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Augmentation of various immune reactivities of tumor-bearing hosts with an extract of *Cordyceps sinensis*

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Abstract

In order to enhance general reactivity of immune system in the tumor-bearing host, we employed *extract* of *Cordyceps sinensis* (CSE) **as** a biological response modifier. *Cordyceps sinensis* is an interesting material produced by a kind of mushroom parasitic to larval moths and was used to hasten recovery from exhaustion in ancient China.
In this experiment, C57BL/6 mice implanted subcutaneously with syngeneic EL-4 lymphoma cells were employed as the host. Oral administration of the extract leads to a reduction of tumor size and prolongation of the host survival time. As judged by plaque-forming cells against T-independent (sheep erythrocytes) and T-independent (bacterial lipopolysaccharide) antigens, CSE showed to augment the antibody responses. As for the activities of peritoneal macrophages, chemotaxis was dramatically depressed within a few days after EL-4 transplantation up to the end of life, but treatment with CSE at -14, -7, -4, +4, +7 an +/10 days after the tumor transplantation augmented the activity about four times stronger than that of control. Phagocytic activity of macrophages was also decreased in tumor-bearing mice treated with cyclophosphamide (100mg/kg) 3 and 5 days after tumor transplantation. But administration of CSE restored the activity to more than the normal level. The overall efficacy of CSE was tested with protective activity against systemic infection by *Salmonella enteritides.* The tumor-bearing mice receiving this medicine lived significantly longer than any other groups without CSE.

Introduction

It has been reported that in tumor-bearing animals, both tumor-specific [l] and non-specific immunities are depressed [2]. The former allows tumor cells to proliferate within the host, and the latter permits serious infectious diseases to occur, especially as opportunistic infections due to normally non-pathogenic microorganisms. A number of natural and synthetic biological response modifiers [3-8] have been used to treat the compromised immune state of tumor-bearing hosts Unfortunately, these effective BRMs tend to exhibit serious side effects. Therefore, attempts were made to find a substance having good direct and/or indirect effects on the tumor cell but the fewest side

effects. One famous oriental medicine, Cordyceps Sinensis (Be& Sacc., with the history longer than 3000-years of medical use, was selected in this study in the hope that it would exert an effect on a hemopoietic system and cause rapid recovery from exhaustion with few side effects. This material is famous for the unique climatic and geographical conditions in which it grows. It is produced by a mushroom parasitic to larval moths which are found only in the highlands of the Himalayan region. In this and subsequent studies, attempts are made to clarify the effect(s) of CSE on physical status of tumor-bearing hosts, especially on the immune system including lymphocytes and macrophages.

Materials and methods

Animals

Female syngeneic C57BL/6 mice at 6 weeks of age were purchased from an animal dealer (CLEA **JAPAN** Co. Ltd., Tokyo) and reared in our facility of Kanazawa Medical University in an environment free from specific pathogens (SPF).

Preparation of CSE

Dried samples were ground into powder using an electric mill. Then extraction was performed using Tris-HCI buffer (50 mM) containing 0.3 M NaCl (pH 7.0) at 40 C for 2 hr. After centrifugating at 15000 xg for 15 min. the supernatant was collected and dialysed with phosphate buffered saline (pH 7.0, 0.15 M) and aseptically prepared by a millipore filter. This extract was found to contain 28.5% of protein and 64.2% of sugar. The administered doses were adjusted according to the protein content.

Tumor and transplantation

Mouse lymphoma cells (EL-4) were maintained *in vivo* in C57BL/6 mice in ascites form. Tumor cells were collected from ascites. fluid seven days after intraperitoneal (1 p.) inoculation with I x 10^6 tumor cells, then washed twice with RPMI-1640 medium (Nissui Co. Ltd., Tokyo) and resuspended in the same medium at a concentration of 1 x 10^5 or 1 x 10^6/ml. To establish a tumor-bearing host, 0.1 ml of the tumor cell suspension was injected subcutaneously into the lumbar region.

Determination of tumor growth

Mice subjected to subcutaneous implantation of the tumor cells were sacrificed every week. Growth of their tumors was recorded by measuring the short and long diameters. The survival time of tumor-bearing mice was also monitored until 60 days after transplantation.

Estimation of macrophage activity by chemotaxis

Peritoneal macrophages were collected and purified in **a** conventional manner using fetal calf serum (FCS)-coated Petri-dishes [9]. The adherent cell population contained macrophages of about 97% uniform as judged by functional and morphological spection. These cells were applied to the nuclepore-membrane (pore size: 5 *microns,* Neuro Probe Co. Ltd., California. USA). of a chemotaxis chamber (Neuro Probe Co. Ltd., California, USA). After 90 min. incubation the membrane was washed vigorously with RPMI-1640 and saline (37 C), then fixed and stained with methylene blue dye solution [10]. After counting by a microscope the average number of migrating cells **was** expressed as cell number/mm^2.

