Immunological responses of human peripheral blood mononuclear cells (PBMCs) derived from healthy donors cultured with β-1.3-1.6 glucan produced by *Aureobasidium pullulans*

Nobunao IKEWAKI *Noboru FUJII **Takashi ONAKA *** Hiromichi YAMAO ***Hiroshi INOUE

Abstract

We have succeeded in purifying a new β-1.3-1.6 glucan produced by the black yeast *Aureobasidium pullulans* (*A. pullulans*) FO68, and reported that this β-1.3-1.6 glucan has unique immunological actions such as interleukin-8 (IL-8) production in the human monocyte-like cell line U937 and the acquisition of natural killer (NK) activity in mouse peritoneal cells. In this study, we investigated some immunological responses of the β-1.3-1.6 glucan on human peripheral blood mononuclear cells (PBMCs) derived from healthy donors. The ^{3}H-thymidine incorporation rates (cell proliferation) of the human PBMCs cultured with β-1.3-1.6 glucan were significantly enhanced, and this enhancement was completely blocked in the presence of the CD11a (lymphocyte-function associated antigen-1: LFA-1) monoclonal antibody, mNI-58A. Furthermore, production of IL-8 into the culture supernatants from the human PBMCs cultured with β-1.3-1.6 glucan was also significantly augmented. These findings strongly indicate that the β-1.3-1.6 glucan produced by *A. pullulans* FO68 sufficiently enhanced some immune responses of human PBMCs.

Key words : *Aureobasidium pullulans*, β-1.3-1.6 glucan, CD11a, cell proliferation, IL-8, PBMCs

Introduction

Glucans formed by linking of many glucose molecules have several interesting properties, and are structurally divided into the two types of α-glucan and β-glucan depending on the mode of bonding. Glucan is contained in extracts of many species of mushrooms, and has some immunological activity capability [1-3]. In particular, β-glucan has been found to activate an anti-cancer action accompanied by the production of interleukin-2 (IL-2), interferon-γ (IFN-γ), interleukin-12 (IL-12) or tumor necrosis factor-α (TNF-α), and to lead cytotoxic activity against cancer cells [4-6]. These findings indicate that β-glucan enhanced the immunomodulating activity underlying the activation of human peripheral blood mononuclear cells (PBMCs) including lymphocytes, monocytes, macrophages, granulocytes, and NK cells. In general, the action of the β-glucan type of glucans is not due to their direct action on cancer cells as is the case with chemical anti-cancer drugs, which depends on the immunological enhancement of organisms such as a biological response modifier (BRM) [7].

Recently we have succeeded in purifying a β-1.3-1.6 glucan produced by the yeast *Aureobasidium pullulans* FO68 [8], which contains about ten fold the quantity of β-1.3-1.6 glucan compared with mushrooms. In this study, to refine the immunological actions of β-1.3-1.6 glucan as a BRM to human PBMCs, we investigated the

Department of Occupational Therapy, Kyushu University of Health and Welfare School of Health Science, and Institute of Immunology, 1714-1 Yoshino-cho, Nobeoka, Miyazaki 882-8508 JAPAN
*Seishin Ursura Gakuen Mission Junior College, 5-1-12 Midorigaoka, Nobeoka, Miyazaki 882-0863 JAPAN
**Sophy Corporation, 752-1 Ibe, Bizen, Okayama 705-0001 JAPAN
***Inoue Hospital, 1-990-1 Hirabaru-machi, Nobeoka, Miyazaki 882-0866 JAPAN

immunological responses of human PBMCs cultured with β-1.3-1.6 glucan such as ^{3}H-thymidine incorporation rates (cell proliferation) and IL-8 production.

Materials and Methods

Donors

We examined the human PBMCs derived from 20 healthy donors (mean age 38.3±8.6, 4 males and 16 females). Informed consent was obtained from all donors.

Reagents

A monoclonal antibody (mAb), mNI-58A[9] was established in our laboratories. An enzyme-immunoassay (EIA) kit for IL-8 assay was purchased from TFB Co. (Tokyo, Japan).

Preparation of human PBMCs

The human PBMCs obtained from 20 healthy donors were isolated by Ficoll-Paque (Pharmacia, Uppsla) density sedimentation as previously described[9].

Purification of β-1.3-1.6 glucan produced by *A.pullulans*

Purification of the β-1.3-1.6 glucan produced by *A. pullulans* FO68 was performed following Hayashi's method [10].

