The effect of Celosian, a water extract from *Celosia argentea* L., on NK activity in rats with galactosamine/LPS-induced acute hepatic injury

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Abstract

Natural killer (NK) cells may be involved in the important defense system against viral infections. It has been found that the intraperitoneal injection of Celosian (CE), a water extract of *Celosia argentea* L., had hepatoprotective effects on hepatitis models. Recently, we have shown that CE augments NK activity both in the liver and spleen. In the present study, we determined the effect of CE administration on hepatic and splenic NK activities in rats with D-galactosamine (GalN)/LPS-induced hepatitis. Intraperitoneal administration of CE protected the rats from chemically induced hepatic injury in terms of serum GOT and GPT levels. CE administration also significantly augmented NK activity both in the liver and spleen whereas the GalN/LPS treatment decreased NK activity in the liver. Furthermore, there was a strong negative correlation between the serum transaminase activity and NK activity both in the liver and spleen. Therefore, NK augmentation by CE in the liver, and probably in the whole body, prior to the injection of GalN/LPS might be critical for the hepatoprotective effect.

Key words NK activity, Chinese medical plant, hepatoprotective effect, liver, spleen, rat.

Abbreviations CE, Celosian ; GalN/LPS, D-galactosamine/lipopolysaccharide ; GOT, Glutamic oxaloacetic transaminase ; GPT, Glutamic pyruvic transaminase ; IFN, interferon ; LDH, lactate dehydrogenase ; LGL, large granular lymphocyte ; NK, natural killer.

Introduction

Natural killer (NK) cells have been morphologically defined as large granular lymphocytes (LGLs) containing dense azurophilic granules in the cytoplasm.[1,2] Functionally, NK cells possess non-major histocomatibility complex-restricted cell mediated cytotoxicity.[1,2] Several studies support that NK cells, as well as other natural resistance mechanisms, work as a mediator or early host resistance to virus infections.[3-5] The antiviral activity of NK cells has been well studied in animal models.[6] In human study, it has been shown that the NK cytotoxic activity in a group of hepatitis C virus (HCV)-infected individuals was four-fold lower than in normal donors.[7] Recently, there is more attention paid to organ-associated NK cells. Previous studies have indicated that NK cells reside in various organs other than the spleen and peripheral blood. Lauzon *et al.* demonstrated that rat lung lymphocytes showed significant levels of cytotoxicity compared to spleen or peripheral blood lymphocytes.[8] Mouse NK activities in the liver and the lung were augmented to a greater extent than the peripheral blood and splenic NK activities by some biological response modifiers (BRMs).[9] Several studies have indicated that pit cells which reside in liver are different from lymphoid NK cells in both morphology and cytotoxicity.[10-12] In our previous study, the NK activity of rat liver was

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higher than that of spleen after administration of a water extract from *Celosia argentea* L. (CA).[13] These studies suggest that the NK cell response is different among various organs.

Many bioactive polysaccharides from Chinese medical plants possess a variety of immunomodulating activities including stimulation of NK activity.[14,15] Several studies have indicated that NK activity is stimulated by poly- or oligosaccharides.[16-19] *Celosia argentea* L., a member of the Amaranthaceae family, is a Chinese medical plant and known to have an anti-inflammatory effect especially on inflammation in the liver.[20] Celosian (CE) is an acidic polysaccharide isolated from the water extract of the seeds of *Celosia argentea* L. It has been reported that CE had hepatoprotective effects on liver injury models.[21,22] Intraperitoneal administration of CE inhibited the elevation of glutamic pyruvic transaminase (GPT) activity in the fulminant hepatitis induced by D-galactosamine/lipopolysaccharide (GalN/LPS) in mice and in the hepatic injury induced by carbon tetrachloride in rats. Recent work from our laboratory has demonstrated that the hepatic and splenic NK activities were augmented by intraperitoneal injection of CE in rats.[13] However, it is not known whether the NK augmentation by CE is involved in the hepatoprotective mechanisms or not. Therefore, in the present study, we investigated the effect of CE on the hepatic and splenic NK activities using the GalN/LPS-induced hepatitis model in rats.

