Full Length Research Paper

Neutralization effects of egg yolk immunoglobulin (IgY) and Fab' fragment against lipopolysaccharide (LPS) in burned mice model

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The aim of this study is to evaluate the neutralization effects of egg yolk immunoglobulin (IgY) and Fab' fragment against lipopolysaccharide (LPS), and identify possible approaches to prevent and treat LPS related injuries. Mice with third-degree burns covering 30% of the total body surface and exposed to LPS were orally administered with the IgY or Fab' fragment of egg yolk immunoglobulin at 6, 12, 24 and 48 h after burning. The mortality rates and circulating levels of LPS, tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) were determined. The mortality rate of mice in the IgY-treated groups and Fab'-treated groups were significantly lower when compared with the control groups (without treatment). The levels of LPS, TNF-α and IL-6 in the IgY-treated group and Fab'-treated group were lower than those in the control group, and Fab'-treated group posses much lower levels of LPS, TNF-α and IL-6 than IgY-treated group. The IgY and Fab' fragment of egg yolk immunoglobulin raised against LPS may be used to inhibit and treat tissue injury caused by LPS. IgY technology has significant future opportunities for the prevention and treatment of LPS related infectious gastrointestinal diseases in humans and animals.

Key words: Lipopolysaccharide, egg yolk immunoglobulin, interleukin-6, TNF-alpha.

INTRODUCTION

Lipopolysaccharide (LPS), a cytoderm component of gram-negative bacteria, also termed endotoxin is associated with severe tissue injury and high mortality rates, particularly when administered in combination with extensive burning. Furthermore, antibiotic therapy increases the release of LPS and intensifies its injuries. Immunoglobulin Y (IgY) is the predominant egg yolk immunoglobulin in chickens, and is transferred from the serum to the yolk to confer passive immunity to the developing embryo (Larsson et al., 1993). Egg yolk immunoglobulins could be purified simply with low cost and without blood collection. Compared with IgG, IgY has some unique characteristics because of its phylogenetic distance. It is noteworthy that IgY does not cross-react serologically with mammalian immunoglobulins, complement or rheu-

matoid factors (Tini et al., 2002; Lucyna, 2005). IgY also shows good bioactivity across a wide pH range of 4.0 to 9.0 (Lee et al., 2002).

IgY has been used to prevent and treat infectious diseases caused by viruses and bacteria including rotaviruses, coronavirus, *Yersinia ruckeri*, enterotoxigenic *Escherichia coli*, *Staphylococcus*, *Pseudomonas* and *Helicobacter pylori* urease B (Tini et al., 2002; Lee, 2002; Sarker et al., 2001; Smith et al., 2001; Shin et al., 2002; Shin et al., 2003; Nilsson et al., 2007; Zhen et al., 2008). Previous studies have shown that the Fab' fragment of egg yolk IgY, which can be obtained by digesting IgY with pepsin, can bind to antigens (Akita and Nakai, 1993, 1993; Carlander et al., 2000). The titer of Fab' is about 70% higher than IgY, as determined by ELISA. Therefore, the Fab' fragment appears to enhance antigen neutralization in tissues because of its greater tissue penetration than intact IgY (Panacek et al., 2004). The Fab' fragment was also proposed to be more absorbable from the intestinal tract than intact IgY (Xie et al., 2005). There-

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Table 1. Mortality rates in mice 6 to 48 h after inducing a third-degree burn across 30% of the total body surface area and oral administration of LPS, without (control group) or with Fab' (Fab'-treated group) and IgY administration (IgY-treated group).

Group	Time (h)	n	Mortality (%)
Control	6	10	2 (20)
group	12	12	4 (33.33)
	24	10	6 (60)
	48	12	8 (66.67)
Fab'-treated	6	10	0 (0)
group	12	11	2(18.18)
	24	10	2 (20)[a,b]
	48	12	2 (16.67)[a,b]
IgY-treated	6	10	1 (10)
group	12	10	2 (20)
	24	10	3 (30)[a]
	48	12	3 (25)[a]

[a] ($P < 0.01$) versus the control group; [b] ($P < 0.05$) versus the IgY group.

fore, for testing the neutralization effect of anti-LPS IgY or Fab' *in vivo*, we carried out this study to examine whether oral administration of the anti-LPS IgY or Fab' fragment of egg yolk IgY raised against LPS, decreased mortality and tissue damage of burned mice exposed to LPS.