Cell proliferation assay cultured with β-1.3-1.6 glucan

Human PBMCs (5x10^4) in RPMI 1640 medium supplemented with 20% human serum (HS) (referred to as complete medium) were put into 96-well microplates (Sumitomo Co. Japan) and cultured with β-1.3-1.6 glucan (50μg/ml) in the presence and absence of mNI-58A (20μg/ml) for 5 days in a CO_2 incubator. Cell proliferation measured by ^{3}H-thymidine incorporation was assayed by liquid scintillation spectroscopy. Morphological changes of the cells cultured with or without β-1.3-1.6 glucan (50μg/ml) were observed under a phase-contrast microscope (Olympus, Japan).

Assay of IL-8

The amount of IL-8 in the culture supernatants of the human PBMCs cultured with or without β-1.3-1.6 glucan (50μg/ml) was assayed with an EIA kit.

Statistical analysis

The results shown are the mean rates of ^{3}H-thymidine incorporation (counts per minute: cpm)±standard deviation (SD) for the 20 human PBMCs cultures. 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 responses of the PBMCs derived from 20 healthy donors cultured with β-1.3-1.6 glucan (50μg/ml) produced by the black yeast *A.pullulans* FO68. As shown in Figure 1, β-1.3-1.6 glucan significantly enhanced ^{3}H-thymidine incorporation rates (cell proliferation) of the human PBMCs. The difference between the presence and absence of β-1.3-1.6 glucan was significant ($P<0.01$). These findings indicates that β-1.3-1.6 glucan as a mitogenic substance effectively induced the promotion of cell division (DNA-synthesis). Next, to examine the morphological characteristics of the human PBMCs cultured with β-1.3-1.6 glucan, these cells were incubated for 5 days at 37℃, and observed under a phase-contrast microscope. As shown in Figure 2, the morphological changes (blast formation) of human PBMCs cultured with β-1.3-1.6 glucan were dramatically induced. These findings indicate that β-1.3-1.6 glucan also has an ability to augment the immunological actions of the human PBMCs in the morphological level. In addition, the enhancement of ^{3}H-thymidine incorporation rates of the human PBMCs cultured with β-1.3-1.6 glucan was completely blocked in the presence of CD11a (lymphocyte-function associated antigen-1: LFA-1) mAb, mNI-58A (20μg/ml) at the optimum concentration (Fig.3). CD11a is a member of the β-2 integrin family of adhesion molecules. This molecule is mainly expressed on all lymphoid cells and plays an important role in the regulation of various immune responses, such as cell proliferation/differentiation and immunoregulatory cytokine production [11]. Our data strongly reveal that enhancement of ^{3}H-thymidine incorporation rates in the human PBMCs cultured with

β-1.3-1.6 glucan is mainly mediated by the CD11a (LFA-1) molecule.

We also measured IL-8 in the culture supernatant of the human PBMCs cultured with or without β-1.3-1.6 glucan with EIA kits. Figure 4 shows that β-1.3-1.6 glucan significantly enhanced IL-8 production (about two-fold) at 96 hrs, and the difference between the presence and absence of β-1.3-1.6 glucan was significant ($P<0.01$). By contrast, no other cytokines such as IL-1β, IL-6 and IL-12 (p70+40) were detected in the culture supernatant under the same conditions (data not shown). IL-8 is one of the most potent chemoattractants for neutrophils, and it is released by endothelial cells, human alveolar macrophages, bronchial epithelial cells, fibroblasts, mast cells, and neutrophils stimulated by various substances [12]. Although the detailed mechanism of IL-8 production from the human PBMCs in response to β-1.3-1.6 glucan remains largely unknown at the present time, it was recently reported that the synergistic action of fungal (1→3)-β-D-glucan schizophyllan (SPG) and platelets strongly induced IL-8 production by human PBMCs [13]. This finding suggests that platelets play a key role in the IL-8 production from the human PBMCs cultured with β-glucan.

The β-glucan isolated from the mashroom family has been medically investigated [14], and medicinal mushrooms are recognized as a source of anti-tumor, immunostimulation compounds, and pharmacological agents for clinical applications [15]. In this study, we provided the evidence that the β-1.3-1.6 glucan produced by *A. pullulans* enhanced some immunological responses of the human PBMCs as a BRM. Further studies will be necessary to analyze the detailed mechanism(s) of β-1.3-1.6 glucan at the molecular, cellular and clinical levels.

References

1 Hong, F., Hansen, R.D., Yan, J., et al.: Beta- glucan functions as an adjuvant for monoclonal antibody immunotherapy by recruiting tumoricidal granulocytes as killer cells. Cancer Res. 63: 9023-9031, 2003.

