Properties of the mouse peritoneal cells immunized with β-1.3-1.6 glucan produced by *Aureobasidium pullulans*

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Abstract

We previously succeeded in purifying β-1.3-1.6 glucan produced by the black yeast *Aureobasidium pullulans*, and reported that this β-1.3-1.6 glucan has unique immunological actions such as inducement of CD11b antigen expression and interleukin-8 (IL-8) production in the human monocyte like cell line, U937. In this study, we investigated some immunological properties of mouse peritoneal cells immunized with β-1.3-1.6 glucan. The peritoneal cell number immunized with β-1.3-1.6 glucan was significantly enhanced accompanied by morphological changes (spread formation). Furthermore, the peritoneal cells immunized with β-1.3-1.6 glucan acquired natural killer (NK) activity against YAC-1 lymphoma cells. In addition, it was found that double positive cells expressing both Mac-1 (macrophage specific antigen) and NK1.1 (natural killer cell specific antigen) on the peritoneal cells immunized with β-1.3-1.6 glucan were significantly enhanced using two-color flow cytometric analysis. These findings indicate that peritoneal cells immunized with β-1.3-1.6 glucan produced by *Aureobasidium pullulans* sufficiently acquired immunological activities.

Key words : *Aureobasidium pullulans*, β-1.3-1.6 glucan, NK activity, peritoneal cells

Introduction

Glucan is constituted from only glucose combined, and is divided into two types of α-glucan and β-glucan depending on the mode of bonding. β-glucan is contained in large amounts in mushrooms, cellulose and microbes such as black yeast [1, 2]. In particular, β-glucan produced by the black yeast *Aureobasidium pullulans* is found in quantities about ten fold or more than mushrooms, and plays a significant role in human health maintenance. The features of β-glucan consist of some unique physical properties and physiological activities, and are utilized partially as anticancer drugs by applying their physiological activities [3-5]. In general, the action of β-glucan is not due to their direct action on cancer cells as is the case with chemical anticancer drugs, but is due to the immunological enhancement of organisms as a biological response modifier (BRM) or biotherapeutic agent.

We previously succeeded in purifying β-1.3-1.6 glucan produced by the black yeast *Aureobasidium pullulans* with the latest cultural technology, and reported the

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immunological actions of this β-1.3-1.6 glucan using a human monocyte like cell line, U937 *in vitro* [6]. In this study, to refine and confirm the immunological actions of β-1.3-1.6 glucan *in vivo*, we investigated some immunological properties of mouse peritoneal cells immunized with β-1.3-1.6 glucan.

Materials and Methods

Animals

Eight to 10- week-old of male C57BL/6 mice were purchased from Japan SLC, Inc. (Hamamatsu, Shizuoka), and maintained in the animal care facilities at the Department of Laboratory Animal Science, Kitasato University School of Medicine.

Purification of β-1.3-1.6 glucan produced by *Aureobasidium pullulans* and immunization

Purification of β-1.3-1.6 glucan produced by *Aureobasidium pullulans* FERM-P4257 was performed by the method of Hayashi [7]. In immunization, β-1.3-1.6 glucan was injected *i.p.* (1.4 mg/mouse) at a volume of 0.3 ml for 5 successive days. Controls (non-immunized β-1.3-1.6 glucan) were injected *i.p.* with saline under the same conditions.

Cell line

The mouse lymphoma cell line, YAC-1 used in this study was cultured in RPMI 1640 medium (GIBCO) supplemented with 10 mM Hepes buffer, 2 mM glutamine,10% fetal calf serum (FCS) (GIBCO), and antibiotics (100 μg/ml streptomycin, 100 U/ml penicillin)(referred to as complete medium). The cells were used as target cells in the assay of natural killer (NK) activity.

Antibodies

Two monoclonal antibodies (mAbs), fluorescence isothiocyanate (FITC)-conjugated NK1.1 (natural killer cell specific antibody) and phycoerythrin (PE)-conjugated Mac-1 (macrophage specific antibody) were purchased from Cosmo Bio Co. (Tokyo, Japan).

Analysis of cell surface antigens

Cell surface antigens were analyzed by a direct immunofluorescence method. Cells were incubated with a PE-conjugated Mac-1 and an FITC-conjugated NK1.1 mAbs at optimum concentration for 40 min at 4 ℃, and then cells were washed twice with phosphate-buffered saline (PBS). The percentage of the positively stained cells was analyzed with the two-color flow cytometric method (FACScan, Becton Dickinson).

