Effect of Celosian, a water extract from *Celosia argentea* L., on NK activity in rat liver and spleen

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Abstract

Celosian (CE), a water extract from *Celosia argentea* L., is an acidic polysaccharide which has been reported to have a hepatoprotective effect. In the present study, we investigated effects of CE administration on the hepatic and splenic natural killer (NK) activities in rats. Results showed that CE augmented NK activities in both the liver and spleen. This augmentative effect of CE was greater on hepatic NK activity. However, CE administration caused no increase in serum GOT or GPT activity. These results suggest that CE may act as a biological response modifier (BRM) and that the response of hepatic NK activity to CE may differ from that of splenic NK activity. Interestingly, an inverse relationship was seen between the NK activity and the serum GPT activity after CE administration in the liver. Since NK cells play an important role for host defense against hepatic cell damage, the augmentation of NK activity by CE administration may relate to this enzyme activity.

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

Abbreviations BRM, biological response modifier ; CE, Celosian ; GOT, glutamic oxaloacetic transaminase ; GPT, glutamic pyruvic transaminase ; LDH, lactate dehydrogenase ; NK, natural killer.

Introduction

Natural killer (NK) cells are a sub-population of large granular lymphocytes (LGLs) and contain dense azurophilic granules in the cytoplasm.[1] NK cells are considered to be involved in an important self-defense system : they are able to recognize and lyse various virally infected cells or neoplastic cells without previous sensitization or major histocompatibility complex expression.[2] Most studies have been focused on the function of NK cells isolated from the peripheral blood or spleen. Recently, however, there is more attention paid to organ-associated NK cells such as hepatic NK cells. Several studies have shown that these NK cells play important roles in defense mechanisms such as viral diseases and cancer.[3,4] Hepatic NK cells, also known as pit cells, were first described in 1976 by Wisse *et al.*[5] They are located within the lumen of the sinusoids, and are of the same cell type as LGLs or NK cells. Moreover, pit cells are larger and contain more rod-core vesicles and dense granules compared to other NK cells.[6,7] Several studies have indicated that pit cells were different from lymphoid NK cells in both morphology and cytotoxicity.[1,8,9]

Immunostimulating agents, also called biological response modifiers (BRMs), exert anti-viral or anti-tumor effects by stimulation of the immune system. These BRMs include the streptococcal cell wall preparation OK-432,[10] recombinant interleuken 2 [11] and polysaccharides.[12] Many bioactive polysaccharides from Chinese medical plants possess a variety of immunomodulating activities including stimulation of NK activity.[12,13] Several studies have indicated that NK activity is stimulated by poly- or oligosacchar-

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ides.[14 17]

Celosian (CE) is an acidic polysaccharide isolated from the water extract of seeds of *Celosia argentea* L., a member of the Amaranthaceae family. It has been reported that CE had hepatoprotective effects on liver injury models.[18,19] Intraperitoneal (ip) administration of CE inhibited the elevation of glutamic pyruvic transaminase (GPT) activity in the fulminant hepatitis induced by D-galactosamine/lipopolysaccharide in mice and in the hepatic injury induced by CCl_4 in rats. However, it is not known whether CE has any influence on the activity of pit cells or lymphoid NK cells. In this study, we examined effects of CE administration on the activities of hepatic and splenic NK cells.

Materials and Methods

Animals : 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 (Table I). A 12 hr light-dark cycle and a temperature between 22°C and 24°C were maintained. The animals had free access to food and water.

Table I Composition of the purified diet.

