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Immune stimulating properties of a novel polysaccharide from the medicinal plant *Tinospora cordifolia*

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Abstract

An α-D-glucan (RR1) composed of (1→4) linked back bone and (1→6) linked branches with a molecular mass of >550 kDa and exhibiting unique immune stimulating properties is isolated and characterized from the medicinal plant *Tinospora cordifolia*. This novel polysaccharide is noncytotoxic and nonproliferating to normal lymphocytes as well as tumor cell lines at 0–1000 μg/ml. It activated different subsets of the lymphocytes such as natural killer (NK) cells (331%), T cells (102%), and B cells (39%) at 100 μg/ml concentration. The significant activation of NK cells is associated with the dose-dependent killing of tumor cells by activated normal lymphocytes in a functional assay. Immune activation by RR1 in normal lymphocytes elicited the synthesis of interleukin (IL)-1β (1080 pg/ml), IL-6 (21,833 pg/ml), IL-12 p70 (50.19 pg/ml), IL-12 p40 (918.23 pg/ml), IL-18 (27.47 pg/ml), IFN- γ (90.16 pg/ml), tumor necrosis factor (TNF)-α (2225 pg/ml) and monocyte chemoattractant protein (MCP)-1 (2307 pg/ml) at 100 μg/ml concentration, while it did not induce the production of IL-2, IL-4, IL-10, interferon (IFN)-α and TNF-β. The cytokine profile clearly demonstrates the Th1 pathway of T helper cell differentiation essential for cell mediated immunity, with a self-regulatory mechanism for the control of its overproduction. RR1 also activated the complements in the alternate pathway, demonstrated by a stepwise increase in C3a des Arg components. Incidentally, RR1 stimulation did not produce any oxidative stress or inducible nitric oxide synthase (iNOS) in the lymphocytes or any significant increase in nitric oxide production. The water solubility, high molecular mass, activation of lymphocytes especially NK cells, complement

Abbreviations: BRM, biological response modifier; CTL, cytotoxic lymphocyte; CD, cluster differentiation; FITC, fluorescein isothiocyanate; GSH, reduced glutathione; GSSG, oxidized glutathione; IFN, interferon; LPS, lipopolysaccharide; MCP, monocyte chemoattractant protein; MWCO, molecular weight cut off; NK, natural killer; NMR, nuclear magnetic resonance; NO, nitric oxide; PBS, phosphate-buffered saline; RPMI, Roswell Park Memorial Institute; SARS, severe acute respiratory syndrome; TNF, tumor necrosis factor.

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activation, Th1 pathway-associated cytokine profile, together with a low level of nitric oxide synthesis and absence of oxidative stress confer important immunoprotective potential to this novel α-D-glucan.

Keywords: Immune stimulation; α-Glucan; Th1; *Tinospora cordifolia*; Complement

1. Introduction

The possibility of bioterrorism, outbreak of severe acute respiratory syndrome (SARS) and Bird Flu virus, continuing spread of HIV/AIDS and emergence of resistant pathogenic strains against current medications compel investigators to look for new protective measures against these threats. Immune activation is an effective as well as protective approach against emerging infectious diseases [1]. Although immune system possesses a wide array of microbial detection and host defense mechanisms, pathogen evasion of the immune surveillance and destruction is a frequent occurrence. This escape of immune surveillance is also an important characteristic of several malignancies [2].

The fundamental role of innate immunity in host defense is becoming clearer as analysis of the human genome continues to identify new genes serving innate immune function. The innate immune system detects the pathogens or the nonself intruders using specific receptors and responds immediately by activation of the immune competent cells, synthesis of cytokines and chemokines, and release of inflammatory mediators to eliminate or contain the intruders. Innate immune activation also triggers and paves the way for adaptive immune response by antigen-specific T and B lymphocytes. The natural killer (NK) cells play a decisive role in the innate immune defense against virus-infected and malignant cells by virtue of their ability to recognize and destroy abnormal cells [3,4]. Cytokines play crucial roles in regulating various aspects of immune responses. Among cytokines, interleukin (IL)-12 plays a central role in coordinating innate and cell-mediated adaptive immunity [5]. Immune stimulation can provide both prophylactic as well as postexposure protection [6,7]. Usually, these protective measures are correlated with synthesis of IL-12 and interferon (IFN)-γ, the cytokines of Th1 pathway of T cell differentiation associated with the adaptive immune system [8]. Several compounds that activate immune system such as microbial lipopolysaccharides (LPS), double-stranded RNA and DNA oligonucleotides containing unmethylated CpG motifs, have been reported earlier [1,9]. Stimulation of multiple receptors is reported to exhibit synergistic effect in cytokine production [10]. Complement activation cascade is another integral part of the immune system in which the cellular pathogens like intracellular bacteria are coated with complement components (opsonization) and readily undergo phagocytosis. Thus, complement activation plays an important role in microbial killing and is essential for transport and clearance of immune complexes.

Polysaccharides are known immune stimulants of which β-glucans have recently received considerable attention. β-Glucans or other biological response modifiers (BRMs), because of their structural identity with the conserved "pathogen-associated molecular pattern" activate the immune system by binding to specific receptors (pattern recognition receptors) of the innate immune system and stimulate the phagocytic, cytotoxic, and antimicrobial activities by the synthesis and release of cytokines, chemokines and reactive oxygen and nitrogen intermediates [11,12]. Stimulation by particulate β-glucans can also enhance the ability of macrophages to recognize and clear apoptotic cells through upregulation of the polysaccharide receptors [13]. Specific β-glucan receptors have been reported on various immune cells including monocytes, macrophages, neutrophils, eosinophils, NK cells as well as nonimmune cells including endothelial cells, alveolar cells and fibroblasts [12]. β-Glucans induce proinflammatory and antimicrobial responses such as tumor necrosis factor (TNF)-α and IL-12 synthesis, required for IFN-γ production

and polarization of T helper cells to Th1 pathway [14]. β-Glucans have also been found to synergize with monoclonal antibodies to suppress and/or eradicate tumors [15,16]. Yeast β-glucan has already been demonstrated to have significant degree of protection against anthrax (*Bacillus anthracis*) infection in a mouse model [17]. Furthermore, exogenous IFN-γ and IFN-α/β are reported to modulate host's innate immune response in human macrophages, for improving the cell viability and reducing the number of germinated intracellular spores of anthrax [18].

