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Assessment of two synthesized curdlan derivatives as possible antioxidants and/or modulators of human PMN cells respiratory burst

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Abstract

β-glucans are described as active compounds with immune activity. These polymers demonstrated positive effects on the immune system (anti-tumoral, anti-infectious, protection against fungi, bacteria and viruses infections). The correct selection of β-glucans is essential to identify compounds with favourable clinical effects. There are also evidences that macrophages respond to β-glucans, possibly released from fungal cell walls, via receptors such as Dectin-1 and Toll-like receptors, to induce inflammatory reactions. The aim of this study was to investigate the capacity of two synthesized curdlan derivatives as possible antioxidants and/or modulators of human polymorphonuclear cells respiratory burst. It has been demonstrated that Curdlan did not significantly influence the ROS and superoxide anion production in polymorphonuclear cells, while curdlan derivatives were able to modulate respiratory burst. All the tested compounds did not act as oxygen radicals scavengers. By comparing with Curdlan, the synthesized curdlan derivatives proved to be more attractive and useful compounds able to modulate human polymorphonuclear respiratory burst. It might be hypothesized that curdlan derivatives could act on the cell membrane, possibly by binding to cellular receptors (i.e. Dectin-1 receptor which could recognize 1,3-β-glucan, either alone or by Dectin-1 – Toll-like receptor 2 co-operation). In summary, the data obtained in this study are important because they increase the knowledge on curdlan derivatives containing palmitoyl, carboxymethyl and sulfopropyl groups, the information gathered here having important implications for our understanding of how immune responses to fungal pathogens develop, as well as for the design of immunomodulators containing curdlan derivatives.

Keywords: β-glucan, Curdlan, Pam3Cys, oxidative stress, ROS, PMNs, Dectin-1, TLR2

Introduction

The alleged anti-infective or anti-cancer effects of most non-prescriptional medicinal herbs are mainly based on studies derived from *in vitro* or *in vivo* animal experiments. The current information suggests that these herbal extracts exert their biological effect either through cytotoxic or immunomodulatory mechanisms. One of the active compounds responsible for the immune effects of herbal products is a polysaccharides complex known as β-glucans that have been implicated in the initiation of anti-microbial immune response.

β-1,3-linked glucans are glucose polymers forming cell wall components of plants, fungi and bacteria and, as conserved structures, can be considered to be classical pathogen associated molecular patterns (PAMPs). β-glucan polymers belong to a class of drugs known as biological response modifiers (BRMs) and have a variety of effects on the immune system,

including anti-tumour and anti-infectious activities, protecting against fungal, bacterial, viral and protozoal infections.

Based on *in vitro* studies, β-glucans act on specific immune receptors and trigger a group of immune cells including macrophages, neutrophils, monocytes, natural killer cells and dendritic cells. As a consequence, both innate and adaptive response can be modulated by β-glucans and they can also enhance opsonic and non-opsonic phagocytosis [1]. Taking into account that β-glucans of different sizes and branching patterns may have significantly variable immune potency, a careful selection of appropriate β-glucans is essential if we wish to investigate the clinical effects of these compounds.

Through activation with epichlorohydrine, Curdlan (β-1,3 glucan) can covalently link available amino, hydroxyl and sulfhydryl groups of enzymes, thus it can be used as support matrix for immobilization of enzymes [2]; Curdlan gels can be used as protein drug delivery vehicles [3]. Sulfate derivatives of Curdlan have been intensively studied; they proved to have anti-HIV activity [4-15]; also, they can have anticoagulant [16-18], antioxidant [19] or antitumoral activity [20]. Carboxymethyl curdlan presented antitumoral activity [21,22] and sulfopropyl derivative can have anticoagulant [23] or antitumoral [24] activity.