Preparation of peritoneal cells immunized with β-1.3-1.6 glucan

Peritoneal cells were obtained by lavage using cold PBS containing 10 μg/ml heparin from the peritoneal cavity of mice injected with or without β-1.3-1.6 glucan. After washing three times with PBS, the cells were suspended in the complete medium. The number of peritoneal cells was determined by the trypan blue dye exclusion method.

Cultures of peritoneal cells

Peritoneal cells ($1x10^5$) were suspended in the complete medium and plated on 24-well multi plates (Sumitomo Tokyo Japan), and then cultured for 1 hr at 37 ℃. After 1 hr, morphological characteristics of the peritoneal cells were observed and photographed with an Olympus camera body under a phase-contrast microscope (Olympus Tokyo Japan).

^{51}Cr release assay for natural killer (NK) cell activity

The peritoneal cells immunized with or without β-1.3-1.6 glucan were seeded in 96-well microtiter plates (Sumitomo Tokyo Japan). YAC-1 cells labeled for 2 hrs with ^{51}Cr (with sodium chromate, 37 MBq/ml; Dupon New Research Products, DE, USA) were washed with the complete medium and added to the wells and cultured for 4 hrs at 37 ℃. The amount of radioactivity in the supernatant was measured using a γ-counter (Beckman Instruments Inc., CA, USA) and percent specific lysis was calculated using the formula: % specific ^{51}Cr release=[(mean cpm experimental release - mean cpm

spontaneous release)/(mean cpm total releasable counts - mean cpm spontaneous release)]x100.

All experiments described herein were performed in compliance with the National Institutes of Health (NIH) guidelines for the use of research animals. The Animal Use Committee of the Kitasato University School of Medicine also approved the experimental protocol before the start of the study.

Statistical analysis

The results are shown as means±standard difference (SD). Statistical analysis was performed using the unpaired Student's *t* test. A difference was considered significant when the *P* value was less than 0.05.

Results and Discussion

In this study we investigated some immunological properties of the mouse peritoneal cells immunized with β-1.3-1.6 glucan produced by the black yeast *Aureobasidium pullulans*. As shown in Fig. 1, the number of peritoneal cells immunized with β-1.3-1.6 glucan was significantly enhanced compared with non-immunized cells ($P < 0.01$). This finding indicates that β-1.3-1.6 glucan effectively induced the promotion of cell division *in vivo*. Next, to examine the morphological characteristics of peritoneal cells immunized with β-1.3-1.6 glucan, these cells were incubated for 1 hr at 37 ℃ and observed under the phase-contrast microscope. Figure 2 shows that morphological changes were dramatically induced in the peritoneal cells immunized with β-1.3-1.6 glucan. In addition, these cells also strongly acquired the phagocytic activity of latex beads (data not shown). These data indicate that β-1.3-1.6 glucan has sufficient abilities to augment the immunological actions of peritoneal cells *in vivo*.

We examined whether the peritoneal cells immunized with β-1.3-1.6 glucan had NK activity against the lymphoma cell line, YAC-1. As shown in Fig. 3, the peritoneal cells immunized with β-1.3-1.6 glucan significantly acquired NK activity compared with non-immunized cells ($P < 0.01$). In addition, it was found that the expression of Mac-1 and NK1.1 antigens on the peritoneal cells immunized with β-1.3-1.6 glucan was also enhanced using two-color flow cytometric analysis (Fig.4). Taken together, these findings suggest that the cell population which acquired NK activity is composed of Mac-1+ and NK1.1+ double positive cells, and the β-1.3-1.6 glucan produced by the *Aureobasidium pullulans* possesses abilities of antitumor activity.

The immunological actions of polysaccharide (PS) have been investigated for a long time. In particular, the 6-branched 1.3-β-D-glucan isolated by mashroom (Agaricus blazei) has been medically and widely investigated in Japan [8]. Medicinal mushrooms are recognized as a source of antitumor and immunostimulation compounds, and comprise a vast source of powerful new pharmaceutical products. In particular, most of the clinical evidence for antitumor activity comes from the commercial polysaccharides lentinan, PSK (krestin), and schizophyllan[3, 4], but polysaccharides of some other promising medicinal mushroom species also show good results. Their activity is especially beneficial in clinics when used in conjunction with chemotherapy.