The immunomodulatory effects of β-glucans are influenced by the molecular mass, chain length, degree of branching, tertiary structure and solubility of the polymer. Although no consensus could be made on the structure–activity relationship [19], the (1→3)-β-glycosidic linkage has been described as an explicit requirement for biological activity [20]. However, Bao et al. [21] recently reported the isolation, characterization as well as in vitro and in vivo lymphocyte proliferation and antibody production by a branched (1→3)-α-D-glucan. Water solubility and lower degree of branching were found to be desirable requirements for biological activity based on the immunological properties of its derivatives. This observation along with the immunological properties of α-(2→8) linked sialic acid as well as that of heteropolysaccharides having α conformations in the main chain [22,23] dispute the explicit requirement of (1→3)-β-glycosidic linkage for the biological activity. In this paper, we describe a novel polysaccharide (RR1), an α-glucan, exhibiting unique immune boosting properties. RR1 is isolated from a medicinal herb, *Tinospora cordifolia* (family Minispermaceae), well known in the Indian *Ayurvedic System of Medicine* for anti-inflammatory, antiallergic, antiarthritic, antioxidant and immune stimulating properties [24–28].

2. Materials and methods

2.1. Isolation and characterization of RR1

Our preliminary investigations with extracts of the *T. cordifolia* powder, purchased from Garry and Sun, Reno, NV revealed that the immune stimulating principle is confined in the aqueous fraction. The aerial part of the plant was air dried and powdered for RR1 extraction. The procedure adapted for the isolation is illustrated in the flow chart (Fig. 1). The final compound RR1 was isolated in about 0.1% yield of the total dry material used for extraction as a puffy solid that dissolved in water. The initial analysis of the final product in our laboratory and the ^{13}C nuclear magnetic resonance (NMR) spectra revealed that it is a polysaccharide. Therefore, detailed polysaccharide analyses such as the glycosyl composition, linkage, molecular weight and conformation of the glucose units at the anomeric center were performed at the Complex Carbohydrate Research Center, University of Georgia, Athens, GA. The glycosyl composition analysis was done by the combined Gas Chromatography/Mass Spectrometry (GC/MS) of the per-*O*-trimethylsilyl derivatives of the monosaccharide methyl glucosides obtained from RR1 by acidic methanolysis according to the method of York et al. [29]. Inositol was used as internal standard in this analysis. The monosaccharide derivatives were identified by their characteristic retention time and further authenticated with their mass spectra. To perform glycosyl linkage analysis, the sample was permethylated thrice by the method of Ciucanu and Kerek [30], hydrolyzed with 2 M trifluoroacetic acid, reduced with $NaBD_4$ and acetylated with acetic anhydride/pyridine and the resulted partially methylated alditol acetates (PMAA) were analyzed by GC/MS. The sugar residues were identified by their characteristic retention times and mass spectral data. The conformations at the anomeric center of the glucopyranosyl units were obtained based on the 500-MHz proton NMR spectra recorded in D_2O and by comparison with the anomeric proton signals of standard samples. The molecular mass was obtained from the size exclusion chromatography using Supelco silica column (1.0×30 cm), eluting with 50 mM ammonium formate buffer at a rate of 0.5 ml/min and detected by the refractive index. Dextran samples were used as standards and the molecular mass was obtained by comparing the retention time of the eluted peak with the standards.

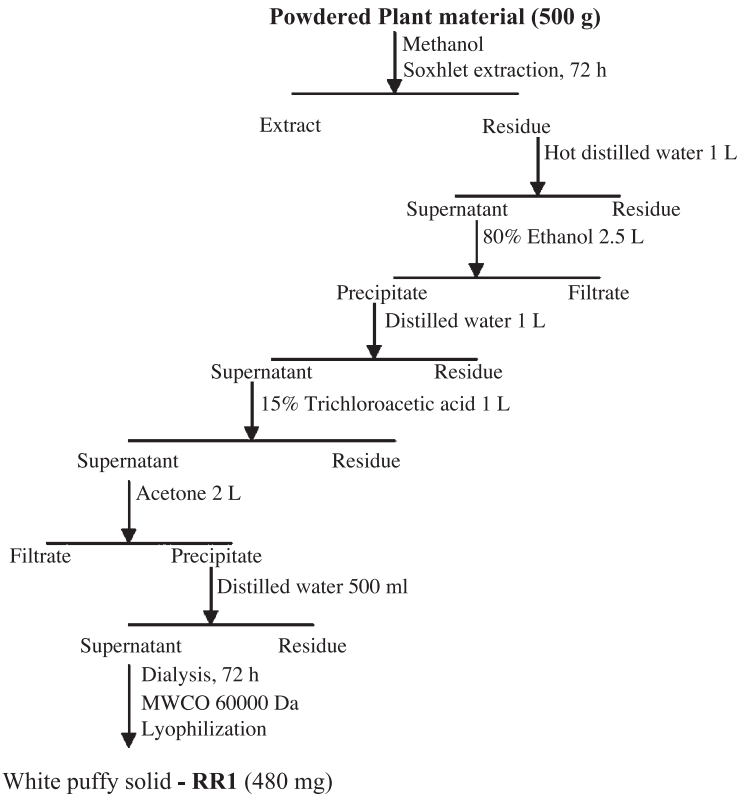

Fig. 1. Flow diagram showing the steps in the isolation of RR1.

2.2. Immune stimulating properties

RR1 samples were tested for any endotoxin contamination by the Limulus Amoebocyte Lysate (LAL) assay before the immune stimulation experiments and tests showed insignificant levels. To measure the immune stimulating property of RR1, we analyzed the activation of the different subsets of lymphocytes, syntheses of cytokines such as interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-10, IL-12 p70 and p40, IL-18, interferon (IFN)-α and γ, tumor necrosis factor (TNF)-α and -β, monocyte chemoattractant protein (MCP)-1, synthesis of nitric oxide (NO) and the extent of oxidative stress elicited in human lymphocytes. Normal lymphocytes were isolated by histopaque 1077 density gradient method from fresh blood drawn from healthy volunteers and utilized for various assays. Human leukemic (CEM) and multidrug resistant (CEM/VLB) cell lines were grown in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum and antibiotics in a 5% CO_2 incubator at 37 °C.