2 Sier, C.F., Gelderman, K.A., Prins, F.A., et al.: Beta-glucan enhanced killing of renal cell carcinoma micrometastases by monoclonal antibody G250 directed complement activation. Int. J. Cancer. 109: 900-908, 2004.

3 Ishurd, O., Zgheel, F., Kermagi, A., et al.: Antitumor activity of beta-D-glucan from Libyan dates. J. Med. Food. 7: 252-255, 2004.

4 Garcia-Lora, A., Pedrinaci, S., Garrido, F.: Protein-bound polysaccharide K and interleukin-2 regulate different nuclear transcription factors in the NKL human natural killer cell line. Cancer Immunol. Immunother. 50: 191- 198, 2001.

5 Young, S.H., Ye, J., Frazer, D.G., et al.: Molecular mechanism of tumor necrosis factor-alpha production in 1--> 3-beta-glucan (zymosan)-activated macrophages. J. Biol. Chem. 276: 20781-20787, 2001.

6 Koguchi ,Y., Kawakami, K., Kon, S., et al.: Penicillium marneffei causes osteopontin-mediated production of interleukin-12 by peripheral blood mononuclear cells. Infect. Immun. 70: 1042-1048, 2002.

7 Brown, G.D., Gordon, S. : Fungal beta-glucans and mammalian immunity. Immunity.19: 311-315, 2003.

8 Ikewaki, N., Fujii, N., Onaka, T.: A polysaccharide produced by *Aureobasidium pullulans* FERM-P4257 up-regulates expression of CD11b antigen and induces interleukin-8 production. J. Kyushu Univ. H. and W. 3: 5-9, 2002.

9 Ikewaki, N. : A novel human monoclonal antibody rapidly induces homotypic cell aggregation and promotes antibody-secreting activity by human B lymphoblastoid cell line IM-9. J. Clin. Immunol. 17: 127-139, 1997.

10 Hayashi, S., Hayashi, T., Takasaki, Y., et al.: Purification and properties of glucosyltransferase from *Aureobasidium*. J. Ind. Microbiol. 13: 5- 9,1994.

11 Shiina, M., Kobayashi, K., Mano, Y., et al.: Up-

regulation of CD11a (LFA-1) expression on peripheral $CD4^+$ T cells in primary biliary cirrhosis. Dig. Dis. Sci. 1209-1215, 2002.

12 Ikewaki, N., Yamada, A., Inoko, H.: Depolymerization of actin filament by cytochalasin E induces interleukin-8 production and up-regulates CD54 in the HeLa epithelial cell line. Microbiol. Immunol. 47: 775-783, 2003.

13 Suzuki, T., Tsuzuki, A., Ohno, N., et al.: Synergistic action of beta-glucan and platelets on interleukin-8 production by human peripheral blood leukocytes. Biol. Pharm. Bull. 140-144, 2002.

14 Ebina, T., Fuzimiya, Y.: Antitumor effect of a peptide-glucan preparation extracted from Agaricus blazei in a double-grafted tumor system in mice. Biotherapy. 11: 259-265, 1998.

15 Wasser, S.P.: Medicinal mushrooms as a souce of antitumor and immunomodulating polysaccharides. Appl. Microbiol. Biotechnol. 60:258-274, 2002.

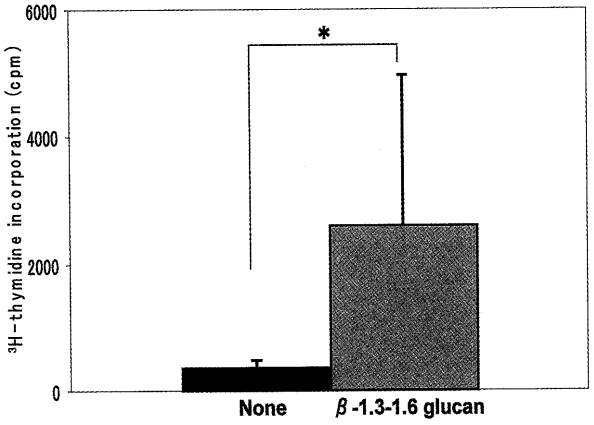

Figure 1. Human PBMCs (5x10^4) in RPMI 1640 medium supplemented with 20% HS were put into 96-well microplates and cultured with β-1.3-1.6 glucan (50μg/ml) for 5 days. Cell proliferation measured by ^{3}H-thymidine incorporation was assayed by liquid scintillation spectroscopy. The difference between the findings in the presence and absence of β-1.3-1.6 glucan is significant. *$P<0.01$

