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Heteroglucan-dendrimer glycoconjugate: a modulated construct with augmented immune responses and signaling phenomena

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ABSTRACT

Background: Newer strategies for augmenting immune responses of pharmacologically active glucans may serve to improve the medicinal potential of these biomolecules. With this aim, the present work was focused on generating targeted high molecular size glucan particles with magnified immune response activity.
Methods: Heteroglucans were conjugated with PAMAM dendrimers using a Schiff base reductive amination reaction to generate a polytethered molecule with multiple glucan motifs. The modulated construct was characterized by FTIR, TEM, ^{1}H NMR and dynamic light scattering (DLS) methods. Effects of conjugated glucans were examined in RAW 264.7 macrophage cells as well as in S-180 murine tumor models.
Results: Dendrimer-conjugated glucans were found to exhibit a two-fold increase in immune stimulation in comparison to unconjugated glucans. This may be corroborated by the predominant enhancement in immunological functions such as nitric oxide production, ROS generation and immune directed tumor inhibition in murine models. Immune cell surface markers (CD4, CD8, CD19, MHC-II) and cytokine levels were also found to be highly up-regulated in the splenocytes of mice subjected to particulate glucan administration. Our study also demonstrated that conjugated glucan treatment to RAW 264.7 cells strongly enhanced the phosphorylation of two downstream signalling molecules of the mitogen activated protein kinase (MAPKs) family: p38 and MEK1/2 relative to single glucans thereby relating molecular mechanisms with enhanced immune stimulation.
Conclusions and general significance: The results obtained thus support that particulate format of soluble heteroglucan will thereby improve its functionality and identify leads in therapeutic competence.

1. Introduction

Glucans (linear or multi-branched polymeric sugar entities), referred to as "biological response modifiers" (BRMs), can modulate the immune system to effectively encounter foreign pathogens, rectify self anomalies as well as maintain normal body homeostasis and may be regarded as 'immune regulatory switchers' in the arena of therapeutics. Unlike other customary antitumor drugs, most glucans isolated from various medicinal fungus have been shown to mediate antitumor efficacies by stimulating the immune response mechanism of the body without evoking harmful side-effects [1]. In contrast to lipopolysaccharides (present in gram-negative bacteria), these biomacromolecules have the additional advantage of mounting a controlled immunostimulatory response, thereby avoiding any chance of septic shock which may be detrimental to the host [2]. Indeed, the immune response induced by glucans retains a balanced limit which is beneficial and protective for the organism. These glucans behave as ligands and bind to their corresponding receptors protruded on the cell membranes of macrophages, monocytes, dendritic cells, NK cells as well as few T-cells. The major receptors of β-glucans in immune cells consist of Dectin-1 [3], TLRs [4], lactosylceramide [5], several scavenger receptors [6] and complement receptor 3 [7]. On recognition, various effector immune responses such as generation of ROS [8], production of NO (nitric oxide), activation of signalling molecules and release of proinflammatory cytokines/chemokines occur [9]. The downstream signalling processes enthused on glucan binding to cell surface receptors have been explored over the last few decades and published reports have revealed that immune cell maturation is an outcome of the up-regulation of NF-κβ, Akt kinase, p38 as well as other mitogen activated protein kinase (MAPK) pathways [10–12].

Recent studies have shown that different forms of glucan elicit varying degree of immune augmentation. It has been observed that particulate glucans such as those derived from *Saccharomyces cerevisiae* (yeast) produce amplified immune up-regulation such as maturation of dendritic cells, phagocytosis by macrophages and differentiation and priming of T_h and T_c cells while soluble glucans augment antibody mediated therapy [13]. The degree of immune response generated is

Abbreviations: BRM, biological response modifiers; PAMAM, poly amido amine; FTIR, Fourier transform infrared spectroscopy; TEM, transmission electron microscopy; NK cells, natural killer cells; ROS, reactive oxygen species; NO, nitric oxide; PBS, phosphate buffered saline; PBST, phosphate buffered saline with Tween 20; TNBS, 2,4,6-trinitrobenzene sulfonic acid; MAPK, mitogen activated protein kinase

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reported to be dependent on the mode of presentation of β-glucan (particulate versus soluble formats) for robust receptor mediated downstream signalling [14]. In this regard, generation of higher biofunctional molecules by using dendrimers as frameworks has gained wide recognition nowadays [15]. Dendrimers, which are synthetic low molecular weight molecules with an inner core and a series of branches, have become attractive tools as carriers of drugs or for conjugating pharmacologically bioactive molecules. The concept of receptor-ligand interactions are related to cell surface based signalling and new effective drugs possessing cooperative and multiple receptor binding capacities modulate this interactive mechanism. The functional groups of dendrimers have been exploited for conjugating with biological molecules possessing novel therapeutic traits to enhance their functionality by regulating the receptor-ligand based downstream machinery [16]. There are numerous reports which define that molecules with multiple side chain functional units enhance their functional potency by increasing molecular interactions at the receptor-ligand level. For example, Papp et al., 2008 have reported that dendritic-polygalactose conjugate exhibited many fold increase in their binding to selectins in comparison to a single galactose unit [17]. Another report has also revealed that polymers such as polyacrylamide-sialioside conjugates increased their binding affinity for Hemagglutinin (HA) present in the Influenza virus to 10^8 fold through multivalent interactions. Such multiple bindings were able to mask the virus particles and prevent their initiation of infection[18].