2.3. Activation of lymphocytes

Normal lymphocytes (10^6/ml) were treated with 0–100 μg of RR1 for 24 h in a CO_2 incubator maintained at 37 °C in RPMI medium. The cells were then stained with specific fluorochrome-conjugated monoclonal antibodies for 30 min at room temperature and analyzed in a Coulter Elite Flow Cytometer by four- and five-color immunotyping assay protocol of Alamo and Melnick [31]. The percentage of activation of lymphocyte subsets such as NK, T and B cells was calculated.

2.4. Cytotoxicity of RR1-treated lymphocytes

To evaluate the enhanced cytotoxicity of activated lymphocytes, a functional assay was adopted

using RR1 activated lymphocytes as effecter cells and human leukemic cells (CEM) as targets [32,33]. Briefly, normal lymphocytes (10^6/ml) were treated with different concentrations of RR1 in RPMI medium for 24 h in a CO_2 incubator at 37 °C. On the next day, target cells (CEM 1×10^6/ml) were labeled with 4.6 μM membrane labeling dye PKH26 (Sigma) in 1 ml phosphate-buffered saline (PBS) at room temperature for 3 min. The labeling was stopped by adding an equal volume of fetal bovine serum (GIBCO, Life Sciences, MD) for 1 min. The labeled tumor cells were then incubated with RR1 treated lymphocytes in an effecter to target ratio 1:1 for 4 h and untreated lymphocytes were used as control. The percentage of cells killed by the activated NK cells was determined by fixing the mixture with 1 ml of 2% Para formaldehyde solution for 30 min on ice followed by suspending in 0.5% Tween 20 in PBS. The cell mixture was stained with 7.5 μl of antiactive caspase-3-fluorescein isothiocyanate (FITC) antibody (BD Biosciences, CA) for 30 min at room temperature, washed with PBS and analyzed in a Coulter Elite Flow Cytometer.

2.5. Quantification of cytokine and chemokine synthesis

Cytokines such as IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12 p40, IL-12 p70, IL-18, IFN- γ and TNF-α, β and MCP-1 were quantified by ELISA procedure using kits from BD Biosciences, according to manufacturer's protocols. IFN-α was assayed using the ELISA kit of Research Diagnostics (NJ). In short, 50 μl of ELISA diluent is pipetted into antibody coated wells of 96-well plates followed by 100 μl of each standard and test samples, shaken for 5 s to mix the contents in the wells, covered with plate sealer and incubated for 2 h at room temperature. After incubation, the contents of the wells were aspirated and washed five times with wash solution. After complete removal of the wash solution in the final wash 100 μl of detection solution was added, covered with plate sealer and incubated for 1 h. The wells were washed seven times with wash solution and added 100 μl of one step substrate reagent and incubated for 30 min in dark. The color development was stopped by adding 50 μl of stop solution and the absorbance were recorded at 450 nm with a reference wavelength of 570 nm in a Bio-Rad Benchmark plate reader.

2.6. Complement activation pathway

Human complement C3a des Arg and C4a des Arg correlate EIA™ Kits (Assay Design, Ann Arbor, MI) were used to measure the cleaved complement components (C3a des Arg and C4a des Arg) according to the manufacturer's protocol. In brief, 1 ml normal blood, drawn from healthy volunteer was incubated with 0–100 μg/ml of RR1 in a CO_2 incubator at 37 °C for 24 h. The treated blood samples were centrifuged at $2000\times g$ at 4 °C and 225 μl of complement reagent 'A' was added to an equal volume of the sample supernatant and vortexed thoroughly. To this mixture, 50 μl of 10 N HCl was added, vortexed again and incubated at room temperature for 1 h. The samples were centrifuged at 10,000 rpm in a micro centrifuge at room temperature for 5 min, 180 μl of the supernatant was transferred to a 15 ml tube, 20 μl of 9 N NaOH was added and vortexed thoroughly. To this mixture 600 μl of complement reagent 'B' was added followed by 10.7 ml of assay buffer, vortexed and used for the analysis. Assay sample (100 μl) was pipetted into wells in a 96-well microplate followed by 50 μl of blue conjugate and 50 μl of yellow antibody. The plates were shaken on a platform shaker at 500 rpm for 2 h. The wells were aspirated to remove the unbound materials, washed thrice with 200 μl of wash solution and added 200 μl of p-Npp substrate solution. The plate was incubated at 37 °C for 1 h without shaking, added 50 μl of stop solution and absorbance was taken at 405 nm with a reference wavelength of 570 nm in a Bio-Rad Bench top plate reader.

2.7. Nitric oxide (NO) and inducible nitric oxide synthase (iNOS)

The NO quantification was performed by Nitric Oxide quantification kit (Active Motif, Carlsbad, CA) as per the manufacturer's protocol. Briefly, lymphocytes (10^6/ml) were incubated with varying concen-

trations of RR1 (0–100 μg/ml) for 24 h in a nitrate-free medium (Dubelco Minimum Essential Medium) at 37 °C. The cell culture supernatant (70 μl) was pipetted into the wells of a 96-well plate along with 20 μl reconstituted cofactor and 10 μl nitrate reductase enzyme solution. The plate was shaken at 150 rpm on a plate shaker for 30 min at room temperature. Griess reagents A and B (50 μl each) were added into each well, allowed the color to develop for 20 min and the absorbance was taken at 540 nm with a reference wavelength of 620 nm in the Bio-Rad plate reader.

Higher and long-lasting release of NO is resulted by the enzyme iNOS from arginine on stimulation. RR1-induced iNOS was assayed by Quantikine iNOS immunoassay kit (R&D systems, Minneapolis, MN) that employs a sandwich enzyme immunoassay technique. The iNOS was assayed from the cytoplasmic extract of the cells treated with different concentrations of RR1. Briefly, the samples and standards were pipetted in to the wells of the 96-well plate and any iNOS present bound to the immobilized antibody. After washing away the unbound substances an enzyme linked monoclonal antibody specific for iNOS was added. After washing away the unbound antibody enzyme reagent, the color developing substrate solution was added, the color development was stopped by stop solution and the absorbance was read at 450 nm with a reference wavelength of 570 nm in the Bio-Rad plate reader.