Recently, it was found that a major 6-branched 1.3-β-D-glucan from Sparassis crispa effectively shows antitumor activity accompanied by interferon-γ (IFN-γ) and interleukin-12p70 (IL-12p70) production[9]. Furthermore, it also became clear that Candida soluble β-D-glucan (CSBG), which is mainly composed of a linear β-1.3 glucan with a linear β-1.6-glucan, has several immunopharmacological actions as follows: (1) interleukin-6 (IL-6) production of macrophages *in vitro*; (2) antagonistic effect for zymosan mediated-tumor necrosis factor-α (TNF-α) synthesis of macrophages; (3) augmentation for lipopolysaccharide (LPS) mediated TNF-α and nitrogen oxide (NO) synthesis of macrophages; and (4) an adjuvant effect on antibody production [10-12]. Taken together, these results strongly indicate that 6-branched 1.3-β-D-glucan possesses various immunopharmacological activities suitable for clinical applications.

In this study, we provided the evidence that microbial polysaccharide (β-1.3-1.6 glucan) produced by the black yeast *Aureobasidium pullulans* also has some immunological actions *in vivo* and may possibly become an extremely important substance for clinical applications. Further studies will be necessary to analyze the detailed mechanism(s) for signal

transduction of β-1.3-1.6 glucan in cytokine production such as IFN-γ, TNF-α and IL-12 under antitumor activity and immunomodulation, and we are actively pushing forward with new studies in our laboratory.

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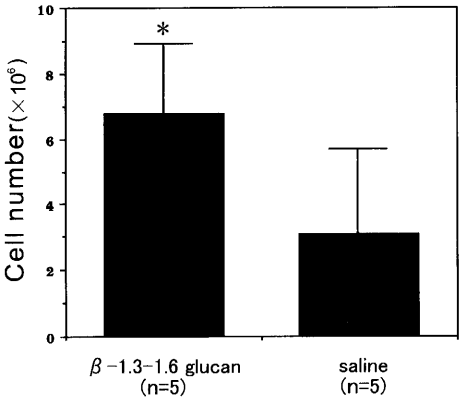

Figure 1. The number of peritoneal cells of C57BL/6 mice (n=5) immunized with β-1.3-1.6 glucan. The number of peritoneal cells was determined by the trypan blue dye exclusion method. The experiments were repeated at least three times. *Significant difference between β-1.3-1.6 glucan and saline ($P < 0.01$).

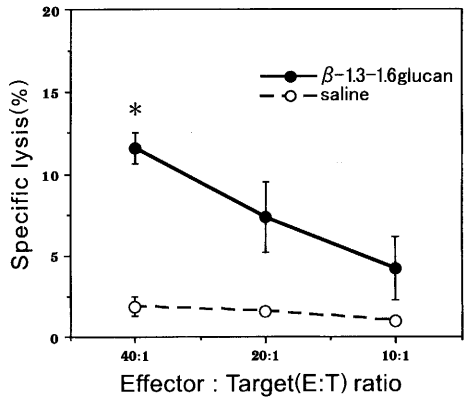

Figure 3. NK activity of the peritoneal cells of C57BL/6 mice (n=10) immunized with β-1.3-1.6 glucan. NK activity was determined by the ^{51}Cr release method. The experiments were repeated at least three times.
* Significant difference between β-1.3-1.6 glucan and saline ($P < 0.01$).