Materials and methods

A. Materials

The extraction procedure from *Celosia argentea* L. was as follows. The dried powder of the seeds of CA (3 kg) was refluxed with distilled water (8L×2) for 3 hours. After centrifugation at 4000 rpm for 15 minutes, the supernatant was filtered through paper (Advantec type 2, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) and partially evaporated. These filtrates were further separated into the dialyzable and non-dialyzable fractions by ultrafiltration against water overnight. The non-dialyzable fraction was evaporated and lyophilized.[21] This non-dialyzable (molecular weight≧100kDa) was called as CE.

RPMI 1460 was obtained from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from Bio Whittaker (Walkersville, USA) and penicillin G potassium from Meiji Seika Co. (Tokyo, Japan). Other tissue culture chemicals were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). The solution for gradient centrifugation methods, Histopaque-1083, was purchased from Sigma Chemicals Co. (St. Louis, USA). GalN was obtained from Wako Pure Chemical Industries Ltd. and LPS was purchased from Defco Laboratories (Detroit, USA). The non-radioactive cytotoxicity assay kit, Cytotox-96, was purchased from Promega (Madison, USA). The GOT-GPT CII kit, other chemicals and reagents were obtained from Wako Pure Chemical Industries Ltd.

B. Methods

Animals and diets : Specific pathogen-free male Fisher 344 rats, aged 3 weeks, were purchased from Japan SLC Inc. (Hamamatsu, Japan). The rats received a purified standard diet from the 4th to the 6th week of age. The composition of the diet was described previously.[13] A 12-hr light-dark cycle and a room temperature between 22°C and 24°C were maintained. The animals had free access to food and water.

Experimental procedures : At the end of the 6th week of age, the rats were divided into two groups, the CE injection group and the saline injection group. CE was administered intraperitoneally (50 mg/kg) 18 hrs and 2 hrs before the induction of hepatic injury. After the second CE injection, each group was further subdivided into two subgroups. The rats in one subgroup were injected with 0.2 ml of a mixed aqueous solution of 300 mg/kg of GalN and 5 μg/kg of LPS (E. coli 055 : B5) and those in the other with saline, both intraperitoneally.

Isolation and cytotoxicity assay of liver LGLs and splenic lymphocytes : Isolation and cytotoxicity assay of hepatic and splenic lymphocytes were described previously.[13] Briefly, the liver was perfused with warm (37°C) Dulbecco's phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} but supplemented with 0.1 % EDTA at the pressure of 50 cm water, and the 100 ml of effluent was collected. Spleens were removed and cut into small pieces in Hanks' buffered

salt solution (HBSS) supplemented with 3 % FBS. The cells were then dissociated between two frosted micro slide glass sheets and filtered through nylon mesh.

The cell suspensions from the liver and spleen were purified by fractionation using Histopaque (density=1.083 g/ml). After centrifugation at 400×g for 30 minutes at room temperature, the interface containing mononuclear cells was collected. To remove adherent cells, the cells were incubated on nylon wool columns with RPMI medium at 37°C for 1 hr in 5 % CO_2 humidified atmosphere. Non-adherent cells were eluted off with warm (37°C) RPMI medium. These cells were used as effector cells in the cytotoxicity assay.

In the cytotoxicity assay of effector cells against NK-sensitive YAC-1 target cells, we quantitatively measured lactate dehydrogenase (LDH), which is released upon cell lysis.[23,24] The kit of Cytotox 96 was used for this assay. The effector-to-target cell ratio was 20 : 1 for the liver and 20 : 1 or 50 : 1 for the spleen. Cytotoxicity was calculated by the following formula :

% cytotoxicity=(A−B−C / D−C) ×100

A : experimental release

B : effector spontaneous release

C : target spontaneous release

D : target maximum release

Serum GOT and GPT activities : Liver injury was assessed by measurement of serum glutamic oxaloacetic transaminase (GOT) and GPT activities. The blood samples were collected from the inferior vena cava before liver perfusion. These activities were assayed by using the GOT-GPT CII kit.