2.8. Oxidative stress (GSH/GSSG levels)

The measure of the reduced (GSH) as well as oxidized (GSSG) levels of glutathione and their ratio are useful indicators for oxidative stress. The levels of GSH and GSSG were assayed by a colorimetric method using Biotech GSH/GSSG-412 kit (Oxis Research, Portland, OR) according to manufacturer's protocol. Briefly, normal blood samples from healthy donors were incubated with different concentrations of RR1 for 24 h in a 5% CO_2 incubator at 37 °C. For GSSG, 100 μl of each of the treated sample was frozen at −70 °C for 4 h, thawed and added 290 μl of 5% meta phosphoric acid (MPA). The samples were vortexed for 15–20 s and centrifuged at 1000×*g* for 10 min. MPA extract (50 μl) was added to 700 μl of GSSG buffer that was used for the colorimetric assay. For GSH measurement 50 μl of the whole blood was frozen at −70 °C, thawed and added 350 μl of 5% MPA, vortexed for 15–20 s and centrifuged at 10,000×*g* for 10 min. MPA extract (50 μl) was added to 3 ml of the assay buffer and used for further analysis. MPA buffer mixture (200 μl) of each standard and sample was transferred to a spectrophotometer cuvette, added 200 μl of chromogen followed by 200 μl of enzyme in the order and incubated at room temperature for 5 min. Afterwards, 200 μl of NADPH was added into the cuvette and changes in the absorbance at 412 nm were recorded in a Beckman spectrophotometer. The reaction rates were plotted using the absorbance values and the levels of GSH and GSSG were determined.

2.9. Statistical analysis

All experiments were repeated thrice with three replications in each. Mean and standard deviation were estimated using Excel software and single-factor ANOVA was used for data analysis. The data was also analyzed by Student's *t* test to compare the effectiveness of RR1 concentrations.

3. Results

3.1. Characterization of RR1

The results of the glycosyl composition and linkage analyses corroborated our initial findings and the ^{13}C NMR spectral data on the carbohydrate composition of RR1. The glycosyl composition analysis showed glucose as the only component in RR1 while linkage analysis revealed three types of glucopyranosyl residues corresponding to three types of linkages: 4-linked glucopyranosyl residue (80%), 4,6-linked glucopyranosyl residue (12%) and terminal glucopyranosyl residue (8%). The ^{13}C NMR spectra (Fig. 2a) showed well-resolved signals for the carbon atoms in the glucopyranosyl moieties—C1 (δ 99.97 ppm), C2 (δ 73.68 ppm), C3 (δ 71.89 ppm), C4 (δ 77.09 ppm), C5 (δ 71.52 ppm) and C6 (δ 60.83 ppm). The downfield shifts in the C1 and C4 signals confirm the (1→4) linkage while the signal at δ 69.57 ppm may be due to the C6 of the (1→6) linkage. The signals at δ 5.44 ppm (not well resolved) and that at δ 5.00 ppm in the 500-MHz proton NMR spectra

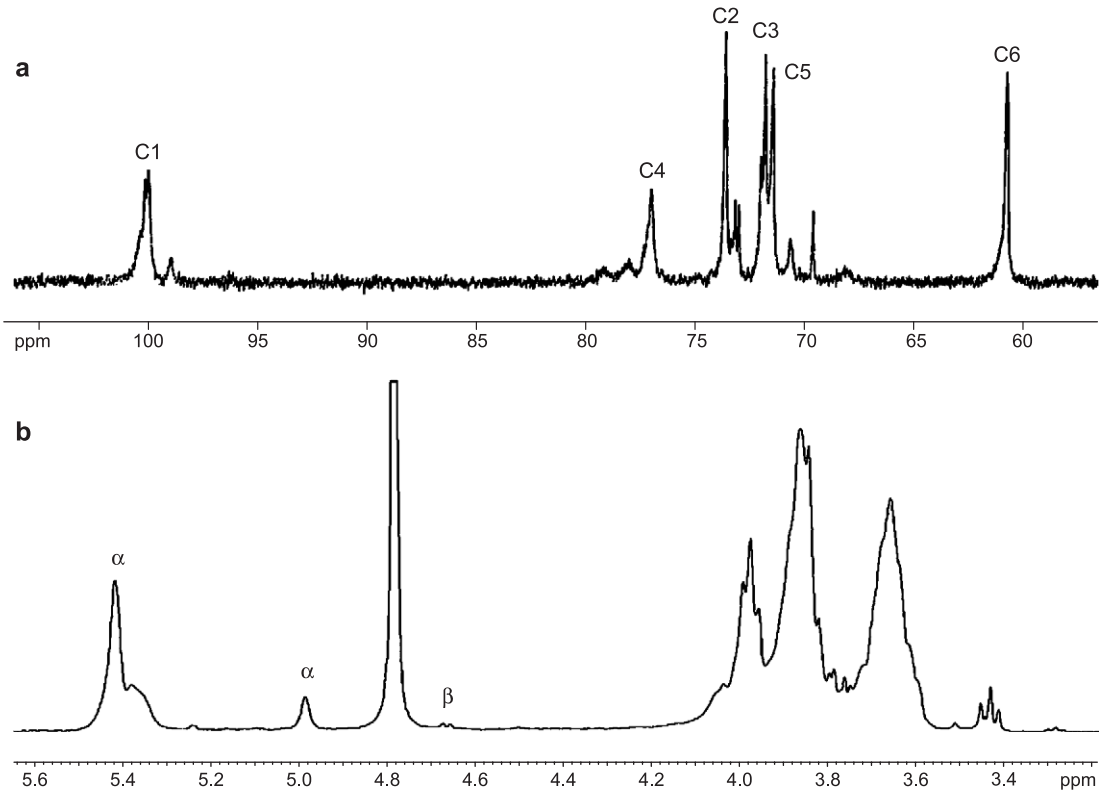

Fig. 2. (a) ^{13}CNMR spectra of RR1 in D_2O showing (1–4) and (1–6) glycosidic linkages, (b) 500-MHz proton NMR spectra of RR1 in D_20 showing the α-conformation.

