EFFECTS OF INTERLEUKIN-18 ANTIBODY ON IMMUNE LIVER INJURY IN MICE

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ABSTRACT

Introduction*: Anti-IL18 antibody was applied to inhibit IL-18, regulate the cytokine network, inhibit liver immune injury, and exert a protective effect against immune liver injury. The present study aimed to explore the immunological mechanism of the effect of anti-IL18 antibody on immune liver injury in mice, and appropriate dose of antibody intervention, in order to provide theoretical and animal experimental evidence for the treatment of immune liver injury.*

Materials and methods*: Bacillus Calmette-Guérin (BCG) and lipopolysaccharide (LPS) were used to establish an animal model of immune liver injury. For the intervention group, the high and low doses of anti-IL18 antibody were intraperitoneally injected on day 2, 7 and 12. The control and model groups were intraperitoneally administered with normal saline. From the following day, the positive control group was intragastrically administered with bifendate pills, daily. Serum levels of alanine transaminase (ALT), aspartate transaminase (AST), IL-18, interleukin-1 (IL-1), and tumor necrosis factor alpha (TNF-α) were measured after 12 days. The liver, spleen and thymus gland were removed, and the weight coefficients were calculated. Liver tissue was collected and sectioned for hematoxylin-esosin staining (H&E) and Van Gieson's staining.*

Results*: The results revealed that the coefficients of the injured liver, spleen and thymus in the high-dose antibody intervention group were significantly decreased compared with those in the model group ($P<0.05$). Furthermore, ALT and AST levels were all significantly lower in the high-dose, low-dose and positive control groups, compared with the model group ($P<0.01$). The high-dose intervention group exhibited a significantly inhibited increase in IL-18, IL-1 and TNF-α ($P<0.05$). Furthermore, in the high- and low-dose antibody intervention groups, and positive control group, the pathological liver damage was reduced to varying degrees, while the cytological grades of liver cell injury were significantly different among these groups ($X2 = 14.667$, $P<0.01$).*

Discussion and conclusions*: These results indicate that immune liver injury in antibody intervention mice could be alleviated, and this was most significant in the high-dose group. High- and low-dose antibody intervention inhibited the immune injury of the liver, thereby playing a protective role in mouse immune liver injury.*

Keywords*: Immune liver injury, Interleukin-18 antibody, Lipopolysaccharide, Bacillus Calmette-Guérin (BCG).*

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Introduction

Bacillus Calmette-Guérin (BCG) and lipopolysaccharide (LPS) are commonly used to co-induce animal models of immune liver injury. These are similar to human viral hepatitis, thereby making it an ideal model for the study of hepatitis[(1)]. BCG and LPS injections in mice cause serious liver injury by inducing the release of inflammatory mediators from macrophages, including the release of inflammatory cytokines, leading to an imbalance in the body's cytokine network and the occurrence of liver injury[(2)].

Interleukin-18 (IL-18) knockout mice were able to resist LPS-induced liver injury[(3)], indicating the important role that IL-18 plays in LPS-induced liver injury. LPS attacks mice to induce liver injury, which manifests as increased levels of serum alanine transaminase (ALT) and aspartate transaminase (AST), while liver damage caused by LPS can be completely blocked by IL-18 antiserum. This result also suggests that IL-18 is an important factor for LPS-induced liver injury[(4)].

The present study focused on the induction of the immune liver injury model in Kunming mice by BCG and LPS. Anti-IL18 antibody was applied to

inhibit IL-18, regulate the cytokine network, inhibit liver immune injury, and exert a protective effect against immune liver injury. The present study aimed to explore the immunological mechanism of the effect of anti-IL18 antibody on immune liver injury in mice, and appropriate dose of antibody intervention, in order to provide theoretical and animal experimental evidence for the treatment of immune liver injury.

Materials and methods

Experimental materials

The animal breeding facility was provided by the Base of Clinical Pharmacology, School of Medicine, Henan University of Science and Technology, Luoyang, China. Healthy Kunming mice (weighing 20 ± 2 g, male, and 6-8 weeks old) and mouse feed were provided by the Experimental Animal Center, Fourth Military Medical University, Xi'an, China.