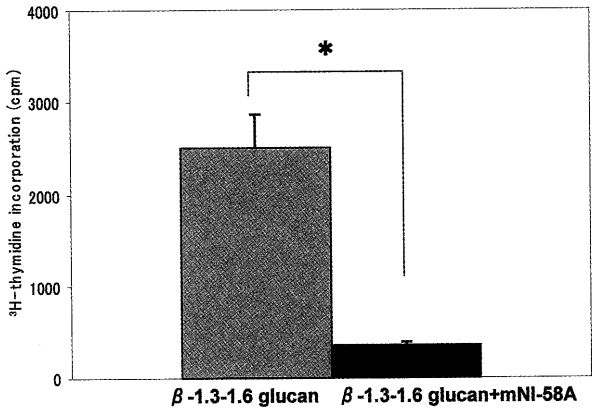

Figure 3. Human PBMCs (5x10^4) in RPMI 1640 medium supplemented with 20% HS were put into 96-well microplates and cultured with β-1.3-1.6 glucan (50μg/ml) in the presence and absence of CD11a (LFA-1) mAb, mNI-58A (20μg/ml). Cell proliferation measured by ^{3}H-thymidine incorporation was assayed by liquid scintillation spectroscopy. The difference between the findings in the presence and absence of mNI-58A is significant. *$P<0.01$

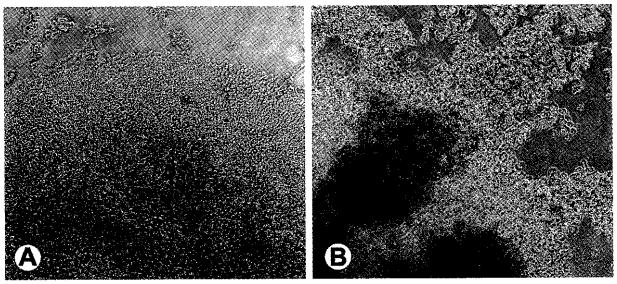

Figure 2. Morphological changes of the human PBMCs cultured with or without β-1.3-1.6 glucan (50μg/ml) for 5 days were observed under a phase-contrast microscope. A: without β-1.3-1.6 glucan. B: with β-1.3-1.6 glucan.

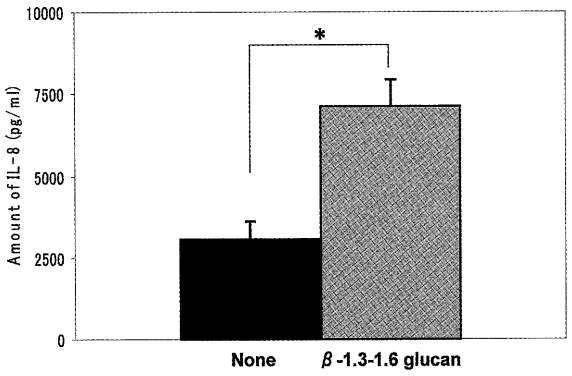

Figure 4. The amount of IL-8 in the culture supernatants of the human PBMCs cultured with or without β-1.3-1.6 (50μg/ml) glucan was assayed with an EIA kit. The difference between the findings in the presence and absence of β-1.3-1.6 glucan is significant. *$P<0.01$

J. of Kyushu Univ. of Health and Welfare. 6：193～198, 2005

*Aureobasidium pullulans*の産生するβ-1.3-1.6グルカンによって培養されたヒト末梢血単核球の免疫応答

池脇　信直　　*藤井　昇　　**尾仲　隆　　***山尾　裕道　　***井上　博

九州保健福祉大学保健科学部作業療法学科・免疫研究所　*聖心ウルスラ学園短期大学
**ソフィ株式会社　***井上病院

要　約

*Aureobasidium pullulans*によって産生されるβ-1.3-1.6グルカンは、ヒト末梢血単核球のトリチウムサイミジンの取り込み（細胞増殖）を有意に増強させた。この細胞増殖の増強は、CD11a（lymphocyte-function associated antigen-1: LFA-1）に対するモノクローナル抗体（mNI-58A）で完全に抑制された。さらに、β-1.3-1.6グルカンはヒト末梢血単核球のインターロイキン-8（IL-8）産生能も有意に増加させた。以上の結果は、β-1.3-1.6グルカンがLFA-1（CD11a）分子の経路を介してヒト末梢血単核球に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 glucan, CD11a, 細胞増殖, IL-8, ヒト末梢血単核球