(Fig. 2b) are due to the α proton associated with the anomeric carbon of the glucopyranosyl units. On the other hand, the very weak signal at δ 4.66 ppm may be due to that of the β anomer. However, the ratio of the signals of α-D-glucose to β-D-glucose is about 99.9:1, and hence almost all glucose units appeared to be in the α configuration. Therefore, RR1 is an α-D-glucan with (1→4) linked glucopyranosyl units in the main chain with (1→6) linked glucopyranosyl unit branches and a 0.15 degree of branching (Fig. 3). In the size exclusion chromatography RR1 eluted as single peak at 12.32 min which was very close to the peak for the 511-kDa dextran sample (retention time 12.72 min). Therefore, RR1 is assigned a molecular weight >550 kDa.

3.2. Noncytotoxic/cell proliferation effect

The results of MTT assay given in Fig. 4a and b show that RR1 has no direct cytotoxic or cell-proliferating effect either on normal lymphocytes or on tumor cell lines (CEM and CEM/VLB) even at a concentration as high as 1000 μg/ml.

3.3. Lymphocyte activation

Lymphocytes are the key effecter cells of mammalian immune system and our studies show that the different subpopulations of lymphocytes are activated by RR1 at varying levels. B cells are activated by 39%, T cells by 102% and NK cells 331% with 100 μg/ml of RR1 (Fig. 5). The higher activation of NK cells is quite important as NK cells are the main effecters of innate immune system that comes into contact with antigens/mitogens before antibody production and recognition by the adaptive immune system. The increased activity of NK cells by RR1 is quite evident from the results of functional cytotoxic assay given in Fig. 6. RR1-treated normal lymphocytes were able to kill a higher percentage of

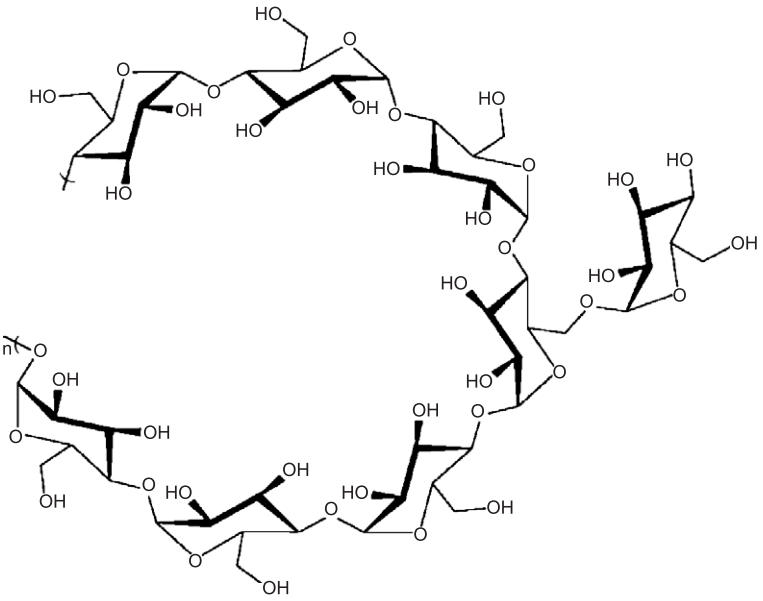

Fig. 3. Chemical structure of RR1.

tumor cells compared to untreated cells and a dose-dependent enhancement of cytotoxicity of activated lymphocytes was evident.

3.4. Complement activation pathway

A stepwise increase in the levels of C3a des Arg of the alternative pathway was noticed with increase in concentrations of RR1 (Fig. 7). However, there was no significant difference in C4 des Arg levels (classical pathway) with increasing RR1 concentrations.

3.5. Synthesis of cytokines

RR1 induced the synthesis of IL-1β (1080 pg), IL-6 (21,833 pg), IL-12 p40 (918.23 pg), IL-12 p70 (50.19 pg), IL-18 (27.47 pg), IFN- γ (90.16 pg), MCP-1 (2307 pg) and TNF-α (2225 pg) (Fig. 8) while it did not induce the production of IL-2, IL-4, IL-10, TNF-β and IFN-α. In general, a dose-dependent increase in the production of cytokines was observed with RR1 except for IL-12 p40 which recorded the maximum at 10 μg/ml ($p<0.05$) and further increase in the RR1 exhibited a decreasing trend with no significant difference between 50 μg and 100 μg/ml concentrations ($p>0.05$). MCP-1 production was very significant up to 10 μg/ml of RR-1 ($p<0.05$) and higher RR1 concentration produced only little increase in MCP-1 level ($p>0.05$). In general proinflammatory cytokines IL-1β, IL-6 and TNF-α and the regulatory cytokine IL-12 p40 exhibited higher levels of production compare to other cytokines.

3.6. Oxidative stress

RR1-treated (0–50 μg/ml) normal lymphocytes have a narrow range in GSH (364.43–367.09 μM), GSSG (22.60–22.77 μM) and GSH/GSSG ratio (14.05–14.56) levels and these estimates were not significantly different from control.

3.7. NO synthesis and iNOS induction

RR1 stimulation caused only <20% increase in NO level compared to untreated cells. In addition, there was no significant induction of iNOS at 0–100 μg/ml of RR1 (data not shown).