Statistical analysis : Data were expressed as mean values±S.E.M. Analysis of variance (ANOVA) and *t*-test were performed to determine the significance ($p<0.05$) of differences. Linear regression analysis was used to determine the degree of correlation between the serum transaminase and NK activities.

Results

Serum GOT and GPT activities

The effect of CE against GalN/LPS-induced hepatic injury is evident from the suppressed serum GOT and GPT activities in the CE- and GalN/LPS-treated rats as shown in Table I. When rats were treated with GalN/LPS alone, the activities of serum GOT and GPT were significantly elevated approximately 30- and 35- fold, respectively, compared to those of the control rats. The activities of serum GOT and GPT of rats treated with GalN/LPS alone were also significantly elevated compared to those of the rats which pre-treated with CE. The elevation of serum GOT and GPT activities by GalN/LPS was not seen when the rats were pre-treated with CE. These results indicated that two times of 50 mg/kg ip injection of CE protected against GalN/LPS-induced liver injury. The serum GOT and GPT activities of the group of CE administration only were not different from that of the control group. This was the same result as in our previous study,[13] indicating that CE did not the cause any hepatic damage.

Table I Protective effect of Celosian against GalN/LPS-induced hepatic injury in rats.

Celosian	GalN/LPS	n	GOT activity (IU)	GPT activity (IU)
−	−(Control)	6	44.9±1.8	9.3±0.7
−	+	12	1296.8±555.6*	343.9±136.7*
+	+	6	64.7±9.4	12.7±2.3
+	−	6	49.7±15.1	8.9±2.1

Data are expressed as the mean±S.E.M.
*Significantly different from control, $p<0.05$

Effects of CE administration and GalN/LPS treatment on NK activity in the liver and spleen

The liver NK activity as influenced by CE administration and GalN/LPS treatment is shown in Fig. 1. There were no significant interaction differences in the NK activity among the four groups (Fig. 1A). However, when the effects of CE pre-treatment were analyzed, the liver NK activity of the CE-treated group was significantly higher than that of CE-untreated group (Fig. 1B). Regarding the GalN/LPS treatment, the liver NK activity of the group treated with GalN/LPS was significantly lower compared to that of the GalN/LPS-untreated group (Fig. 1C).

The splenic NK activity as influenced by CE administration and GalN/LPS treatment is shown in Fig. 2. There were no significant interaction differences in the NK activity among the four groups as in