MATERIALS AND METHODS

Materials and animals

IgY and Fab' fragment of egg yolk immunoglobulin was prepared according to Ma and Zhang (2007). In brief, Roman laying hens were immunized with pure LPS mixed with Freund's Adjuvant, eggs were collected before immunization and stored in the refrigerator at 4°C. IgY against LPS was isolated from yolk of eggs, and then purified by water extraction, salt precipitation with ammonium sulfate and dialysis. Then, the Fab' was prepared by the digestion of the pepsin.

Kunming mice weighing 25 ± 1 g were provided by the Laboratory Animal Center, Third Military Medical University, China. Animals were maintained according to the guidelines of the animal ethical committee of China. The experimental procedure was approved by the Ethical Committee of Southwest Hospital.

Animal groups and treatments

Kunming mice were used as the animal model in this study. To determine the effect of Fab' administration on mortality, we randomly divided Kunming mice into three groups: a control group, an IgY-treated group and a Fab'-treated group. In both groups, after withholding food and water for 8 h, the mice under pentobarbital anesthesia (60 mg/kg body weight) were shaved on their back, weighed and orally administered with 0.2 ml of 10 g/l NaHCO$_3$ followed by 0.5 ml of LPS (1mg/ml) according to a method described by Li and Zhang, (2005) using a remolded syringe needle. Then 3% napalm (1 ml) was applied on the shaved skin and the skin was burned for 14 s to produce third-degree burns.

The total body surface area of the mouse was calculated using the formula S = 0.0913 × W2/3, where S (cm^2) = total body surface area of mouse and W (g) = weight of the mouse. The burn was applied over 30% of the total body surface area (TBSA) or S×30%. After the burn, the mice were intraperitoneally injected with 2 ml of 0.9% isotonic NaCl to prevent shock. Povidone-iodine was administered on the burned area to prevent infection. All mice were returned to their standard cages and provided with food and water.

In the control group, after establishing the burn, the mice were intraperitoneally injected with 0.5 ml 0.9% isotonic NaCl every 6 h. In IgY-treated group and Fab'-treated group, the mice were treated by orally administering to them 0.5 ml (16 mg/ml) IgY or Fab' (16mg/ml) every 6 h via a remolded syringe needle. The mice in all groups were then divided into four subgroups: 6, 12, 24 and 48 h after burning with 10 to 12 mice per group. The mortality rates were determined in each subgroup at the specified time points, that is, at 6, 12, 24 and 48 h after burning. After each time point, the existed mice were abandoned from the experiment.

Changes in serum LPS, NF-α or IL-6 levels were assessed in other mice, including 10 normal/untreated mice, without undergoing burning, 40 mice in a control group, 40 mice in the IgY-treated group and 40 mice in the Fab'-treated group. In the normal group, the mice continued normal feeding and were not exposed to burning. The control and treated mice were prepared as described earlier. The mice in the control and treated groups were divided into four subgroups (10 mice per group) and the survived mice were killed at 6, 12, 24 or 48 h after burning.

Measurement of LPS, IL-6 and TNF-α

Blood samples were collected from mice at the appropriate time points, centrifuged at 3000 rpm for 10 min to obtain serum and stored at -80°C until use. Serum LPS levels were measured using a commercially available kit based on the tachypleus amebocyte lysate (TAL) technique (Chromogenic TAL endpoint assay kits, Chinese Horseshoe Crab Reagent Manufactory Co., Ltd., Xiamen, China), and performed in accordance with the manufacturer's instructions (Xia et al., 2002).

The serum levels of IL-6 and TNF-α were measured using relative ELISA kits (e-Bioscience, USA) in accordance with the manufacturer's instructions. References and concentrations were calculated by comparison with standard IL-6 or TNF-α solutions supplied in the assay kit.