Phagocytic activity

Sheep erythrocytes were used as the target cell. Briefty, the ratio was optimum at 1:10 for macrophages and target cells. Thirty minutes after mixing with phagocytes and target cells, the intracellular erythrocytes were counted and phagocytes which contained more than three erythrocytes were regarded as positive cells. In this way, the phagocytic ability of the

macrophages was monitored.

Antigens used

Sheep erythrocytes (SRBC; 2 x 10^8/mouse) and bacterial lipopolysaccharide (LPS; 1 µg/mouse) were employed as a T-dependent and T-independent antigen, respectively.

Detection of antibody, forming cells in the spleen

Five days after antigenic stimulation, the antibody secreting cells (PFC) were detected by the method of localized hemolysis in agar gel. PFCs were developed by the method of Jerne et al with slight modification [1 l].

Results

Effect of CSE on the survival of tumor-bearing mice

Animals were subjected to oral administration of CSE (50 mg/kg) on day -14, -7, -4, +4, +7 and +10 before and after tumor implantation (1 x 10^4/mouse) on day 0. The survivors were monitored up to 40 days tumor inoculation. Mean survival times in each group were 28.5 and 23 days in the CSE and the control group, respectively, indicating a significant prolongation of survival by CSE administered (Fig. I).

Days after tumor transplantation

Fig. 1. Prolongation of survival times of C57BL/6 mice bearing EL-4 tumor by CSE. CSE was administered orally into mice 6 times (on days -14, -7, 4, +4, +7 and +10), and EL-4 cells (1 x 10') were inoculated subcutaneously on day 0. Survival of mice was traced up to 40 days in experimental group given CSE (0) and control group given the same volume of distilled water (o). Each group consisted of fifteen mice.

Effect of CSE on *the survival of tumor-bearing mice combined with cyclophosphamide*

Four groups of mice were prepared to test the effect of CSE with or without combining cyclophosphamide (CPA) as a chemotherapeutic agent. In case of combination therapy, CPA was injected twice 3 and 5 days after the tumor implantation (100 mg/kg; i-p.). CSE was administered following the schedule shown in Fig. 1. But the number of tumor cells transplanted were 1 x 10^5 cells/animal in this experiment. As shown in Fig. 2, CSE alone was scarcely effective in such a high dose of tumor cell implantation. In spite of this circumstance, CPA treatment was effective for prolongation of the survival time in tumor-bearing mice and combination CSE with CPA was more effective than CPA alone as well as any other groups, indicating additive effect between CSE and CPA (Fig. 2).

Days after tumor transplantation

Fig. 2 Effect of combination of CSE with CPA on the survival of mice implanted with a high dose of tumor cells (1 x 10^5). CSE was given to mice following the schedule in Fig, 1. Cyclophosphamide (CPA) was injected 3 and 5 davs after the tumor implantation. (0) CPA+ CSE, (0) CPA control, (m) CSE and (0) distilled water control.

Augmentation of lymphocyte activity

Effect on antibody response to LPS

In order to test the effect of CSE upon B-cells in the tumor-bearing host, bacterial lipopolysaccharide (LPS; T-independent antigen) was injected into mice 10 days after tumor implantation and the numbers of anti-

LPS IgM and IgG PFC were enumerated 4 and 6 days after the intraperitoneal injection of antigen. CSE was administered 5 times following the schedule shown in Fig. 1. As shown in Fig. 3, CSE enhanced B-cell activity, especially recovered the depressed activity of PFC production by treatment with cyclophosphamide.

Fig. 3. Effect of CSE and PFC response against LPS in tumor-bearing CPA-treated mice. So as to inhibit tumor cell growth, CPA (100mg/kg) was injected i.p. 3 and 5 days after EL-4 cell implantabon. LPS was injected ten days after the tumor cell implantation. The experiment was done following the schedule in Fig. 1. PFCs were enumerated on 4 and 6 days after the antigenic stimulation. Brackts = mean + S.E.M.

Fig. 4. Effect of CSE on PFC response against SRBC in tumor-bearing CPA-treated mice. Experimental schedules were about the same as in Fig. 3 except that antigen was sheep erythrocytes (2 x 10⁷/mouse).

Effect on antibody response to SRBC

To test the effects on antibody response to SRBC(T-dependent antigen), CSE was administered orally 5 times following the schedule in Fig. 1. Then 2 x 108 cells of SRBC were injected intraperitoneally 10 days after tumor implantation. Four and 6 days later, PFCs were enumerated as shown in Fig. 4. On both days, generation of IgM and IgG antibody producing cells was significantly suppressed in the CPA-treated group. CSE recovered the PFC level to normal one in such CPA-treated group.