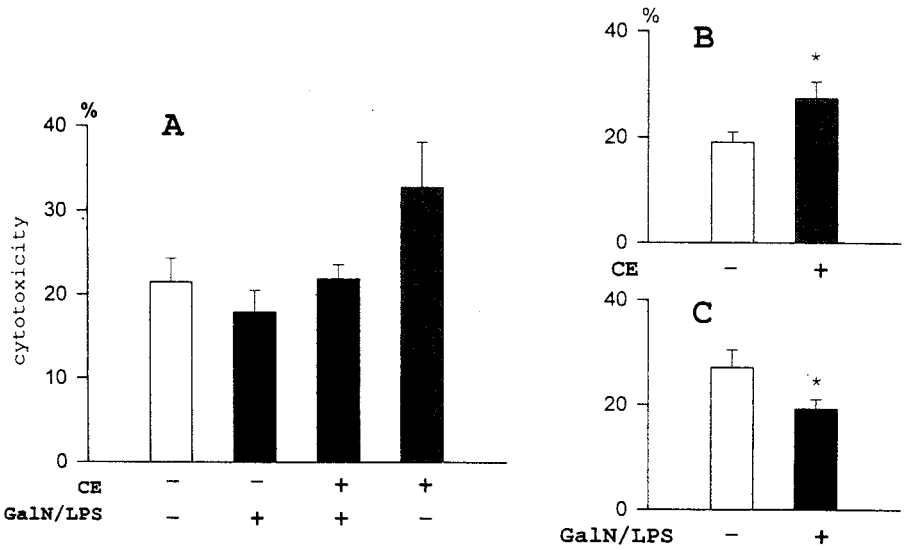

Fig. 1 Effect of CE and GalN/LPS on liver NK activity. (lymphocyte : Yac-1=20 : 1)
Data are expressed as the mean±S.E.M., $*p<0.05$. CE+ ; CE ip administration, GalN/LPS+ ; galactosamine/LPS ip administration. The hepatic lymphocyte-to-Yac-1 cell ratio was 20 : 1. There were no significant differences among the four groups in two-way ANOVA (Fig. A) and subsequently each factor was pooled and analyzed in one-way ANOVA (Fig. B and C).

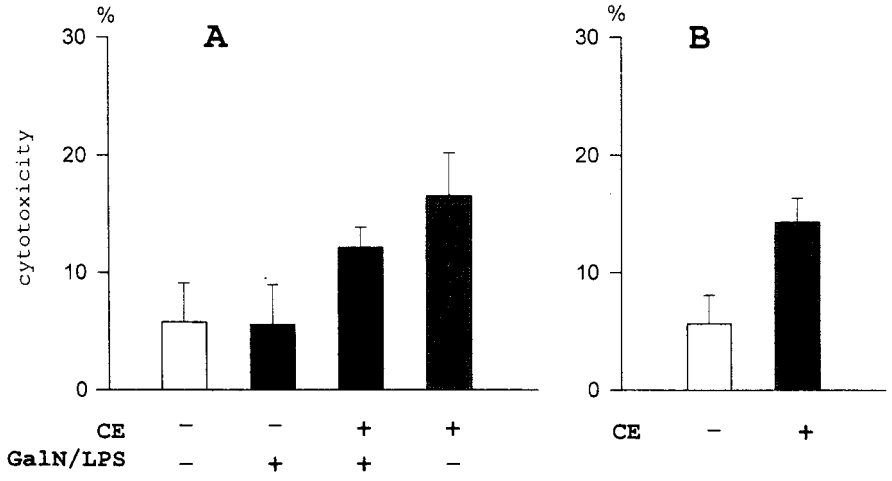

Fig. 2 Effect of CE and GalN/LPS on splenic NK activity. (splenic lymphocyte : Yac-1=20 : 1)
Data are expressed as the mean±S.E.M., $*p<0.05$. CE+ ; CE ip administration, GalN/LPS+ ; galactosamine/LPS ip administration. The splenic lymphocyte-to-Yac-1 cell ratio was 20 : 1. There were no significant differences among the four groups in two-way ANOVA (Fig. A) and subsequently each factor was pooled and analyzed in one-way ANOVA (Fig. B).

the liver NK activity (Fig. 2A). When analyzed separately, the NK activity of the CE administration group was also significantly higher than that of the CE-untreated group (Fig. 2B). However, there was no influence of GalN/LPS treatment on splenic NK activity.

Relationship between the serum transaminase activity and NK activity

Correlation analysis was conducted to examine the relationship between the serum transaminase activity and the NK activity in the liver and spleen. There were inverse relationships between the serum

Table II Correlation coefficients of the serum transaminase activity and the NK activity in control rats and rats treated with either GalN/LPS, CE, or both.

		NK activity	
		Liver	Spleen
Serum	GOT	r=−0.394	r=−0.422
Transaminase		(p=0.03)	(p=0.02)
Activity	GPT	r=−0.354	r=−0.448
		(p=0.055)	(p=0.01)

transaminase activities and NK activities when the data of all groups were plotted. Correlation coefficients of the serum transaminase activity and the NK activity are shown in Table II.