4. Discussion

Mammalian immune system recognizes antigens, pathogens and nonself molecules and trigger defense

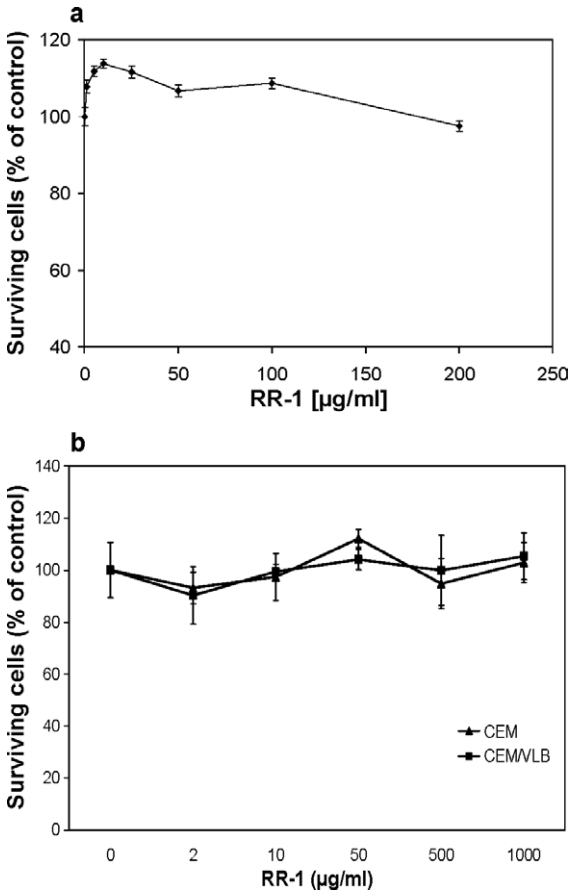

Fig. 4. Cytotoxic analysis of RR1 in normal lymphocytes (a) and tumor cell lines (b). Cells (10^6) were treated with RR1 for 24 h and cytotoxicity assay performed using MTT cell proliferation kit (Roche Biochemicals). The percentage of surviving cells over control was plotted against RR1 concentrations.

mechanism by the activation of immune competent cells, production of chemical messengers (the cytokines and chemokines), activation of complement cascade pathway and synthesis of nitric oxide (NO). Cytokines are a group of low molecular weight regulatory nonantibody proteins secreted by immune component cells in response to stimulation. They bind to specific receptors of target cells triggering signal transduction pathways that ultimately lead to gene expression in target cells. Cytokines regulate the intensity and duration of immune responses by stimulating or inhibiting activation, proliferation and/or differentiation of various cells and by regulating the secretion of antibodies or other cytokines. The pleiotropy, redundancy, synergy and antagonism exhibited by cytokines permit them to coordinate and regulate cellular activities. Inflammatory responses are crucial in controlling and eliminating infectious agents as well as in promoting wound healing for restoration of tissue integrity.

IL-1β and TNF-α induce the production of each other as well as that of IL-6, and act synergistically and regulate several biological actions besides IL-1 being self-inductive [34]. The production of IL-1β and TNF-α in RR1-treated lymphocytes is consistent with that in the LPS stimulated human monocyte cell lines (THP-1) [35]. IL-12 p70 is the bioactive isoform of IL-12 and is an important factor in the differentiation of naïve T cells into effecter T helper type 1 (Th1) CD4+ lymphocytes secreting IFN-γ [36]. Besides, it is also reported to have stimulatory effects on NK cells [2]. Recently, IL-12 p70 has emerged as an efficient and minimally toxic antitumor cytokine due to its ability to elicit the Th1 response [37]. Beyond the immune system, this cytokine is also reported to have the capacity to inhibit UV-induced apoptosis and initiation of DNA repair in UV-damaged keratinocytes which ultimately protect the cells from malignancy [38]. IL-12 p40, the homodimeric isoform, is a receptor antagonist of the bioactive heterodimeric isoform p70 in mouse [39]. In the RR1-treated cells, IL-12 p40, the regulatory cytokine is

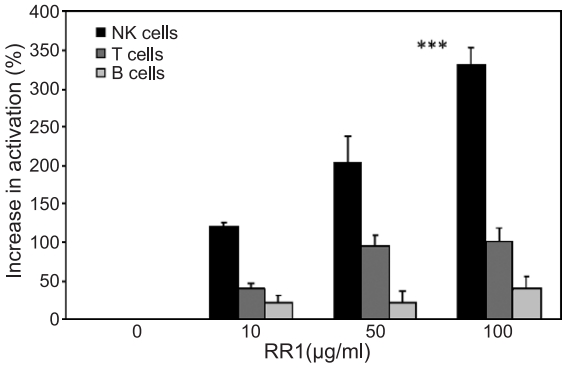

Fig. 5. Flow cytometric analysis of Activation of NK, T and B cells by RR1. Normal lymphocytes were treated with RR1 (0–100 µg/ml) for 24 h at 37 °C in a CO_2 incubator. The cells were stained with a panel of cell-specific antibodies conjugated with different fluorochromes: CD3-FITC, CD16/56-PE, CD19-ECD, CD69-PC5 or CD8-FITC, CD4-PE, CD3-ECD, CD69-ECD. The stained cells were analyzed in a Coulter Elite flow cytometer in a four-color assay (***$p<0.001$).

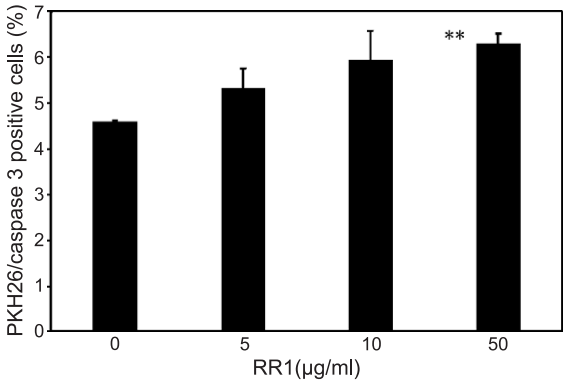

Fig. 6. Flow cytometric assay for cytotoxicity of RR1 activated human lymphocytes. Normal lymphocytes were treated with RR1 (0–100 μg/ml) for 24 h in RPMI medium. The cells were washed and coincubated with PKH26-labeled human leukemic CEM cells for 12 h for the active NK cells to lyse the tumor cells. The cell mixtures were stained with FITC-labeled and activated caspase-3 monoclonal antibody. The percentage of PKH26+ and Caspase-3+ cells were quantified by analyzing in a Beckman-Coulter Elite flow cytometer (**p=0.01).

produced in many-fold excess to the bioactive form which may be natural mechanism to control the over production of the bioactive form p70. IL-18 is another potent inducer of IFN-γ and apparently NK cells [40]. RR1 induction of IL-18 production may be an early response in the development of Th1 response acting in consonance with IL-12 and IFN-γ.