Curdlan derivatives could reduce hyperglycemia and hyperinsulenaemia, controlling the diabetes [25,26] and could reduce hyperlipidemia [27] and hypercholesterolemia [28]. Many studies are devoted also to the immunostimulatory activity [29,30] and to the anti-tumoral effects [31,32] of curdlan derivatives. A recent study of our group showed that several curdlan derivatives differently enhance the cytostatic activity of some drugs such as Actinomycin D, Cyclophosphamide and Doxorubicin on tumor cells (B16 and HEp-2) [33]. Other literature data evidenced some interesting properties of microparticles made of curdlan derivatives containing weakly and strongly acidic anionic and/or hydrophobic groups, which recommend them for interaction with proteins (enzymes, vaccines) [34]. Both particulate and soluble β-glucans mediate these activities by activating leukocytes and stimulating their phagocytic activity, production of reactive oxygen intermediates, inflammatory mediators and cytokines [1,35].

When the reactive oxygen species (ROS) production in the cells becomes aberrant and exceeds the ability of the antioxidant system to eliminate them, oxidative stress results [36]. It is well-documented that significant oxidative stress carries out severe damage to lipids, proteins, sugars and nucleic acid bases which compromises cell viability and functions. The highly reactive species are molecules or ions presenting radical and non-radical structure: superoxide anion, hydrogen peroxide, hydroxyl radical, singlet oxygen etc. These intermediates are formed continuously in cells as a consequence of both oxidative biochemical reactions and external factors. Oxidative stress pathways involve complex biochemical reactions leading to harmful reactive oxygen and nitrogen radicals effects at the cellular level.

In this study, two Curdlan derivatives having anionic carboxymethyl, sulfopropyl or palmitoyl groups on the same macromolecular backbone have been synthesized by using Curdlan from *Agrobacterium biovar*. These compounds have been tested for possible antioxidant and/or modulatory activities on human PMN cells respiratory burst.

Materials and Methods

Obtaining of curdlan derivatives

Curdlan from *Agrobacterium biovar* (Takeda-Kirin Food Corporation, Tokyo Japan) kindly supplied by Mitsui & Co. Deutschland GmbH 20355 Hamburg, Germany has been

used for derivatives synthesis (performed in "Petru Poni" Macromolecular Chemistry Institute, Iasi, Romania) :

- *Sulfopropylation of curdlan* (SP). 1 g curdlan was swollen with 5 ml 10% NaOH solution; 2.26g (18.5 mmoles) propane sultone in 10 ml isopropyl alcohol was added and the reaction continued for 6 h at 50°C. The sulfopropylated curdlan was dialyzed against water and dried through lyophylization. Yield: 1.10g (~ 74% from theoretical).

- *Hydrophobization of curdlan derivative* (Palm CM/SP). For having both weakly and strongly acidic groups and also hydrophobic groups on the same chain, the reaction was carried out in two steps: (i) the curdlan was chemically modified by introduction of carboxymethyl and sulfopropyl groups; the reaction was carried out on 1g curdlan swollen in 5 mL 10% NaOH solution; 1.08g (9.3 mmoles) sodium chloroacetate in 2 mL water and 1.13g (9.3 mmoles) propane sultone, divided to portions, were added alternatively; the reaction was continued for 5h at 60°C; the carboxymethyl/sulfopropyl derivative obtained was dialyzed against water and dried through lyophylization; yield: 1.20 g (~ 83% from theoretical); (ii) 1 g carboxymethyl/sulfopropyl dry anionic derivative was swollen in 10 ml formamide at 50°C; then 10 ml dimethylformamide, 0.5 ml pyridine and the reagent: 0.63 g (2.3 mmoles) palmitoyl chloride were added. The reaction was continued with stirring and heating at 65°C; the obtained Palm CM/SP derivative was dialyzed against water and dried through lyophylization. Yield: ~ 0.9 g (~ 89% from theoretical).

- Reagents: sodium hydroxide, sodium chloroacetate (FLUKA, Switzerland), propane sultone (JANSSEN – Holland), palmitoyl chloride (FLUKA).

Oxygen Radicals Absorbance Capacity (ORAC)

In order to investigate the antioxidant capacity of the studied Curdlan and curdlan derivatives, consisting in their scavenging capacity of peroxyl radicals, we used a modified and validated protocol based on Prior et al. method [37], adapted for 96 well plates. Probes fluorescence has been quantified using an automatic Fluorimeter/Chemiluminometer system - *THERMO Fluoroskan FL* with a dispenser as endowment and connected to a PC. Data acquisition and statistical processing was performed by a dedicated software (Ascent Fluoroskan FL). The results were calculated by using a Trolox (S)-(-)-6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid) calibration curve (3.125-100μM Trolox) and substracting the blank area from the net area under fluorescence decreasing curve in the presence of the sample and relating to net area corresponding to 1 μM Trolox. The obtained value was multiplied with dilution factor.