Discussion

The present study was aimed to examine the effect of CE administration on the hepatic and splenic NK activities in a chemically induced hepatic injury model. Our present study demonstrated that ip administration of CE before GalN/LPS treatment was very effective in protecting the rat hepatocyte from the toxicity of GalN/LPS. Moreover, CE augmented NK activity both in the liver and spleen at the same time (Fig. 1B and Fig. 2B). These NK augmentation effects prior to the injection of GalN/LPS seem to inhibit the chemically induced hepatic injury, because the serum GOT and GPT activities of the CE administration group before GalN/LPS treatment did not differ from the control levels (Table I). On the other hand, the serum GOT and GPT activities of the GalN/LPS treatment group were very high and this indicated that GalN/LPS indeed induced hepatocyte damage. Furthermore, the NK activity treated with GalN/LPS was lower than that of GalN/LPS-untreated group (Fig. 1C). This finding is in agreement of other studies.[6,7] There were inverse relationships between the hepatic and splenic NK activities and the serum transaminase activities (Table II). This observation indicated that the more the NK cells were activated, the less the hepatocytes were damaged. Therefore, these results suggested that NK activation may lead to protection against the GalN/LPS-induced hepatic injury.

NKR-P1, which is a membrane protein on NK cells and has a lectin-like domain, is the receptor protein and has a recognition site for on oligosaccharide ligand, and it triggers NK cells for cytotoxicity.[19,25] Moreover, NK cells produced a great amount of IFN-gamma on cross-linking of NKR-P1.[26,27] Therefore, it is possible that CE has a conformation similar to a certain kind of oligosaccharide which triggers NK cytotoxicity as well as IFN-gamma production. In this study, when the levels of NK activity both in the liver and spleen were elevated by CE administration, CE might be an effective substance for NK augmentation.

In the present study, we administered GalN/LPS to induce acute hepatic injury in rats. This model of liver injury resembles human acute hepatitis.[28-30] In this treatment the amino sugar GalN is metabolized mainly in the liver and it blocks hepatic transcription and protein biosynthesis by depletion of uracil nucleotides. Then, the structure and function of the cell membrane may have been altered because of impaired membrane protein synthesis. LPS seems to sensitize the hepatocyte to the toxicity of GalN up to 100,000 fold when they were co-administrated. Consequently, the animals administered GalN/LPS develop severe acute hepatitis. Therefore, inhibition of the hepatic cellular protein biosynthesis by GalN/LPS seems to create a state similar to the pathophysiological state of human hepatitis. How, then, does activation of NK cells effectively protect the hepatocyte from the injurious effect of GalN/LPS? The study of Orange *et al.*[26] indicated that resistance of host cells to a virus was induced by NK cells after a brief exposure to IFN. Furthermore, Djue *et al.*[31] showed that the NK augmentation was blocked by actinomycin D, which is an inhibitor of new RNA and protein synthesis. Therefore, this study suggested that new RNA synthesis was required for transcription and translation into antiviral proteins or enzymes. From this result, they suggested that the augmentation of NK might induce synthesis of new cellular RNA and proteins which have protecting effects against attack.

In summary, we have shown that CE, an acidic polysaccharide extracted from the *Celosia argentea* L., augmented NK activity both in the liver and spleen, and this NK augmentation prior to the injection of GalN/LPS protect the hepatocytes. Therefore, we

suggest that CE might be an effective substance to protect the hepatocyte against acute hepatitis.

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和文抄録

Celosian (CE) は青箱子からの熱水抽出物である酸性多糖類であり，先の研究において我々は，CE がラットの肝臓および脾臓の Natural killer (NK) 活性を亢進させることを報告した。本研究では，CE によって保護された肝臓に化学誘発肝障害物質を投与したときの NK 活性の変化について検討した。化学誘発肝障害物質であるガラクトサミンと LPS の投与に対して，CE は保護効果を有することが示された。また，CE の投与により肝臓と脾臓の NK 活性はともに上昇し，さらに，肝臓と脾臓の NK 活性と血清 GOT と GPT の活性との間に負の相関関係がみられた。このことより，化学誘発肝障害の発症前に NK 活性を亢進させることが肝臓の保護に深く関与していることを考察した。

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