Ingredient	Composition (%)
Corn starch[1]	63
Corn oil[2]	5
Casein[3]	20
Mineral mixture[4]	5
Vitamin mixture[4]	2
Cellulose[5]	5

The diet components were purchased from [1]Nippon Kako Co., Ltd., Tokyo ; [2]Nihon Oil Co., Ohji ; [3]Oriental Yeast Co., Ltd., Tokyo ; [4]Oriental Mixture, Oriental Yeast Co., Ltd., Tokyo ; [5]Avicel® , Asahi Kasei Co., Osaka

Experimental procedure : After feeding on the purified diet for 2 weeks, 30 rats were equally divided into 5 groups of 6 animals each. CE, which was isolated from seeds of *Celosia argentea* L., was injected ip both 18 hrs and 2 hrs before the liver perfusion. CE was given at the dose of 200, 150, 100, and 50 mg/kg body weight to 4 experimental groups each time, and saline was injected to the control group. Isolation and characterization of CE has been described by Hase *et al.*[18]

Isolation of LGLs : LGLs were isolated from the liver using the modified procedure of Boewens *et al.*[20] 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. In this procedure, the portal vein and the inferior vena cava were cannulated with a 16G and a 18G catheter, respectively. The portal cannula was connected via a silicone tube to a Master Flex flow pump (Cole-Parmer Instrument. Co., Chicago, USA). First, the liver was perfused with a few ml of PBS at physiological pressure (10 cm water). The perfusion pressure was then increased to 50 cm water and 100 ml of effluent was collected.

The splenic lymphocyte isolation procedure[21] was as follows. Spleens were removed and cut into small pieces in Hanks' buffered salt solution (HBSS) supplemented with 3 % fetal bovine serum (FBS, Bio-Whittaker, Walkerville, USA). The cells were then dissociated between two frosted micro slide glasses and filtered through nylon mesh.

The cell suspensions from the liver and spleen were centrifuged at 250 × g for 5 minutes and resuspended in 3 ml of HBSS and carefully layered onto 3 ml of the Histo-Paque (density = 1.083 g/ml ; Sigma, St. Louis, USA). After centrifugation at 400 × g for 30 minutes at room temperature, the interface containing mononuclear cells was collected, washed twice in HBSS and resuspended in 2 ml of RPMI 1460 medium supplemented with 10 % FBS.

Nylon wool columns : Pre-washed 0.5 g of nylon wool (NW) was packed into 10 ml syringes. After these columns were washed with RPMI 1460 medium supplemented with 10 % FBS, the cells were incubated on the NW 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.

Cell line : The murine lymphoma cell line, YAC-1, was provided by Dr. Ken-ichi Takahashi of Osaka Prefectural Junior College of Health Sciences. The cells were maintained in RPMI 1460 medium supplemented with 10 % FBS, 3 % glutamine, and 3 % antibiotic solution (5,000 U penicillin and 5,000 μg

streptomycin per milliliter).

Cytotoxicity assay : The cytotoxicity assay of effector cells against YAC-1 target cells quantitatively measured lactate dehydrogenase (LDH), which is released upon cell lysis.[22,23] This enzyme assay has advantages over other methods of reliability, simplicity, speediness and avoidance of radioactivity. Released LDH in the supernatant was measured colorimetrically by coupled enzymatic assay which resulted in the conversion of tetrazolium salts into red formazan products. The amount of color formed was propotional to the number of lysed cells. The kit of Cytotox 96 (Promega Co., Madison, USA) was used for this assay. Briefly, 0.1 ml of effector cells were mixed with 0.1 ml of target cells on a 96-well round micro-titer plate (Iwaki glass, Chiba, Japan). The effector-to-target cell ratio was 20 : 1 for the liver and 20 : 1 or 50 : 1 for the spleen. After incubation at 37°C for 4 hrs in 5 % CO_2 humidified atmosphere and centrifugation at 250×g for 4 minutes, 0.05 ml of the supernatant was transferred to a 96-well flat micro-titer plate and LDH activity was quantified. To each well of the plate, 0.05 ml of the substrate mix was added and incubated at room temperature for 30 minutes. After adding 0.05 ml of stop solution to each well, the absorbance was measured at 490 nm using a micro plate reader. Cytotoxicity was calculated by the following formula :

$$\% \text{ cytotoxicity} = \frac{A - B - C}{D - C} \times 100$$

A : experimental release
B : effector spontaneous release
C : target spontaneous release
D : target maximum release

Serum GOT and GPT activities : Serum glutamic oxaloacetic transaminase (GOT) and GPT activities were determined as indicators of liver injury. Blood samples were collected from inferior vena cava before liver perfusion. These activities were assayed using the GOT-GPT CII kit (Wako Pure Chemical Industries, LTD. Tokyo, Japan.).