MCP-1 is a potent chemoattractant for monocytes and activated CD4 and CD8 T cells that is reported to induce granule release from NK and CD8+ cells, activate NK function in CD56+ cells, and act as a potent releasing factor for histamine from basophiles [41]. Furthermore, it is reported to exhibit antitumor effects by enhancing tumor specific immunity, presumably in a T-cell-dependent manner [42]. Our observations indicate that RR1 induces the production of this cytokine significantly at 10 μg/ml. The significant synthesis of TNF-α by RR1 stimulation can play a critical role in host resistance to infections and to the growth of malignancy. TNF-α and its receptors are essential for protection against tuberculosis and for NO synthesis in macrophages early in infection [43]. RR1-induced IFN-γ production may function in part to promote the activity of the components of the cell-mediated immune system such as cytotoxic T lymphocytes (CTLs), macrophages and NK cells in addition to its inhibitory role in Th2 response. It stimulates the bactericidal activity of phagocytic cells and, therefore, boosts the innate immune response [5]. Moreover, it may modulate MCP-1 synthesis in macrophages by LPS [44].

CD4+ T cells contribute to the regulation of antigen specific (adaptive) immune system through the recognition of antigens and consequent production of cytokines. The distinct pattern of cytokine production by CD4+ cells form a dichotomy, Type 1 (Th1) characterized by IFN-γ production and promotes elimination of intracellular pathogens and Type 2 (Th2) characterized by IL-4 production, involves IgE and eosinophils suitable for elimination of extra cellular pathogens. Cytokines act directly on T cells during primary activation and appears to be the most direct mediator among the factors influencing the terminal differentiation. The presence of IL-12 leads to Th1 response while IL-4 leads to Th2 development and the two pathways express mutually suppressive effect as well. IL-1 is identified as an inducer of IL-12 [41] while IL-18 is an early response in the development of Th1 cells by induction of IFN-γ. The cytokine profile, IL-12, IL-18, IFN-γ together with IL-1 by RR1 stimulation and the dose-dependent synthesis of these cytokines clearly demonstrate the Th1 pathway which is essential for cellular immunity and killing of intracellular pathogens and malignant cells. This observation is in conformity with IL-12 induced IFN-γ-dependent T cell development to Th1 and

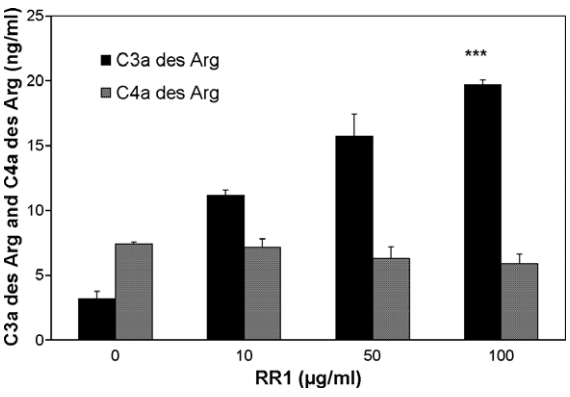

Fig. 7. Analysis of RR1-induced activation of complement pathways. Normal blood samples from healthy volunteers were incubated with RR1 (0–100 μg) for 24 h. Human complement C3a des Arg and C4a des Arg correlate EIA™ Kits (Assay Design) were used for quantification of classic and alternative pathway of complement activation using an ELISA procedure (***p<0.001).