Final data were expressed as micromoles of Trolox equivalents (TE) per mg, (1 TE = 1μM Trolox). Quantification limit of Fluoroskan FL system was 0.01 TE/mg. The measurements were performed three times in triplicate and final results represent percent values against the corresponding control. Chemicals were purchased from FLUKA.

Total antioxidant capacity (TAC)

For Curdlan and each curdlan derivative, Total Antioxidant Capacity has been measured using the Preito modified spectrophotometric method [38]. Briefly, 0.1 ml sample was put in 0.9 ml Reagent A (H_2SO_4 0.6 M, Na_2HPO_4 x $2H_2O$ 28mM and $(NH_4)_2MoO_4$ 4 mM). After an incubation period of 90 minutes at 95ºC, the sample was cooled at room temperature and then centrifuged (1300 rpm, 25°C, 5 minutes). Finally, the supernatant absorbance was spectrophotometricaly measured at 695 nm; results were calculated using a standard curve made for ascorbic acid. Reagents: H_2SO_4 and Na_2HPO_4 x $2H_2O$ (Merck), $(NH_4)_2MoO_4$ (ALDRICH).

Human PMNs isolation

Human polymorphonuclear cells (PMNs) were isolated from 5 healthy donors (who gave their consent), by using the adapted method of Roos and DeBoer [39]. The protocol was approved by the Ethics Committee of "Cantacuzino" National Institute of Research/Development for Microbiology and Immunology. Briefly, heparinized venous blood was diluted 1:1 with RPMI-1640 Medium (SIGMA) supplemented with antibiotics, 2 mM L-glutamine (SIGMA) and were carefully layered onto Ficoll-Paque Plus (AMERSHAM-PHARMACIA), then centrifuged at 2500 rpm for 40 minutes at 20^{o}C. After removal of mononuclear cells, the sediment was collected and lysated for 30–35 minutes with ammonium chloride 0.83 % at 4^{o}C; after total lysis of red blood cells, PMNs were centrifuged at 1400 rpm for 15 minutes. The next wash of cells was achieved with ammonium chloride 0.83 % by centrifugation at 1200 rpm for 10 minutes and the last wash was made in Hanks' Balanced Salt Solution (HBSS); finally, PMNs were suspended in the same buffer at a concentration of $3x10^6$cells/mL. The viability of separated PMNs (measured by trypan blue exclusion) exceeded 98% (data not shown).

Luminol/Lucigenin-enhanced Chemiluminescence

Chemiluminescence method can be useful to evaluate the action of Curdlan and curdlan derivatives on the ROS production and release from non-activated or *in vitro*-activated human PMNs. Lucigenin- or luminol-enhanced chemiluminescence methods [40] have been used. Lucigenin allows the detection of superoxide anion levels, while luminol measures ROS production.

Fresh human PMN cells were seeded into 96 wells cell culture plates (COSTAR) ($3x10^5$ cells/well) in 100 µl Hank's Balanced Sault Solution (HBSS) buffer (reagents were purchased from SIGMA). Then, two experimental models have been performed, as follows:

- System 1: Cells were incubated for 15 minutes with the tested compounds; then the cells were activated with Pam3Cys lipopeptide (Pam3CysSKKKK, EMC Microcollections GmbH), 0.6 µM.
- System 2: Cells were incubated for 15 minutes with Pam3Cys (0.6 µM); then the tested compounds have been added.

Pam3Cys is a known Toll-Like-Receptor-2 (TLR2) specific agonist.

In each experimental model, after the cells were treated as mentioned above, a chemiluminometric assay was run in order to quantify ROS or superoxide anion production and release by human PMNs. Oxygen species release was measured after signal amplification was achieved with luminol (5-Amino-2,3-dihydro-1,4-phthalazinedione) 0.13µM or lucigenin (bis-N-methylacridinium nitrate) 0.3µM; the 2 reagents were purchased from SIGMA.