Statistical analysis : Data are expressed as mean values±S.E.M. Analysis of variance and t-test were performed to determine the significance ($p<0.05$). Pearson's correlation coefficient (r) was also used to evaluate associations.

Results

Serum GOT and GPT activities

CE administration at the doses of 50, 100, 150, and 200 mg/kg caused no significant increases in serum GOT and GPT activities (Table II). Since high levels of GOT and GPT are indications of hepatic injury, these findings suggest that CE probably caused no hepatocyte damage.

Table II Serum GOT and GPT activity in effect of different doses of Celosian.

Celosian treatment(mg/kg rat)	n	GOT activity (IU)	GPT activity (IU)
200	6	50.6±6.0	9.0±0.8
150	6	53.2±5.7	9.8±0.8
100	6	49.5±5.0	9.0±0.6
50	6	54.2±7.7	10.6±1.0
0 (control)	6	48.1±3.4	9.3±0.8

Data are expressed as mean±S.E.M.

Effect of CE administration on NK activity in liver and spleen

Because no dose-dependency in the effect of CE administration on the NK activity was seen in both liver and spleen (data not shown), the NK activities

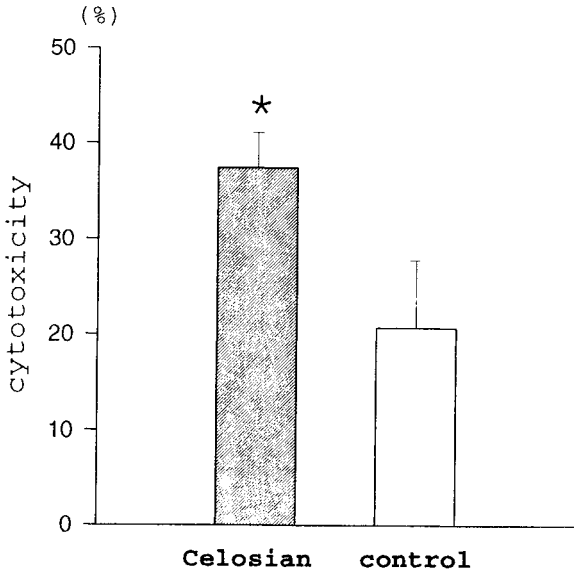

Fig. 1 Effect of Celosian treatment (pooled) on NK activity in liver.
*Significantly different from the control at $p<0.05$.

measured at the 4 different doses of CE were pooled and compared with that of the control group. The liver NK activity after administration of CE was higher than that of the control (Fig.1). The splenic NK activity after CE administration was also higher than that of the control group in both 20 : 1 and 50 : 1 effector-to-target ratios (Fig. 2). When NK activity of the liver was compared to that of the spleen, the hepatic NK activity was higher than the splenic NK activity after CE administration. However, the difference did not reach statistical significance when CE was not administered.

Relationship between serum GPT and NK activity

An inverse relationship was found between the serum GPT and hepatic NK activity levels in the CE administration group ($r=-0.62$). In contrast, such a relationship was not present in the control group (Fig. 3). There was no correlation between the serum GPT and splenic NK activities (data not shown). The serum GOT and NK activities were unrelated in both