Effect on macrophage chemotaxis

The combination of C57BL/6 mice and EL-4 leukemia cells was an excellent model for monitoring compromised immune status especially in regard to macrophage function. In just two days after subcutaneous tumor cell transplantation there was a drastic depressure of macrophage chemotaxis. The depressure was constant up to the final stage of tumor growth. When CSE was administered orally, the chemotactic ability of macrophages was increased by about 4 times as compared to the normal level (Fig. 5).

Chemotactic activity(%)

Fig. 5. Effect of CSE on chemotactic activity of macrophages in tumor-bearing mice. The experimental schedule followed that of Fig. 1. One million cells of peritoneal macrophages were prepared from each group of mice 14 days after tumor implantation. They were applied to the upper room of a chemotactic chamber which was separated by a micropore membrane (pore size 5 urn). After 90 min incubation in the presence of human normal serum (as the chemotactic factor), migrated cells were counted in 10 entire fields of a microscope.

Effects on phagocytosis

In order to test the phagocytic abilities of macrophages, sheep erythrocyte was employed as the target cell. Plastic adherent cells consisting of 97% macrophages were mixed with erythrocytes for 30 min and then washed with EDTA-saline. As shown in Table 1, phagocytic activity was depressed in tumor-bearing and CPA-treated mice. But CSE administration to CPA-treated mice augmented the activity more than the normal level (Table 1).

Effects of CSE derivatives on the overall defense immunity

It has been suggested that the immune-compromised state of mice raised by tumor transplantation is directly related to failure of some abilities of macrophages such as chemotaxis, phagocytosis and/or intracellular killing ability. According to the above tests, treatment with CSE enhanced these macrophage activities to above the normal. Furthermore, the effect of CSE on overall defense ability was evaluated by the level of defense against bacterial infection in tumor-bearing animals.

Days after bacterial inoculation

Fig. 6. Effect of CSE on survival times after the infection with *Salmonella enteritides* in tumor-bearing mice. Then thousands of EL-4 cells were implanted subcutaneously into the lumbar region. CSE was orally administered by the same schedule in Fig. 1. Ten days after the tumor cell implantation, one thousand cells of Salmonella enteritides were injected into the peritoneal cavity. N=10 per group. The experiment included normal control (A), tumor-bearing (0) and CSE treated tumor-bearing group (0).

The infectious agent chosen ws a virulent strain of *Salmonella enteritides.* According to the result shown in Fig. 6., the tumor-bearing controls died earlier than the tumor-free controls. After oral administration of CSE, tumor-bearing mice lived longer than any other control groups of mice. The mean survival time in each group was 8.7 days as compared with 3.5 days in the tumor-bearing controls ($p < 0.01$).

Discussion

Attempt was done to clarify the nature of this Chinese medicine as a biological response modifier and host-mediated effect(s) on certain tumor-bearing hosts. This water soluble extract (CSE) mainly consisted of 64.2% of sugar and 28.5% of protein, suggesting glycoprotein in nature. Many interesting reports have been published about Chinese medicine or its purified materials showing that plant-glycoside, ginsenoside Rh2 and saikosaponin inhibit the growth of tumor cells when these drugs are mixed directly *in vitro* with the tumor cells [12]. It is interesting to note that these substances act not only as an inhibitor of cell growth but also act as an inducer of dedifferentiation for certain tumor cells, rendering certain tumor cells into normal functioning cells [5].

In this experimental system with EL-4 murine lymphoma cells, an interesting phenomenon is the drastic depression of macrophage abilities especially the chemotactic and digestive capacities. This may be the main reason for the susceptibility of the tumor-bearing host to opportunistic infection. Treatment with cyclophosphamide results in a more serious immunodeficient state. Other cancer therapies such as irradiation or surgery are regarded also as the cause of the immunosuppressive states [13]. Thus. there is an urgent need to find immuno-modulating substances with few side effects. When CSE derivatives were administered to immuno-compromised mice, macrophage chemotaxis was enhanced even by oral administration. It

was interesting to note that this medicine elevates macrophage activity above the normal. This effect may have some relationship to PFC production both for T-dependent and T-independent antigens [14]. As to the possible mechanism of this medicine, it may act as a stimulating factor for white blood cells leading to proliferation of both immunocompetent lymphocytes and macrophages. Further studies will be necessary to support this idea.

These results suggest that CSE may be of benefit in enhancing the immunity of tumor-bearing patients without significant side-effects.

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