Statistic analysis

Data are expressed as means ± standard deviation. SPSS 13.0 was used for statistical analysis. Statistical significance was evaluated by χ^2 test for mortality and analysis of variance for LPS, TNF-α and IL-6 levels. P-values ≤ 0.05 were considered statistically significant.

RESULTS

Mortality rates

The mortality rate of mice in the IgY-treated groups and Fab'-treated groups were significantly lower ($P < 0.01$) when compared with the control groups at 24 and 48 h after the burn, for the two treated groups, Fab'-treated groups had lower mortality rate than IgY-treated groups ($P < 0.05$) at 24 and 48 h, after the burn (Table 1).
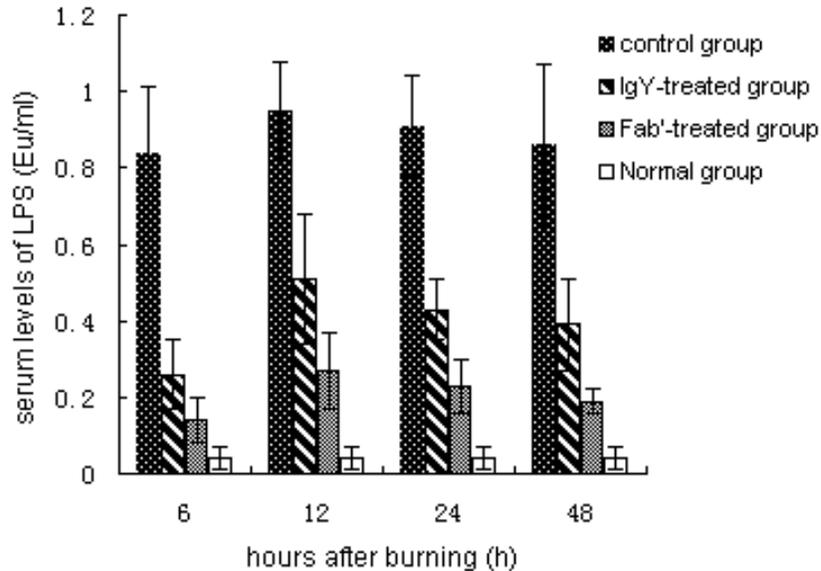
Figure 1. Serum lipopolysaccharide (LPS) levels in mice at 6 to 48 h after inducing a third-degree burn across 30% of the total body surface area and oral administration of LPS. After inducing the burn, the serum levels of LPS were significantly higher (P < 0.01) in the control group (burned mice exposed to LPS but without IgY or Fab' treatment) and in the Fab'-treated or IgY-treated group (burned mice exposed to LPS and treated with IgY or Fab') than in the normal group (untreated mice without burning or exposure to LPS). The serum level of LPS was significantly lower in the IgY-treated group and Fab'-treated group than in the control group (P < 0.01). Fab'-treated group had a lower serum level of LPS than IgY-treated group (P < 0.05).

Serum LPS levels in different groups

The serum LPS level in each group was detected by a chromogenic TAL endpoint assay, as described earlier. The level of LPS was lowest in the normal/untreated group (mice without burning or administration of LPS) of the four groups and was highest in the control group. The serum level of LPS was significantly decreased after administration of the IgY or Fab' fragment when compared with the control group (P < 0.01) and the Fab'-treated groups had lower level than IgY-treated groups (P < 0.05) (Figure 1).

Serum levels of TNF-α and IL-6

The serum TNF-α level increased significantly in the control group and in the treated groups in response to the burn, and increased to a peak at 24 h after the burn. After a further 24 h, the TNF-α levels had slowly decreased, but remained significantly higher than in the normal/ untreated group throughout the 48-h study period (P < 0.01). The TNF-α level remained significantly lower in the treated groups when compared with the control group (P < 0.01) and the Fab'-treated groups had lower level than IgY-treated groups (P < 0.05) (Figure 2a).

Changes in the serum IL-6 level showed a similar tendency to the TNF-α level described earlier. After the burn, the serum IL-6 level increased markedly in the control and treated groups, reaching a peak at 24 h. After a further 24 h, the IL-6 levels had begun to decrease, but remained at a significantly higher level than in the normal/untreated mice throughout the 48-h study period (P < 0.01). The IL-6 level remained significantly lower in the treated groups when compared with control group (P < 0.01) and the Fab'-treated groups had lower level than IgY-treated groups (P < 0.05) (Figure 2b).