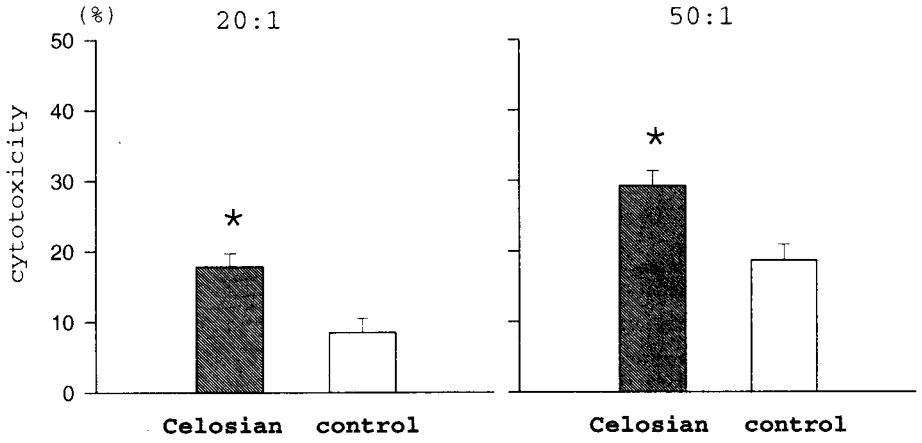

Fig.2 Effect of Celosian treatment (pooled) on NK activity in spleen.
(splenic lymphocyte : Yac-1 =20 : 1 and 50 : 1)
*Significantly different from the control at $p<0.05$

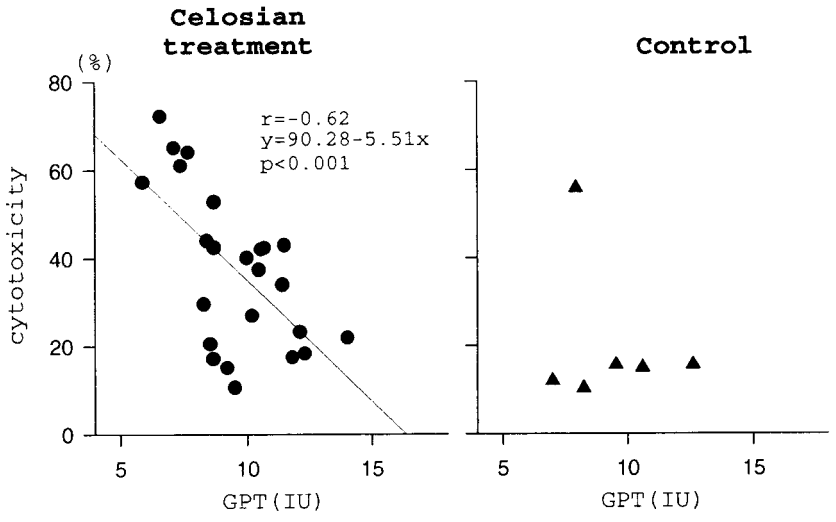

Fig.3 Relationship between serum GPT and Liver NK activity.

CE administration group and control groups (data not shown).

Discussion

The objective of this study was to investigate the effect of CE, an acidic polysaccharide isolated from the water extract of seeds of *Celosia argentea* L., on hepatic and splenic NK cell activities and serum hepatic enzymes. NK cells have been shown to be involved in important self-defense mechanisms.[2] It is well known that NK cells, including organ-associated NK cells, are activated by BRMs.[1] Several researchers have indicated that polysaccharides are immuno-stimulating agents and are thus considered to be BRMs. Many polysaccharides isolated from Chinese medical plants have been shown to possess a variety of immuno-modulating activities.[14,24,25] One of these activities is augmentation of NK activity.[25] Our study demonstrated that ip administration of CE augmented both liver and splenic NK activities (Fig. 1 and 2). However, this augmentation effect was not dose-dependent. The NK activity after administration with 50 mg/kg of CE was similar to that with 200 mg/kg in our study. This result indicated that the doses of CE we used were sufficient to induce NK augmentation in the liver and probably in the whole body. Moreover, the serum GOT and GPT activities after administration with 200 mg/kg of CE were not different from the control values (Table II). The absence of change in these transaminase activities indicated that the above doses of CE were not toxic to the liver.

