL-6, and glucocorticoids (17, 29, 33). Two types of acute phase genes with different
cytokine controls have been described as follows: those that respond to IL-1β and TNF-α such as AGP and those that respond to IL-6 such as A2M and fibrinogen for the rat (8, 29, 33). Moreover, to achieve maximally regulated expression of some proteins, a combination of cytokines and glucocorticoids is often required.

The qualitative pattern of the regulated positive acute phase proteins observed with PX treatment during the acute phase in our study was characteristic of that generally described in vitro. The first protein affected both at the transcriptional level and plasma concentration by the treatment was AGP, which is recognized to be regulated mainly by TNF-α and IL-1β (8, 29, 33). These results were consistent with the findings of Sharma et al. (42) using TNF-α monoclonal antibody treatment of endotoxemia in rats. Glucocorticoids alone are able to induce AGP (31), but there was no difference in the corticosterone level in treated and nontreated infected rats. Although AGP is minimally affected by IL-6 alone (31), some synergistic action with TNF-α and IL-1 can occur (8).

By contrast, A2M and fibrinogen plasma levels were not initially decreased by PX treatment, which did not modify A2M mRNA levels but slightly decreased those of fibrinogen. Because IL-6 secretion was not abolished by PX, these results are in agreement with numerous studies attributing a predominant role of IL-6 in inducing these two proteins (3, 31, 37). Moreover, in vitro data have shown that TNF-α and IL-1 did not alter either the expression or the synthesis of A2M (4). The regulation of fibrinogen gene expression seems more complex, since IL-1 inhibits its stimulation and this inhibitory effect is reversed by endogenous IL-1ra (37). Moreover, glucocorticoids are required to achieve a maximal IL-6 response for A2M but not for fibrinogen (31).

The decline in plasma albumin and mRNA levels appears not to be affected by PX treatment of septic rats, as shown with anti-TNF-α antibody treatment of endotoxemic rats (42). Anti-IL-1 receptor antibody administration before turpentine injection in mice failed to restore albumin plasma concentration (21). However, in vivo administration of TNF-α, IL-1β, or IL-6 is able to decrease albumin synthesis (5, 10, 20), and the combination of these cytokines, especially IL-1β and IL-6, resulted in an additive downregulation of albumin synthesis in vitro (4). On the other hand, glucocorticoids are known to increase albumin synthesis (24) and could partially antagonize the inhibitory effect of cytokines. Taken together, these data suggest that a small amount of any cytokine is enough to inhibit albumin synthesis and/or that unknown mediators or mechanisms play a predominant role in determining hypoalbuminemia.

During the chronic phase, the levels of AGP, A2M, and fibrinogen were significantly decreased in infected rats treated with PX compared with nontreated animals, indicating perhaps the rapid recovery of treated animals. No evidence of return to normal levels of albumin appeared at the end of the study, showing that albumin constitutes a poor index of outcome. However, mRNA levels tend to increase at the end of the experiment. The function of the decreased plasma level of albumin, and more generally of the negative acute phase proteins, is not yet clear and deserves further studies (2).

In summary, in a rat model of long-lasting sepsis, the administration of PX before infection inhibited circulating TNF-α, depressed plasma IL-1β and IL-6 levels, but had no effect on corticosterone levels. Moreover, PX treatment reduced anorexia and body weight loss, suppressed muscle protein wasting, and modulated the acute phase response. Our results suggest that glucocorticoids exert their action not directly, but mainly in

Table 3. Effect of PX treatment on α1-acid glycoprotein, α2-macroglobulin, fibrinogen, and albumin levels 2 and 6 days postinfection

<table>
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<th>Days Postinfection</th>
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<th>6</th>
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<tbody>
<tr>
<td></td>
<td>PF</td>
<td>INF</td>
</tr>
<tr>
<td>α1-Acid glycoprotein, µg/ml</td>
<td>10 ± 1</td>
<td>586 ± 69*</td>
</tr>
<tr>
<td>α2-Macroglobulin, µg/ml</td>
<td>40 ± 3</td>
<td>1,140 ± 131*</td>
</tr>
<tr>
<td>Fibrinogen, mg/ml</td>
<td>2.7 ± 0.2</td>
<td>6.3 ± 0.4*</td>
</tr>
<tr>
<td>Albumin, mg/ml</td>
<td>16.4 ± 0.5</td>
<td>9.1 ± 0.3*</td>
</tr>
</tbody>
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Values are means ± SE for 4–6 animals. *P < 0.05, one-way ANOVA vs. respective pair-fed controls. †P < 0.05, one-way ANOVA vs. respective infected animals.
combination with other mediators. Moreover, our results underline in vivo that regulation of acute phase protein expression can occur at different levels according to the protein (17, 33).

The effects of PX treatment of infected rats shown in this study support the hypothesis of an important role of TNF-α in the regulation of protein metabolism during sepsis. However, further experiments are needed to understand the link between the very early and transient TNF-α secretion and the long-lasting effects observed in muscle and liver protein metabolism. Despite the complexity of the cytokine and hormonal network, the present study demonstrates in vivo that individual mediators have specific effects on particular acute phase proteins, making AGP a better index of recovery after PX treatment than A2M. This emphasizes again that acute phase proteins do not always respond in unison in disease states. Clearly, the role of the various acute phase proteins and the regulation of the acute phase response have to be evaluated in detail in various diseases before acute phase proteins become a practical clinical diagnostic and prognostic tool.

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![Graph A](image)

![Graph B](image)

**Fig. 5.** Effect of PX treatment on mRNA levels for α1-acid glycoprotein (A) and α2-macroglobulin (B). RNA was extracted from liver of infected rats (INF) and their pair-fed controls (PF) and of infected rats treated with PX (PX-INF) and their pair-fed control treated with PX (PX-PF). Samples (20 µg) were electrophoresed, transferred to nylon membranes, and hybridized with [32P] cDNAs encoding α1-acid glycoprotein and α2-macroglobulin. After stripping of the probes, blots were rehybridized with an 18S ribosomal oligonucleotide. Data were corrected for 18S rRNA abundance to take into account variations in RNA loading. Values are means ± SE for 4 or 5 rats. All data were referred to pair-fed day 2 value, which was arbitrarily chosen to equal 1. Representative Northern blots are also shown. d2, 2 days postinfection; d6, 6 days postinfection. *P < 0.05 vs. respective PF animals. †P < 0.05 vs. respective animals without PX treatment.

![Graph A](image)

![Graph B](image)

**Fig. 6.** Effect of PX treatment on mRNA levels for fibrinogen (A) and albumin (B). RNA was extracted from liver of infected rats (INF) and their pair-fed controls (PF) and of infected rats treated with PX (PX-INF) and their pair-fed control treated with PX (PX-PF). Samples (20 µg) were electrophoresed, transferred to nylon membranes, and hybridized with [32P] cDNAs encoding fibrinogen and albumin. After stripping of the probes, blots were rehybridized with an 18S ribosomal oligonucleotide. Data were corrected for 18S rRNA abundance to take into account variations in RNA loading. Values are means ± SE for 4 or 5 rats. All data were referred to pair-fed day 2 value, which was arbitrarily chosen to equal 1. Representative Northern blots are also shown. *P < 0.05 vs. respective PF animals. †P < 0.05 vs. respective animals without PX treatment.
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