DISCUSSION

Lipopolysaccharide (LPS) is a component of the cytoderm and is released by gram-negative bacterium during the growth and death, which can lead to severe tissue injury in humans and animals (Michael et al., 2003). But in healthy animals, LPS could be cleared by themselves, and cannot lead to LPS injuries when orally administrated a small quantity of LPS. During the initial period of time after a burn, particularly, extensive burns and excess LPS translocates into the circulation from the intestinal tract and the wound, led to tissue damage in the intestinal mucosa and whole-body infection. These
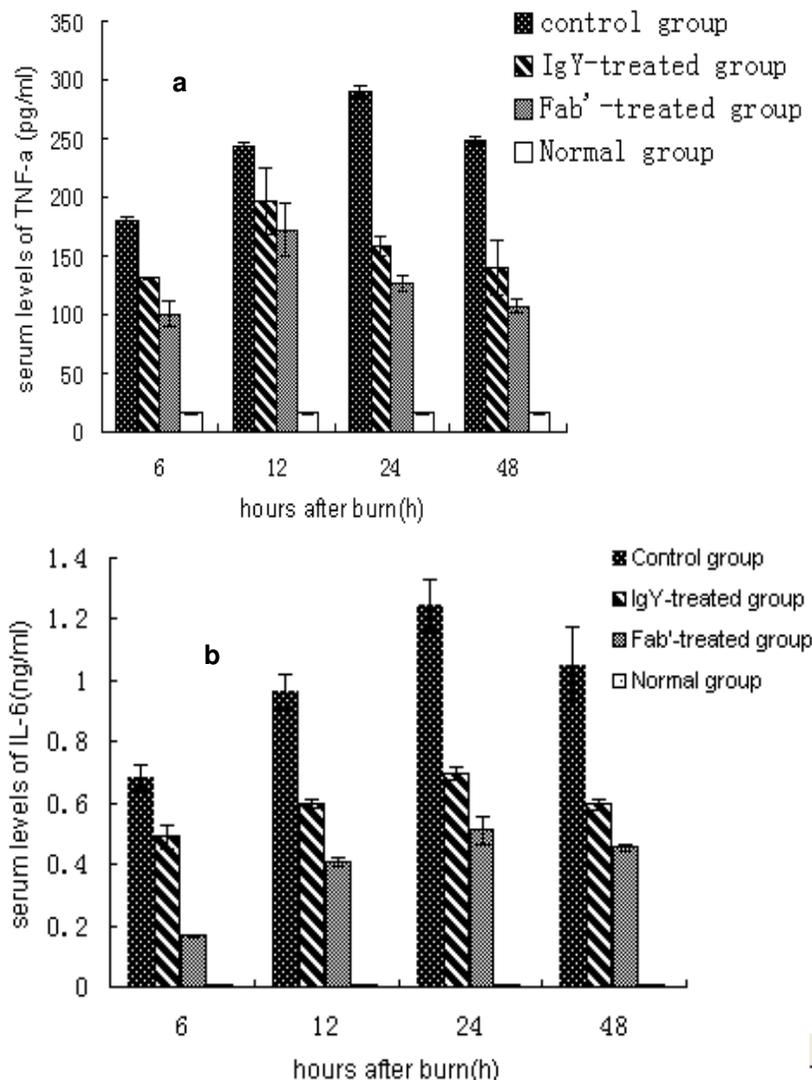
Figure 2. Serum TNF-α (a) and IL-6 (b) levels in mice at 6 to 48 h after inducing a third-degree burn across 30% of the total body surface area. After inducing the burn, the serum levels of TNF-α (a) and IL-6 (b) were significantly higher ($P < 0.01$) in the control group (burned mice exposed to LPS but without IgY or Fab' treatment) and in the Fab-treated group and IgY-treated group (burned mice exposed to LPS and treated with Fab') than in the normal group (untreaed mice without burning, exposure to LPS of Fab' treatment). The serum TNF-α (a) and IL-6 (b) levels were significantly lower throughout the study period in the IgY-treated group and Fab'-treated group than in the control group ($P < 0.01$). Fab'-treated group had a lower serum level of TNF-α and IL-6 than IgY-treated group ($P < 0.05$).