Preparation of the mouse immune liver injury model

In order to determine the appropriate dose of BCG and LPS, the Kunming mice in the primary experiment were randomly divided into seven groups: blank control group, low-concentration BCG group (one injection of BCG + 3 ml of self-contained dilute solution, dissolved as the test solution), medium-concentration BCG group (one injection of BCG + 2 ml of dilute solution), high-concentration BCG group (one injection of BCG + 1 ml of dilute solution), low-concentration BCG+LPS group, medium-concentration BCG+LPS group, and high-concentration BCG+LPS group. Eight mice were placed into each group.

With the exception of the blank control group, which only used an equivalent volume of saline, 0.2 ml of the BCG test solution at high, medium, or low concentrations was injected into the tail of mice in each group. After 12 days, mice in the BCG+LPS groups were additionally injected with 100 μg of LPS (diluted with PBS buffer to 0.2 ml).

During the experiment, the general status of the mental state, activity, posture and hair of mice were observed. After 12 hours, mice in the BCG+LPS groups were treated with LPS, and blood was sampled from the orbital veins of all the mice. Then, serum was collected and stored at -20°C for testing. Liver tissues were taken from the same site of the same liver lobe and fixed in 10% formaldehyde. Conventional sectioning, hematoxylin and eosin (H&E) staining and Van Gieson's staining were performed for the collagen fibers (assisted by the Department of Pathology, Henan University of Science and Technology), and histopathological changes were observed under a microscope. Then, ALT and AST levels in each group were measured (assisted by the Biochemistry Laboratory of the First Affiliated Hospital of Henan University of Science and Technology).

Experimental grouping and steps

Kunming mice were randomly divided into five groups (n=10, each group): blank control group, model group (BCG+LPS group), positive control group (bifendate pill group), low-dose anti-IL18 intervention group, and high-dose anti-IL18 intervention group. Mice were fed in laboratory conditions at room temperature (22 ± 3°C) with 60-75% humidity, and were allowed to acclimate to their new environment for three days.

On the day of the experiment, with the exception of the blank control group, which only used an equivalent volume of saline, mice in other groups were injected with 0.2 ml of the BCG test solution of the medium concentration into the tail vein. Mice in the high- and low-dose antibody intervention groups were intraperitoneally injected with 100 μg and 50 μg of IL-18 antibodies (diluted with PBS buffer to 0.2 ml) on day 2, 7 and 12. After 12 days, mice in the BCG+LPS groups were injected with 100 μg of LPS (diluted with PBS buffer to 0.2 ml). Mice in the control and model groups were intraperitoneally injected with 0.2 ml of normal saline. Furthermore, mice in the positive control group were intragastrically administered with 0.2 g·kg−1 of bifendate (diluted with normal saline to 0.2 ml) daily, beginning the next day. With the exception of the blank control group, mice in the other groups were injected with 100 μg of LPS (diluted with PBS buffer to 0.2 ml) into the tail vein after the last administration on day 12.

Specimen collection and treatment

After 12 hours, blood was collected from the orbital veins of all mice, and serum was routinely separated and stored in a refrigerator at -20°C for testing. The liver, spleen, and thymus were harvested and weighed, and the weight coefficient was calculated. Liver tissues were cut from the same site of the same liver lobe and fixed in 10% formaldehyde.

Then, serum ALT and AST levels in each group were measured. Serum IL-18, IL-1 and TNF-α were determined in strict accordance with the instructions of the manufacturers.

The pathological sections of liver tissues were prepared and underwent H&E staining and Van Gieson's staining for collagen fibers (assisted by the Department of Pathology, School of Medicine, Henan University of Science and Technology).

Statistical analysis

Statistical data were expressed as mean ± standard deviation (SD). Variance analysis and q-test were used. Rank sum test was performed for the statistical analysis of pathological sections using the number of injured cases. Data were analyzed using SPSS 19.0 statistical software.

Results

Comparison of BCG and BCG+LPS-induced immune liver injury models

Mice in the blank control group were active, fast and powerful, and had bright eyes, a good mood, a healthy appetite, wooly and shiny coats and sturdy leg muscles. In contrast, mice in the BCG and BCG+LPS model groups became less active, withered and bow-backed in appearance, had poor appetites, and had hairs that were sparse, dry, messy, or fluffy. Two mice in the high-concentration BCG group died within one week, while three mice in the high-concentration BCG+LPS group died at the night after LPS injection (Table 1).