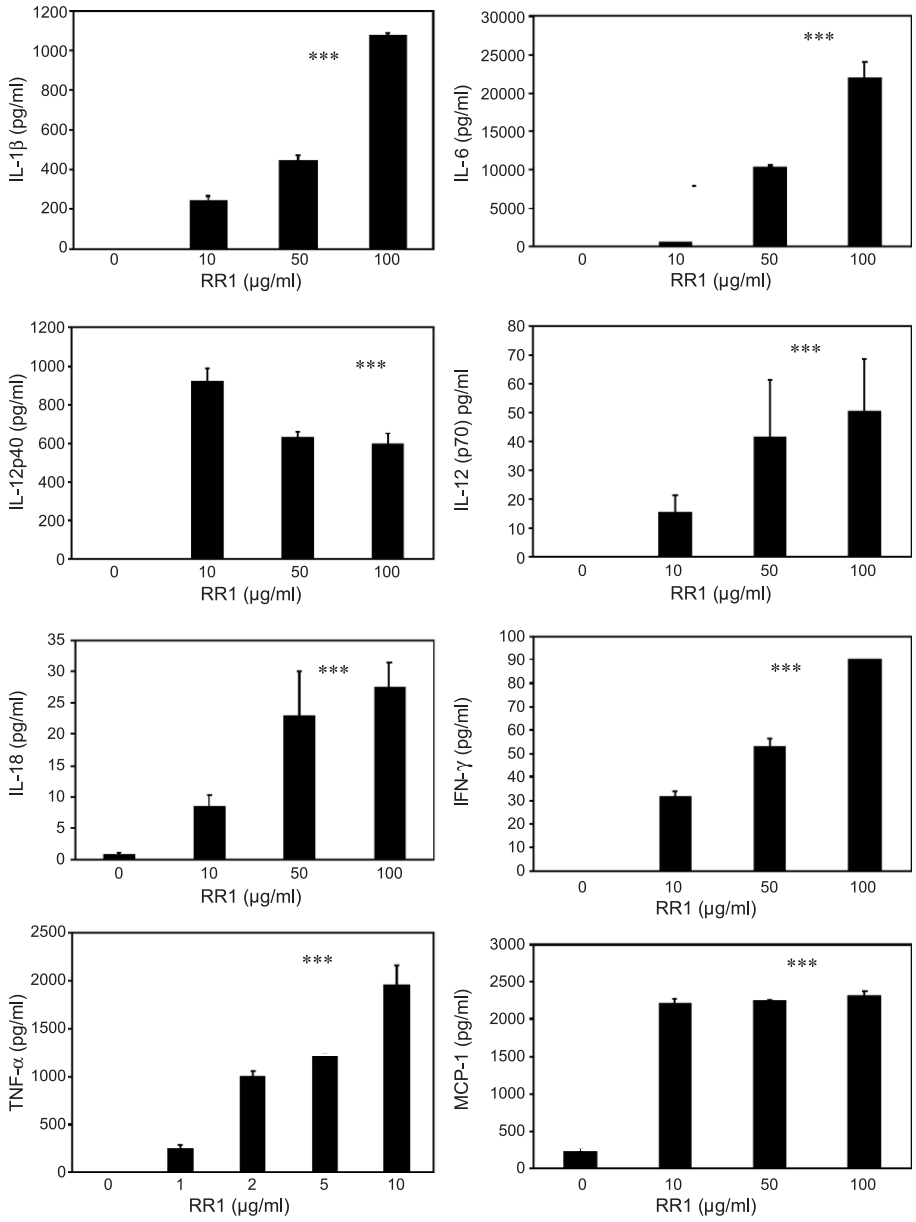

Fig. 8. Synthesis of cytokines and chemokine in RR1-induced normal lymphocytes. Normal lymphocytes from healthy volunteers were treated with RR1 (0–100 μg) at 37 °C for 24 in a CO_2 incubator in RPMI medium. The supernatant medium was analyzed for the production of cytokines and chemokine in an ELISA procedure using reagent kits BD Biosciences. RR1 induced the synthesis of IL-1β, IL-6, IL-12 p40, IL-12 p70, and IL-18, TNF-α, IFN-γ, and MCP-1 significantly (***$p<0.001$).

CD8+ cytotoxic effector cells [45–47]. Concurrent signaling as well as synergistic action by IL-12 and IL-18 induces prolonged IFN-γ production and the continuous strong expression of IL-18R mRNA in T cells [46]. The early inflammatory events, such as T cell adhesion to inflammatory sites, were also reported for IL-12 and IL-18 [48]. The higher level of the synthesis of the regulatory isoform IL-12 p40 may be a natural mechanism to contain the excessive production of Th1 response.

The iNOS enzyme controls the production of NO in macrophages. The insignificant level of iNOS induction by RR1 in the present investigation supports the insignificant increase in the production of NO. Our studies with monocytes isolated from peripheral blood mononuclear cells by Monocyte Isolation kit II (Miltenyi Biotec, Germany) or with human monocyte cell line THP-1 (ATCC) also did not induce any high level of NO with RR1 treatment (data not shown). NO is reported to play an important role in the modulation of T helper cell differentiation and polarization. Yamasaki et al. [49] and Taylor-Robinson et al. [50] reported an inhibitory role of NO on Th1 cytokines while Bauer et al. [51] reported the same for Th1 as well as Th2 cytokines by NO from activated T cells. The low level of NO synthesis by RR1 does not appear to exert any inhibition of Th1 pathway. IFN-α, IFN-γ and TNF-α are known upregulators of NO synthesis [52,53]. As RR1 stimulation resulted in the synthesis of IFN-γ and TNF-α and very little IFN-α, it is reasonable to assume that IFN-α priming may be a necessary step for triggering the production of higher levels of NO by polysaccharides [54].

RR1 stimulation does not produce any oxidative stress in lymphocytes, indicated by the levels of the GSH, GSSG and their ratio, corresponding to the NO synthesis data. In addition, we could not observe any significant elevation in the amount of hydrogen peroxide in our investigations (data not shown). In this context, the low level of NO synthesis by RR1 suggests an immune mediatory role for NO. The noncytotoxic nature of RR1, even at fairly high concentrations (1000 μg/ml), may be attributed to the low level production of NO together with the ability of this polysaccharide not to induce oxidative stress in the cells.

In the present investigation, RR1 has activated the alternate pathway (C3a) of complement activation and there was very little effect on classical pathway (C4a). This observation is analogous with several reports on complement activation by other polysaccharides. C3a and C4a are bioactive cleavage products released from plasma components C3 and C4 during complement activation cascade in alternative and classical pathways [55] which are quickly converted to less active C3a des Arg and C4a des Arg forms and are involved in the mediation of cellular immune responses . The alternative pathway is self-amplifying and is important in the clearance and recognition of pathogens in the absence of antibodies [56]. β-Glucans are reported to activate the alternative pathway and the host mediated antitumor activity exhibited by these polymers were correlated with the activation of the complement system [57]. Lipopolysaccharides activate the complement system via alternative as well as classical pathways; the lipid part activates the classical pathway while the polysaccharide moiety activates the alternative pathway [58].

Immune stimulation by induction of cytokines and synthesis of NO, activation of macrophages, induction of phagocytic, cytotoxic and antitumor activities have been reported recently in polysaccharide or polysaccharide containing fractions of *Phanax ginseng*, *Morinda citrifolia*, and *Echinacea* [52,53,59]. RR1 is a branched α-D-glucan structurally distinct from amylopectin and pullalan as no immune stimulation is reported by either amylopectin or pullalan. The similarity of its structure to the conserved molecular pattern of the cell wall components of fungal β-glucans may be the reason for the activation of immune system. On the other hand, the differences, the α conformation and the (1→4) linkages, may account for the non-induction of iNOS [49] and the consequent noncytotoxicity and the absence of oxidative stress. This water soluble neutral α-glucan has molecular mass and branching sequence well within the range of the polysaccharides exhibiting significant immune stimulating properties [11,12,19,21]. The water solubility can overcome the granuloma formations exhibited by particulate β-glucans while the high molecular mass, being in the most potent range, may enable it to be retained by the host's organs for longer period without degradation [60,61]. While the noncytotoxic nature, the significant activation of the lymphocytes, especially the NK cells and the alternate pathway of complement activation demonstrate the stimulation of the innate immune system, the cytokine profile resulting from the activation proclaims the stimulation of the antigen specific cell mediated (adaptive) immunity (Th1 pathway) with a self-regulating mechanism of its overrun. The absence of IL-4 synthesis (Th1 suppressor cytokine) and IL-10 (the inhibitor of IL-12) on stimulation with RR1 is in good agreement with the Th1 pathway of T cell differentiation. The IFN-γ production by the concurrent signaling of IL-12 and IL-18 may serve as a

potent anti anthrax agent, devoid of side effects, if any, of the exogenous IFN treatment as well. A stimulated innate immune system can fight the entry of any pathogens into the host and has the capacity to prevent primary infections from actually causing disease [62]. The unique immune stimulating properties of RR1 without exerting oxidative stress and any direct cytotoxic effect, thus far described, may make it a potent biodefense agent against a number of pathogens and human malignancies.

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