processes may lead to systemic inflammatory response syndrome (SIRS), multiple organs dysfunctional syndrome (MODS) and death, due to increased intestinal permeability and growth of gram-negative bacteria (Niu and Wang, 2007). In order to evaluate the neutralization effects of IgY and Fab' fragment *in vivo* more effectively, the burned mice was set as the model in our study. Our results showed encouraging results that administration of the Fab' fragment derived from egg yolk immunoglobulin raised against LPS reduced the mortality of burned mice exposed to LPS.

The intestinal tract is the largest endotoxin and bacteria library. Endotoxins translocate into the circulation from the intestinal tract much more easily after the body have experienced extensive burning, and activate mononuclear macrophages to promote the release of TNF-α and IL-6, which are important mediators of inflammation. As a result, activation of the inflammatory cascade causes severe cell and tissue injury (Niu and Wang, 2007). The primary aim of our study was to determine

whether neutralization of LPS by early administration of the Fab' fragment of egg yolk IgY raised against LPS after extensive burning reduces the uptake of LPS into the circulation, inhibits the production of TNF-α and IL-6 and decreases mortality associated with LPS.

Egg yolk IgY obtained from hens immunized by antigens neutralizes the related antigens specifically with high efficiency (Brunda et al., 2006; Sunwoo et al., 2006; Paul et al., 2007). As a result, egg yolk immunoglobulins have attracted increasing attention because of their unique characteristics. They are now widely used for pro-phylaxis and to treat diseases, particularly infective diseases caused by bacteria such as *E. coli* and *Salmonella enteritidis*, (Zhen et al., 2008; Casswall, 1999). Therefore, the IgY and Fab' fragment from egg yolk that was specifically raised against LPS was obtained.

Tumor necrosis factor alpha (TNF-α) can activate multiple downstream inflammatory pathways to trigger the release of pro- and anti-inflammatory mediators, activate coagulation and inhibit fibrinolysis. Release of TNF-α can be stimulated by LPS, and is the most important host-derived mediator molecule in the inflammatory cascade (Hack et al., 1997; Suffredini et al., 1989). Accordingly, reducing the level of circulating TNF-α can limit the activation of multiple downstream inflammatory pathways and improve survival (Panacek et al., 2004). IL-6 is released in response to TNF-α, and plays an important role in the inflammatory cascade, and its serum levels are thought to be associated with adverse outcome (Thijs and Hack, 1995; Casey et al., 1993). In this study, we used mice with 30% TBSA third-degree burns and oral administration of LPS. After oral administration of the IgY or Fab' fragment, it was found that the mortality of the mice decreased significantly when compared with the control mice treated with saline. It was also found that the serum LPS, IL-6 and TNF-α levels were decreased significantly in IgY-treated mice and Fab'-treated mice. Based on these results, it is believed that administration of the IgY or Fab' fragment of egg yolk raised against LPS decreased the mortality of model mice by neu-tralizing the circulating LPS. Accordingly, it seems likely that the Fab' fragment was more effective to clear LPS in the intestine to inhibit the uptake of LPS into the bloodstream, and thus reduce the ability for LPS to stimulate inflammatory cells and ultimately limit the secretion of inflammatory mediators such as TNF-α and IL-6. So, why did the Fab' fragment gain was more effective than intact IgY in this study? We thought the Fab' fragment could enter the circulation through intes-tinal mucosa like previous report shown (Xie et al., 2005), and then neutralize the circulating LPS, but the intact IgY did not posses this ability to get through the gastro-intestinal tract into circulation. Nevertheless, the possi-bility that there are some direct effects among the Fab' fragment, LPS and inflammatory cells, cannot exclude. For example, Fab' or IgY may stimulate the mononuclear

phagocytic system to remove LPS via a hitherto unknown process. Studies to further investigate these possibilities are ongoing in our laboratory.

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