Group	N	BCG concentration	ALT (U·L-1)	AST (U·L-1)
Control	8	Vehicle	15.13±5.17	54.50±20.17
BCG	8	Low	19.75±2.60▲	70.13±15.70▲
BCG	8	Medium	49.38±10.35•	85.88±12.37•
BCG	6	High	54.83±13.32●	98.83±9.22•
BCG+LPS	8	Low	17.50±7.37▲	67.50±14.97▲
BCG+LPS	8	Medium	**76.25±12.24**•□	85.13±10.29••
BCG+LPS	5	High	**77.20±15.64**•□	117.20±18.34••

Table 1: Effects of BCG and BCG+LPS-induced immune liver injury on serum ALT and AST levels in mouse models (means ± SD).

Note: Compared with blank control group: ▲P > 0.05; •P < 0.01; ◇P < 0.05. Compared with same concentration group: □P < 0.05; •P > 0.05. BCG: Bacillus Calmette-Guérin; LPS: Lipopolysaccharide; ALT: Alanine transaminase; AST: Aspartate transaminase; SD: standard deviation

Effects of anti-IL18 intervention on liver, spleen and thymus coefficients in the mouse immune liver injury models

The effects of anti-IL18 intervention on the liver, spleen and thymus coefficients in mice with immune liver injury are shown in Table 2. Compared antibody intervention group and Bifendate group with model group, big dosage antibody intervention group remarkably inhibitited the liver, spleen and thymus weighing indexes (P<0.05). Small dosage antibody intervention group and Bifendate group also inhibitited the liver, spleen and thymus weighing indexes, but had not statistics significance (P>0.05).

Group	Liver coefficient (mg·g-1)	Spleen coefficient (mg·g-1)	Thymus coefficient (mg·g-1)
Blank control group	115.33±22.94	56.16±16.91	2.15±0.91
Model group	200.29±31.06▲	77.69±11.22▲	3.53±1.10▲
High-dose antibody intervention group	151.05±27.88•	58.27±8.86•	**2.30±0.56**◇
Low-dose antibody intervention group	**190.46±25.27**□	**76.10±13.96**□	**2.73±0.87**□
Positive control group	**186.77±36.39**□	**68.51±7.66**□	**2.61±0.60**□

Table 2: Effects of anti-IL18 intervention on the liver, spleen, and thymus coefficients in mice with immune liver injury (n = 10, means ± SD).

Note: Compared with blank control group: ▲P < 0.01. Compared with model group: •P < 0.01; ◇P < 0.05; □P > 0.05. IL-18: Interleukin-18; SD: standard deviation

Effects of anti-IL18 intervention on serum ALT and AST in the mouse immune liver injury models

The effects of anti-IL18 intervention on serum ALT and AST in the mouse immune liver injury models are shown in Table 3.

Group	ALT (U·L-1)	AST (U·L-1)
Blank control group	15.60±11.04	54.40±29.70
Model group	61.70±11.48▲	201.31±65.47▲
High-dose antibody intervention group	19.20±5.53•	72.70±13.00•
Low-dose antibody intervention group	28.90±10.62•	76.00±12.52•
Positive control group	19.20±6.70•	129.10±32.16•

Table 3: Effects of anti-IL18 intervention on serum ALT and AST in mice with immune liver injury (n = 10, means ± SD).

Note: Compared with blank control group, ▲P < 0.01. Compared with model group, •P < 0.01. ALT: Alanine transaminase; AST: Aspartate transaminase; SD: standard deviation

Compared with model group, big and Small dosage antibody intervention group and Bifendate group all remarkably inhibitited the serum level of ALT and AST (P<0.05).

Effects of anti-IL18 intervention on serum IL-18, IL-1 and TNF-α in the mouse immune liver injury models

The effects of anti-IL18 intervention on serum IL-18, IL-1 and TNF-α in the mice with immune liver injury models are shown in Table 4. Compared with model group, big dosage antibody intervention group remarkably inhibitited the serum level of IL-18, IL-1, TNF-α (P<0.05). Small dosage antibody intervention group and Bifendate group also inhibitited the serum level of IL-18, IL-1, TNF-α, but had not statistics significance (P>0.05).