Pleurotus ostreatus is a well known edible mushroom which is easily cultivable and possesses high medicinal benefits. Bioactive compounds isolated from this organism exhibit properties such as immune stimulation as well as anti-tumor, anti-inflammatory and cholesterol lowering properties, among various others [19,20]. The mycelia of *Pleurotus ostreatus* may be considered as a better source for the isolation of therapeutic compounds as they can be grown in a defined temperature and pH conditions without any environmental influences. Our group has previously reported the isolation and characterization of heteroglucans derived from *Pleurotus ostreatus* mycelia. The isolated glucan was found to be of high molecular weight (~2.7×10^6 Da), water soluble in nature and structurally composed of heterosaccharide repeating units of glucose, mannose and fucose (3:2:1) with (1 → 2), (1 → 3), (1 → 4) and (1 → 6) linkages [21,22]. In this report, we describe a strategy to generate particulate heteroglucan molecules from the soluble form (isolated from the above mentioned source) by applying conjugation chemistry to enhance its immune stimulating property without crossing the immunological balance of causing septic shock. Hence, with an aim to incorporate higher immune stimulative effects, the amino groups of the dendrimer (PAMAM dendrimer) were conjugated to a number of aldehyde groups of the soluble glucan by creating a covalent secondary amide linkage between the two molecules. The conjugated glucan behaved as a particulate form with high molecular size and up-regulated immune reactions. Furthermore, probing into the augmented effects of particulate glucan in animal models and in signalling mechanisms, it was revealed that elevated immune competency was due to high cellular and molecular processes. To the best of our knowledge, this is the first description where a soluble glucan has been modified into particulate form using biocompatible polymeric dendrimers to enhance immune responses.

2. Materials and methods

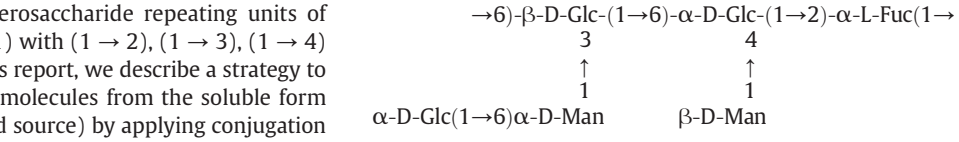
2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from Himedia while Roswell Park Memorial Institute (RPMI) and Foetal Bovine Serum (FBS) were from Gibco company. Essentials such as trypsin EDTA and antibiotics were obtained from Himedia. Other requirements included MTT ((3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium) which was procured from Loba chemicals, India while DEAE-Sephadex beads, PAMAM dendrimers, sodium cyanoborohydride, RNase, propidium iodide dye and lipopolysaccharides were obtained from Sigma, USA. Cytokines and fluorescent tagged antibodies against cell surface markers such as CD4, CD8, CD19 and MHC-II were purchased from BD Bioscience.

2.2. Glucan isolation

The mycelia of *Pleurotus ostreatus* was obtained as a kind gift from Agriculture Department, IIT Kharagpur and have its geographic origin in West Bengal, India. The fungal mycelium was nurtured in potato dextrose (PD) broth at pH 6.5 with regulated temperature conditions of 25 ± 2 °C in an incubator. The mycelial biomass was collected after every 21 days of culture and new inoculums were maintained regularly.

Heteroglucans were isolated from the mycelium of *Pleurotus ostreatus* by alkali extraction method as described in the earlier reports [21]. Briefly, mycelial biomass (~1 Kg) was crushed in a grinder with distilled water and the residual fractions were separated from the aqueous portion by centrifugation at 8000 rpm for 30–45 min. The residual mass obtained was further subjected to 2% KOH treatment overnight followed by centrifugation and neutralization of the supernatant. Alkali soluble polysaccharides were obtained by ethanol precipitation of the neutralized supernatant solution. The dry pellet/extract obtained after ethanol treatment was then dissolved in 20 mM Tris Buffer with pH 8.0 and finally passed through an ion-exchange DEAE-Sephadex column for further purification. The unbound flow through of the column (0.5 ml/min flow rate) was collected and lyophilized to obtain the purified glucan. Physiochemical characterization of the extracted glucan revealed its nature to be water soluble with a heterogenous composition of glucose, mannose and fucose in a ratio of 3:2:1 and having terminal D-glucopyranosyl, terminal D-mannopyranosyl, (1 → 6) linked D-mannopyranosyl, (1 → 2) linked L-fucopyranosyl, (1 → 4,6) linked D-glucopyranosyl and (1 → 3,6) linked D-glucopyranosyl moieties (1:1:1:1:1:1) [21,22]. The repeating unit of the glucan molecule may be represented as follows:



2.3. Conjugation methodology

Heteroglucans were conjugated to branched dendrimers by generating a secondary amide bond between the aldehyde group of glucan and the amine functional group of the dendrimer. About 50 mg of heteroglucan was initially dissolved in 10 ml of an aqueous solution containing 0.4% sodium acetate trihydrate (w/v) and 0.2% boric acid (w/v). This was followed by the addition of a strong reducing agent such as sodium cyanoborohydride (1 M) in the above solution. Amine functionalized dendrimers (PAMAM, 4th generation) were then added to the above conjugating solution at varying glucan:dendrimer (w/w) ratios of 25:1; 50:1; 75:1; 100:1 and the derivatization reaction was carried out at 70 °C for one hour in hot water bath. The mixture was then cooled to room temperature and about 5 volumes of acetonitrile: water (97:3 v/v) solution added to precipitate the dendrimer conjugated glucans. Precipitates were collected by centrifugation at 5000 rpm for 10 min. Washing of the precipitates with acetonitrile/water was repeated two to three times to remove unconjugated dendrimers and other solvent mixtures. The final precipitate obtained was further dissolved in water and dialysed to purify the conjugated glucans from other undesired reagents and ultimately lyophilized.

The endotoxin levels in the soluble glucan as well as the dendrimer conjugated forms was found to be less than 0.01 ng/ml as ascertained by limulus amoebocyte lysate (LAL) test.