Abstract. Objective: Curdlan, an extracellular bacterial polysaccharide, is a linear β-1,3-glucan. Previously, we developed Curdlan-oligo (CRDO). We investigated its effect on the production of cytokines in leukocytes from mice, and compared its activity with that of SCG, a 6-branched 1,3-β-glucan.

Methods: Splenocytes from DBA/2 mice were cultured with CRDO or SCG (0, 1, 10 or 100 μg/ml) in vitro, and then the supernatants were collected to measure cytokines. Bone marrow-derived dendritic cells (BMDCs) were cultured with CRDO (0, 1, 10 or 100 ng/ml) in vitro, and then the supernatant was collected to measure cytokines.

Results: SCG stimulated splenocytes in DBA/2 mice to produce GM-CSF, IFN-γ and TNF-α. CRDO induced production of GM-CSF and IFN-γ, but not TNF-α. The amounts of GM-CSF and IFN-γ were small compared with those produced in response to SCG. The effect of SCG on TNF-α production was partially inhibited by CRDO. In bone marrow-derived dendritic cells, CRDO induced production of TNF-α and IL-6.

Conclusion: Taken together, these results suggest that CRDO stimulated mouse leukocytes to induce the production of cytokines, and the mechanism of the effect of CRDO on leukocytes is different from that of SCG.

Key words: Glucan – Curdlan-oligo – TNF-α, IFN-γ

Introduction

Glucans are natural polysaccharides found in a wide variety of plants, algae, bacteria, fungi and yeast sources. Some β-glucans are well-known biological response modifiers. We and others have demonstrated that the immunomodulating activity of β-glucans is mainly related to their effects on immune effector cells, such as macrophages, mononuclear cells, and neutrophils involved in innate immunity, resulting in the production of cytokines [1, 2]. The body’s defense against microbial attack and against spontaneously arising malignant tumor cells comprises a dynamic orchestrated interplay of innate and acquired immune responses, and the effectors of innate immunity can initiate these systems. These findings indicated that β-glucan is an important player in both host defense against fungi and cancer immunotherapy.

SCG is a major 6-branched 1,3-β-d-glucan in Sparassis crispa Fr. showing antitumor activity [3, 4]. SCG enhanced the hematopoietic response in cyclophosphamide-treated leukopenic mice [5, 6]. SCG stimulated leukocytes to produce cytokines in preparations of human peripheral blood mononuclear cells [7], splenocytes [8] and bone marrow-derived dendritic cells (BMDCs) [9] from mice. These results demonstrated that SCG could enhance immune responses in vivo and in vitro. The study of glucans has been confounded by the presence of endotoxin in glucan preparations. No endotoxin was detected in SCG with endospecy (<30 pg/mg) [10]. So SCG is also used as a purified soluble β-glucan in investigations on cellular receptors and molecular mechanisms [11].

Curdlan from Alcaligenes faecalis var. myxogenes, has unique rheological and thermal gelling properties, with applications in the food industry and other sectors. Curdlan is structurally the simplest of the β-1,3-α-glucans with no glycosyl side chains [12]. Curdlan is slightly soluble in water when its molecular weight is relatively high (ca. >8000) and insoluble in alcohols and most organic solvents, but dissolves in dilute basic (0.25 M NaOH), dimethylsulphoxide (DMSO), formic acid and aprotic reagents such as N-methylmorpholin-N-oxide and lithium chloride in dimethylacetamide [13]. Curdlan’s conspicuously unusual rheological properties among natural and synthetic polymers underlie its
use as a biothickening and gelling agent in foods. Its safety has been assessed in studies with animals and tests in vitro and it is approved for use in Korea, Taiwan and Japan as an inert dietary fiber and is registered in the United States as a food additive [14]. Curdlan, like other β-1,3-glucans, has medical and pharmaceutical potential. The reported immunomodulating and pharmacological responses include antitumorigenicity, anti-infective activities against bacterial, fungal, viral and protozoal agents, anti-inflammatory activity, wound repair, protection against radiation, and anti-coagulant activity [13]. The effectiveness of curdlan in eliciting these responses depends on chemical structure, molecular mass, and conformation [1]. Previously, we have successfully developed Curdlan-oligo (CRDO) which is soluble in water (Japanese patent No. 10-194977). CRDO could induce proliferation of T cells and B cells in the presence of ConA or LPS (Japanese patent No. 10-194977). These results suggested that CRDO has immunomodulating activities.

We have found that there are strain differences in the reactivity of mice to β-glucan, and that DBA/1 and DBA/2 mice are highly sensitive to β-glucan in vitro [4, 8] and in vivo [15]. SCG induced leukocytes from DBA/1 and DBA/2 mice to produce large amounts of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-12p70 (IL-12p70), and granulocyte macrophage – colony stimulating factor (GM-CSF) in vitro, and GM-CSF is one of the key factors in the reactivity to β-glucan [16, 17]. The expression level of the β-glucan receptor, dectin-1, on BMDCs in DBA/2 mice was highest in DBA/2 among C57BL/6, BALB/c, C3H/HeN and C3H/HeJ mice [9]. The finding suggests DBA/2 mice to be a useful model for studying β-glucan. We also reported that the pattern of the response of the leukocytes differed between soluble glucan and particulate glucan [4]. In this study, we investigated the activity of CRDO in leukocytes from DBA/2 mice, and compared it with that of SCG.