Group	IL-18 (pg·ml-1)	IL-1 (pg·ml-1)	TNF-α (pg·ml-1)
Blank control group	122.45±19.92	628.16±71.75	697.17±77.65
Model group	196.52±16.01•	739.90±24.76•	1544.05±345.68•
High-dose antibody intervention group	154.12±23.41▲	641.94±27.84◇	990.46±77.68◇
Low-dose antibody intervention group	**171.80±37.11**□	**720.00±14.16**□	**1256.46±161.25**□
Positive control group	**180.70±37.43**□	**724.12±32.96**□	**1478.26±190.91**□

Table 4: Effects of anti-IL18 intervention on the serum IL-18, IL-1, and TNF-α levels in mice with immune liver injury (n= 10, means ± SD).

Note: Compared with blank control group, •P < 0.01. Compared with model group: ▲P < 0.05; ◇P < 0.01; □P > 0.05. IL-18: Interleukin-18; IL-1: Interleukin-1; TNF-α: Tumor necrosis factor alpha; SD: standard deviation

Effects of anti-IL18 intervention on the pathomorphology of liver tissue in the mouse immune liver injury models

According to the liver lobular structure, fibrous hyperplasia, the formation of a fibrous space, the arrangement of liver cell cords, the extent of liver cell degeneration and necrosis, and the interstitial infiltration of inflammatory cells, mice in each group were classified into four levels: (-) normal liver lobular structure, no fibrous hyperplasia or fibrous space formation, normal liver cell cords and sinus proportions, normal liver cell structure, and no inflammatory cell infiltration; (+) the majority of hepatic lobular structures are clear, small amount of blurred structure, partial connective tissue hyperplasia in the portal area and around small veins, liver cells arranged clearly or basically clearly, and a small amount of inflammatory cell infiltration around small veins and in the portal area; (++) most of the hepatic lobular structures were not clear, connective tissue hyperplasia in the portal area and around small veins, liver cell cord arrangement was not clear, swelling hepatocytes, loose cytoplasm, degenerated vacuoles, dot-sheeted focal necrosis, and inflammatory cell infiltration around the small veins and portal area; (+++) the liver lobular structure was blurred, extensive fibroblast and connective tissue hyperplasia, disorderly arranged liver cell cords, sinusoidal dilatation, extensive hepatic cell focal necrosis of different sizes and a large range, and extensive inflammatory cell infiltration (Table 5).

Group	Extent of fibrous hyperplasia, inflammatory cell infiltration, and hepatocellular degeneration and necrosis			
	-	+	++	+++
Normal control group	10	0	0	0
Model group	0	2	4	4
High-dose antibody intervention group	7	3	0	0
Low-dose antibody intervention group	1	5	4	0
Positive control group	1	3	5	1

Table 5: Effects of anti-IL18 intervention on the pathomorphology of liver tissue in mice with immune liver injury (n = 10).

Note: χ^2 = 14.667, P < 0.01. IL-18: Interleukin-18

Pathomorphological observation of liver tissues

The pathological manifestations of the liver in mice in each group were observed under light microscopy (Table 6).

Discussion

The coefficients of the liver, spleen and thymus significantly increased in the model group, indicating that BCG and LPS caused the liver damage. In mice with immune liver injury, there was a wide range of liver cell vacuolar degeneration, loose cytoplasm, extensive lobular dot-sheeted necrosis, inflammatory cell infiltration around the portal area and hepatic lobules, hyperplasia of portal area fibroblasts, bile duct epithelial cells and connective tissue, and diffuse degeneration and swelling of liver cells. This resulted in an increase in liver volume, which in turn led to liver enlargement and spleen and thymus swelling due to hyperplasia.