It is not known how BRMs derived from bioactive polysaccharides stimulate NK cells. There are several hypotheses concerning the relationship between the activity and their chemical properties such as structure, molecular weight or acidity.[13] Among these, the structure of these polysaccharides and their interactions with NK cell receptors were investigated by several researchers. Vetvicka *et al.* found that the lectin site of the complement receptor type 3 on NK cells functioned as a β-glucan receptor and this interaction event triggered NK cell cytotoxicity without any proinflammatory activation.[25] There is another study that NK-P1, which is a membrane protein on NK cells and has an extracellular Ca^{2+}-dependent lectin domain, has an affinity site for oligosaccharide ligands.[17,26] The interaction of this membrane protein on NK cells with oligosaccharides on the target cells may play important roles in the activation of NK-mediated cytotoxicity.[17] Since CE also consists of a polysaccharide, there is the possibility that CE might have a similar conformation to these saccharide ligands which interact with the lectin site on NK cells. Although the sugar components of CE have been already analyzed,[18] their structures have not been elucidated.

Comparison has been made between the NK activities of lymphocytic and organ-associated cells after administration of BRMs. The study of Wiltrout *et al.* showed that NK activities of liver and lung were several-fold higher than those of blood and spleen in mice after administration with a BRM called maleic anahydride divinyl ether (MVE-2).[4] Xiao-chai-hu-tang (Shosaiko-to ; 小柴胡湯), a traditional Chinese medicine, augmented hepatic and peripheral blood NK activities but not splenic NK activity in mice.[14] In our study, hepatic NK activity was more than twice compared to that of the spleen when CE was administrated. Even though there was no statistical significance, liver NK activity was also three times higher than splenic NK activity in the control group. Vanderkerken *et al.*[8] found that hepatic LGLs were different from peripheral blood LGLs phenotypically and functionally, and were 5 to 8 times more cytotoxic than peripheral blood LGL against YAC-1 cells in normal rats. They considered that circulating LGLs, on entering the liver, were further differentiated or activated in the sinusoidal environment. Furthermore, Wiltrout *et al.* indicated that a major mechanism of augmentation of NK activity by BRMs in liver is through a rapid accumulation of LGLs derived from the bone marrow. Therefore, they suggested that BRMs, directly or indirectly, might regulate LGL production and activation.[4] It is highly possible that different BRMs may act differently on hepatic NK cells and lymphoid NK cells. We already mentioned that CE might interact with NK cells and stimulate their activity. If hepatic NK cells have been already differentiated or activated, CE administration might amplify their activity.

An interesting finding was that an inverse rela-

tionship was seen between the NK activity in liver and the serum GPT activity after CE administration, although the levels of GPT activity were all within a normal range (Fig.3). Furthermore, recent study from our laboratory also found that there was an inverse correlation between serum transaminase activity and NK activity in chemically induced hepatitis model (data not shown). Since NK cells have an important function for first line of host defense against hepatic cell damage,[6] it is hypothesized that the activated NK cells which are administrated CE might protect hepatocyte directly or indirectly.

In summary, we have shown that CE, an acidic polysaccharide which extracted from the Chinese medical plant, augmented NK activity in both the liver and spleen. Therefore, we suggest that CE may act as a BRM. Furthermore, hepatic NK activity was higher than splenic NK activity when CE was administrated. It is also suggested that hepatic NK cells might be more sensitive to BRMs than splenic NK cells.

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和文抄録

Celosian (CE) は青葙子からの熱水抽出物である酸性多糖類で，肝臓の保護作用を有する。本研究では，ラットの肝臓および脾臓の Natural killer (NK) 活性に及ぼす CE の影響を検討した。CE は肝臓と脾臓の NK 活性をともに上昇させ，脾臓よりも肝臓の NK 活性に対して影響が大きかった。これらの結果から (1) CE は biological response modifier の働きを有すること，(2) CE による NK 活性の上昇は臓器によって異なること，が示唆された。また，CE 投与時に肝臓の NK 活性と血清 GPT 値との間に負の相関関係がみられたことから，肝臓の NK 活性は血清 GPT 活性に関与している可能性について考察した。

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