**Materials and methods**

**Animals**

DBA/2 male mice between 6 and 7 weeks of age were purchased from Japan SLC, Shizuoka. The experimental protocol was approved by the Committee for Animal Care and Use of Tokyo University of Pharmacy and Life Science. Mice were maintained under specific pathogen-free (SPF) conditions, at 23 ± 1 °C, with a constant humidity and light and 12 h of light and 12 h of dark, and had free access to food and tap water according to the Guidelines for Experimental Animal Care issued by the Prime Minister’s Office of Japan.

**Preparation of CRDO**

To prepare CRDO, a suspension of Curdlan (30 g) derived from *Alcaligenes faecalis var myxogenes* in a 85 % formic acid solution (3L) was heated at 90 °C for 20 min. After cooling until room temperature, the resulting solution was concentrated in vacuo and neutralized by 5N NaOH. The reaction mixture was centrifuged to collect the soluble fraction, soluble fraction were boiled for 120 min to remove formyl groups, then neutralized by 2N NaOH. The resulting solution was dialyzed against distilled water, and then lyophilized (Japanese patent No. 10-194977). The CRDO solution was prepared by dissolving the lyophilized powder in saline and autoclaving. The average molecular weight of CRDO was 340 ~ 4000 when estimated by HPLC.

**Preparation of SCG**

Fruit bodies of *Sparassis crispa* were cultured by Minahalth Co. (Saitama, Japan). SCG was prepared as previously described [5]. Briefly, air-dried and powdered *S. crispa* was extracted with cold alkali (10 % NaOH/5 % urea, 4 degrees, 2 days). The extract dissolved in 8 M urea was applied to a DEAE Sephadex A25 (Cl-) column equilibrated with 8 M urea, and the pass-through fraction was collected and extensively dialyzed against tap and distilled water, and then lyophilized (elemental analysis C : H : N = 40.06 : 6.77 : 0.08). The SCG solution was prepared by dissolving the lyophilized powder in 0.5 N NaOH, followed by immediate dialysis against saline for 3 days. The dialyzed fraction was then autoclaved and frozen until used.

**Materials**

Twen 20 was purchased from Wako Pure Chemical Co. (Osaka, Japan). Hank’s balanced salt solution (HBSS) was purchased from Nissui Seiyaku Co., Ltd. (Tokyo, Japan). Gentamycin sulfate, RPMI 1640 medium, and bovine serum albumin (BSA) were from Sigma Chemical Co. (St. Louis, MO). Fetal calf serum (FCS) was from Sanko Junyaku Co., Ltd. (Tokyo, Japan). Recombinant mouse GM-CSF and recombinant mouse IL-4 were from BD Biosciences (USA).

**Preparation of bone marrow-derived dendritic cells (BMDCs)**

Bone marrow was removed from mice. Bone marrow cells obtained by flushing femoral shafts were suspended in HBSS containing 50 μg/ml gentamycin sulfate. After centrifugation, the single cell suspension was treated with an ACK-lysing buffer (8.29 g/l NH₄Cl, 1 g/l KHCO₃, 37.2 mg/l EDTA/2Na) to lyse the red blood cells. After further centrifugation, the cells were maintained in RPMI 1640 medium supplemented with 50 μg/ml gentamycin sulfate containing 5 % heat-inactivated FCS, 10 ng/ml recombinant mouse GM-CSF, and 5 ng/ml recombinant mouse IL-4 and cultured in 24-well flat-bottomed plates at 1 × 10⁵ cells per well in 1 ml of culture medium at 37 degrees in a humidified 5 % CO₂: 95 % air atmosphere. Nonadherent and loosely adherent cells were removed by pipetting on day 2 and replated with fresh cytokine-containing media in the plate. CRDO was added on day 5. On day 7, culture supernatant was collected.

**Preparation of splenocytes**

Splenocytes were prepared as previously described [8]. Briefly, the spleen was teased apart in RPMI 1640 medium, and after centrifugation, the single cell suspension was treated with ACK-lysing buffer (8.29 g/l NH₄Cl, 1 g/l KHCO₃, 37.2 mg/l EDTA/2Na) to lyse the red blood cells. After further centrifugation, the cells were maintained in RPMI 1640 medium supplemented with 50 μg/ml gentamycin sulfate and 10 % FCS. Cells were cultured in 24-well flat-bottomed plates, and stimulated with CRDO or SCG.

**CellTiter 96 (R) AQ⃣ aqueous One Solution Cell Proliferation Assay**

Splenocytes were cultured in 96-well flat-bottomed plates, and stimulated with CRDO or SCG. After 24 h incubation, CellTiter 96 AQ⃣ aqueous One Solution Reagent (Promega, USA) was added to each well. After 1, 2, 3 and 4 h at 37 degrees in a humidified 5 % CO₂: 95 % air atmosphere, the absorbance at 490 nm was recorded.