Group	Liver lobule structure	Fibrous hyperplasia and fibrous space	Liver cell cord arrangement	Liver cell degeneration and necrosis	Interstitial inflammatory cell infiltration
Blank control group	Normal structure	No hyperplasia or fibrous space formation	Normal liver cell cord and sinus proportion	Normal structure	No inflammatory cell infiltration
Model group	Blurred structure	Hyperplasia of portal area fibroblast, bile duct epithelial cell, and connective tissue	Arranged disorderly, with sinusoidal dilatation	Focal necrosis with a large amount, different size, and large range	Extensive inflammatory cell infiltration, most significant around the liver sinus and small veins
High-dose antibody intervention group	Normal structure	No hyperplasia or fibrous space formation	Arranged normally	Liver cell structure largely normal	Small amount of inflammatory cell infiltration around small veins
Low-dose antibody intervention group	Most structure clear, with small amount of structure blurred	Small amount of fibroblast hyperplasia around small veins	Arranged clearly or basically clearly	Some cell swelling, loose cytoplasm, and focal necrosis	Small amount of inflammatory cell infiltration around small veins
Positive control group	Structure less clear	Fibrous connective tissue hyperplasia around portal area	Arranged basically clearly	Cell swelling, loose cytoplasm, extensive vacuolar degeneration, and dot-sheeted cell necrosis	Small amount of inflammatory cell infiltration around small veins and portal area

Table 6: Microscopic observations of liver pathomorphology in mice of different groups.
Note: $\chi^2 = 14.667$, $P < 0.01$. IL-18: Interleukin-18

This damage was similar to the acute process of viral hepatitis. The high-dose antibody intervention group exhibited significantly reduced coefficients of the liver, spleen and thymus, indicating that it exerted a protective effect on the liver, spleen and thymus, in addition to inhibiting immune liver injury in mice. The low-dose antibody intervention and positive control groups exhibited a less obvious decrease in coefficients of the injured liver, spleen and thymus, indicating that the interventions in these two groups did not inhibit immune liver injury in mice. Hence, this did not play a protective role against liver injury[5].

ALT and AST were elevated in the model group, compared with the blank control group, while those in the high- and low-dose antibody intervention groups and the positive control group were lower, compared with the model group, indicating that the high- and low-dose antibody intervention could antagonize immune liver injury in BCG- and LPS-treated mice to different a degree. The liver is rich in enzymes, ALT is mainly distributed in the cytoplasm, and AST is distributed in the cytoplasm and mitochondria. When tissue damage, destruction, or necrosis occurs in the liver, the enzymes escape from cells into the blood, leading to an increase in ALT and AST activity. In patients with viral hepatitis, elevated levels of ALT and AST were an important indicator of hepatitis activity. The mechanism of immune liver injury may be mediated by cytokines, causing liver cell damage. Anti-IL18 antibody can inhibit the activity of IL-18, thereby regulating the cytokine network and reducing the production of IL18-induced proinflammatory cytokines, such as IL-1 and TNF-α[6]. This would eventually play a protective role in inhibiting immune liver injury in mice, although the specific mechanism remains to be further elucidated. The bifendate pills used in the positive control group were intermediates in the synthesis of schisandrin C, which can reduce liver damage and the elevated ALT level caused by carbon tetrachloride (CCl4), exert an inhibitory effect on CCl4-induced liver microsomal lipid peroxidation and the transformation of CCl4 into CO, and reduce the consumption of coenzyme II and oxygen in CCl4 metabolism[7]. The enzyme-reducing effect of bifendate does not directly inhibit ALT activity in serum or the liver, nor does it accelerate the inactivation of serum ALT. Bifendate has a significant inductive role in cytochrome P450 enzyme activity, thereby strengthening its detoxification capacity against carbon tetrachloride and some carcinogens. All these eventually play a role in protecting the liver cell membrane, thereby decreasing ALT and AST activity, although the mechanism is different from anti-IL18[8].

In mice with induced immune liver injury, the levels of IL-18, IL-1 and TNF-α were significantly elevated in serum, when compared with the blank control group[9], suggesting that cytokines IL-18, IL-1 and TNF-α are involved in liver injury. The high-dose antibody intervention group exhibited a significantly inhibited increase in IL-18, IL-1 and TNF-α. It has been suggested that the high-dose antibody intervention group can inhibit the production of cytokines IL-18, IL-1 and TNF-α, and that its role against liver injury may be related to the inhibition of increased levels of cytokine IL-18. Although the low-dose antibody intervention and positive control groups also exhibited a reduction in the elevated levels of IL-18, IL-1 and TNF-α, the specific role remains unclear[5].