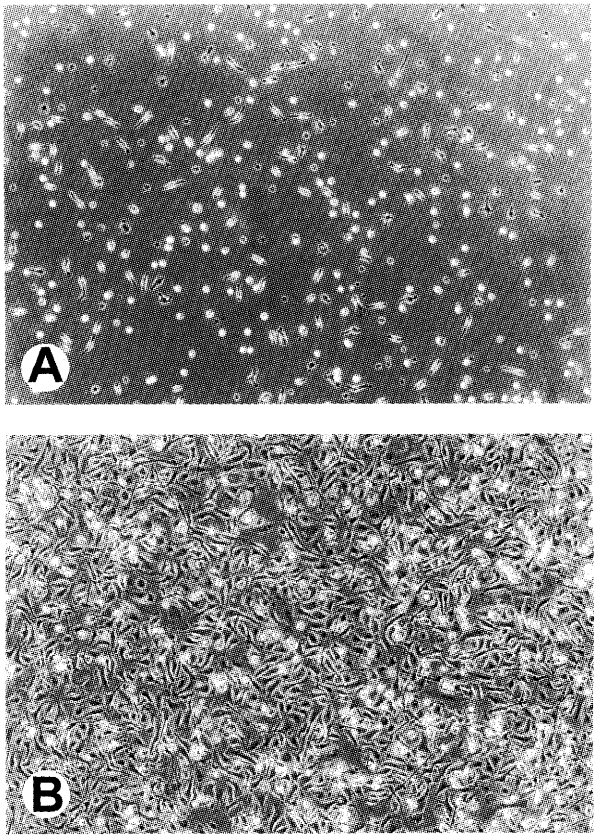

Figure 2. Morphological changes of the peritoneal cells immunized with β-1.3-1.6 glucan. Cells were cultured for 1 hr at 37 ℃. Morphological changes of the cells were observed and photographed under a phase-contrast microscope. The experiments were repeated at least three times. (A): immunized with saline, (B): immunized with β-1.3-1.6 glucan.

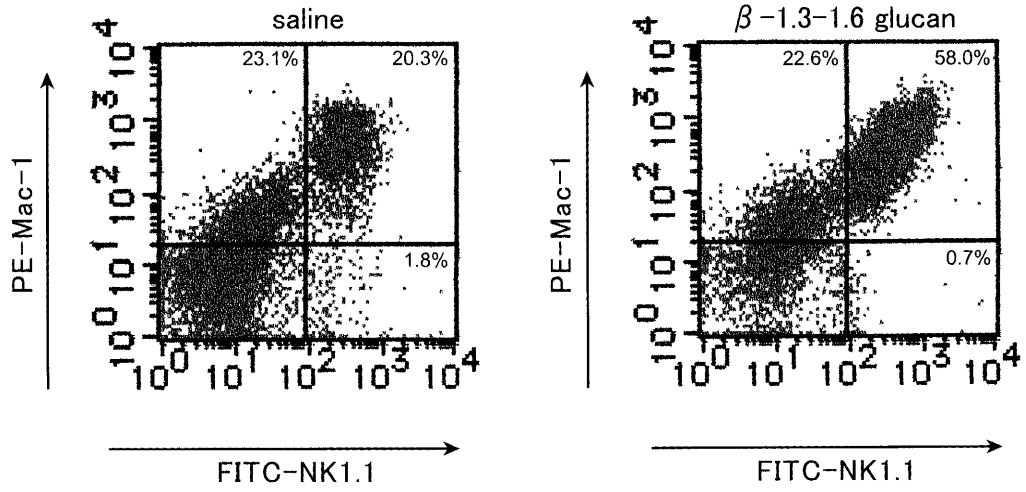

Figure 4. Expression of cell surface antigens on the peritoneal cells of C57BL/6 mice (n=5) immunized with β-1.3-1.6 glucan. Expression of Mac-1 and NK1.1 antigens were analyzed by two-color flow cytometry. Average percentages of positively stained cells are indicated in each panel. The experiments were repeated at least three times.

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Aureobasidium pullulans の産生するβ-1.3-1.6グルカンを免疫して得られたマウス腹膜腔内浮遊細胞の特徴

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要旨

我々は，β-1.3-1.6グルカンが免疫系の調節に密接に関わるインターロイキン8(IL-8)の産生を誘導することを見出し，本誌（3:5～9, 2002）に報告した．今回は，β-1.3-1.6グルカンをマウスに免疫して得られた腹膜腔内浮遊細胞の特徴について検討したところ，細胞数の増加，形態変化，さらにはNK活性（抗腫瘍作用）の上昇が認められた．また，two-color flow cytometry法からNK活性を獲得した細胞は，Mac-1+ NK1.1+の細胞群であることも判明した．この結果は，β-1.3-1.6グルカンがマウス腹膜腔内浮遊細胞に対して免疫能を付与したことを示すものである．

キーワード：*Aureobasidium pullulans*，β-1.3-1.6グルカン，NK活性，腹膜腔内浮遊細胞