IL-18 is an important inducing factor for liver injury caused by endotoxin. LPS can induce liver injury in mice. This presents as an increase in serum ALT, AST, IL-18, IL-1 and TNF-α levels, as well as an increase in other cytokines[10]. Furthermore, IL-18 can induce the production of IFN-γ, activate NK cells and stimulate Th1 cells in the presence of CD3

monoclonal antibody. Moreover, IL-18 can also stimulate T-cell proliferation, and enhance the cytotoxicity of lymphocytes. After the enriched T cells are activated by CD3 monoclonal antibody and co-incubated with IL-18, the number of cells significantly increased in an IL-18 dose-dependent manner[11]. IL-18 can also induce the production of IL-2. Through these effects, and the mediation of the cytokine network, IL-18 is involved in liver autoimmune injury. It has been reported that IL-18BP can inhibit endotoxin-induced liver injury[12], and the effect of IL-18BP is similar to the function of the IL-18 antibody[13]. Therefore, anti-IL18 may block or partially block IL-18, thereby regulating the cytokine network, inhibiting immune liver injury, and playing a protective role against immune liver injury in mice[14].

Tumor necrosis factors are a class of cytokines produced by macrophages, T cells, NK cells and other cells, and have anti-tumor, anti-virus and inflammation-inducing effect[15]. TNF-α is an important mediator of liver injury, with endotoxin-induced liver injury mediated by TNF-α as one of its factors, and TNF-α activity has a positive correlation with the severity of liver cell necrosis[16]. More importantly, TNF-α can be used as the first mediator of liver injury, since its presence would cause the production of many secondary mediators associated with liver necrosis, such as NO, IL-1, IL-6, IL-8 and sIL-2R, and IL-1 can enhance the action of TNF-α in liver injury[17], with tissue damage occurring in the host even if only a small amount of TNF-α is present. It has been reported that TNF derived from liver macrophages plays an important role in BCG+LPS-induced liver injury[18]. The results of the present study revealed that TNF-α was significantly elevated in the model group, compared with the blank control group, while the high-dose anti-IL18 antibody group exhibited a significant decrease in TNF-α level in the model group, suggesting that the protective role of IL-18 antibody in the liver is related to the reduction in TNF-α level[19].

Due to the dose effect, low-dose IL-18 antibody cannot play a role in inhibiting immune liver injury, and has no protective effect against the increase in IL-18, IL-1 and TNF-α caused by immune liver injury in mice[20]. Regarding the immune mechanism, the bifendate pills used in the positive control group exhibited no effect on immune liver injury in mice or any protective effect on the increase levels of IL-18, IL-1 and TNF-α.

Histopathological examination of the liver revealed that the liver structure of mice in the blank control group was normal. In the model group, liver injury was severe, with blurred hepatic lobules, hyperplasia of portal area fibroblasts, bile duct epithelial cells, and connective tissues occurred, the arrangement of liver cell cords was disordered and the liver sinus was dilated, focal necrosis of liver cells occurred, with a large number, different sizes and a large range, and extensive interstitial inflammatory cell infiltration occurred, which was most significant around the liver sinus and small blood vessels. The pathological liver injury of mice in the high- or low-dose intervention groups and positive control group were all alleviated to different extents, indicating that mice in the model group had obvious liver injuries, and that therapy in the anti-IL18 intervention groups and positive control group was meaningful, since it played a protective role in varying degrees.

Conclusion

In mice in the model group, liver injury was obvious and mood was poor, mice were less active and had hair that was thin and fluffy, the coefficients of the liver, spleen and thymus increased, the levels of serum transaminases, IL-18, IL-1 and TNF-α were significantly elevated, and liver cells were seriously damaged, with fibrous tissue hyperplasia, inflammatory cell infiltration and extensive cell necrosis. The high-dose antibody intervention significantly reduced ALT and AST levels, significantly reduced the elevated coefficients of the liver, spleen and thymus, and significantly inhibited the increase in IL-18, IL-1 and TNF-α. Furthermore, mice with high-dose intervention also presented with significantly reduced liver pathological damage, and were protected against immune liver injury. For the low-dose antibody intervention, due to the dose effect, BCG+LPS-treated mice were protected against immune liver injury to some degree, but the difference was not statistically significant. The positive control group was able to temporarily reduce the level of serum transaminases, but was unable to reduce the serum levels of IL-18, IL-1 and TNF-α, thereby showing no immunoprotective effect.

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