The chemiluminescence (CL) tests were performed using an automatic Fluorimeter/Chemiluminometer system - *THERMO Fluoroskan FL*. The CL signals were registered for 50 min. Data acquisition and statistical processing were performed using the dedicated Ascent software. The values were expressed as Relative Luminescence Units (RLU). The measurements were performed three times in triplicate and final results are expressed as percent of ROS or superoxide anion release.

Statistical analysis

All values were expressed as means of minimum 3 parallel samples ± SD.

For the standard curves (Trolox, Ascorbic acid and sodium nitrite), the correlation factor (R^2) was ≥ than 0.95. The coefficient of variation (CV) for each sample was less than 10% in the case of techniques applied in non-cellular systems (ORAC, TAC) and less than 20% for the chemiluminometric method. Final results represented the average of minimum 3 independent experiments.

Results and discussion

Curdlan is a linear nonionic homopolymer of D-glucose with β-(1→3) glucosidic linkages (Figure 1) [41]; its synthesis was reported by Harada in 1966 as a polysaccharide produced by the microorganism *Agrobacterium biovar* through glucose fermentation and has been commercially produced since 1989 year, in Japan.

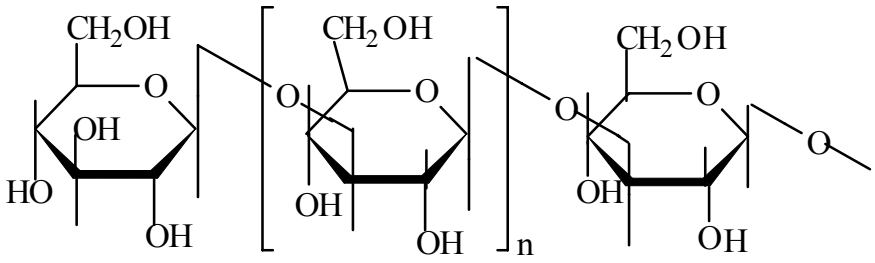


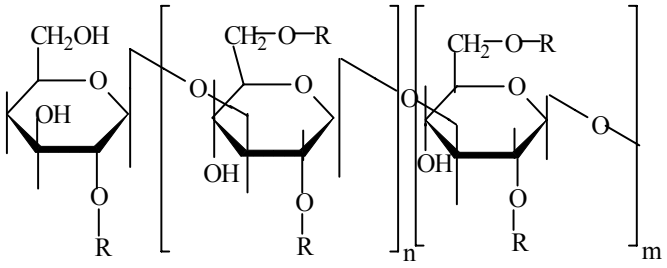
Figure 1. Molecular structure of Curdlan

Curdlan from *Agrobacterium biovar* – (Takeda Chemical Industry) is a tasteless powder, stable in dry state, insoluble in water and alcohol, soluble in alkaline conditions. It is a beta-1,3-Glucan hydrate possessing immunomodulatory activity.

Curdlan being soluble only in alkaline solution (NaOH 1N), a stock solution of 10 mg/ml has been prepared (pH 13). Due to the fact that in cellular experimental models all the tested products must have a physiologic pH (7.2 – 7.4), this pH was achieved only for a Curdlan solution of 0.01 mg/ml made in phosphate buffer saline. This solution has been used in non-cellular systems too.

Synthesis and characterisation of curdlan derivatives

The chemical structure of curdlan derivatives is presented in Figure 2.



R: — OH

—CH_2COONa — carboxymethyl

—$(CH_2)_3\,SO_3\,Na$ — sulfopropyl

—$CO\,(CH_2)_{14}\,CH_3$ — palmitoyl

Figure 2. Structure of synthesized curdlan derivatives

The substitution degree with carboxymethyl groups was determined through conductimetric titrations [42]; the substitution degree with sulfopropyl groups was determined from sulfur analyses, performed according to Schöniger's method [43]. The substitution degree with palmitoyl groups was determined after NaOH hydrolysis, as in the case of other polysaccharide esters [29]. Some physico-chemical characteristics of Curdlan and its synthesized curdlan derivatives are presented in Table 1.

Table 1. Characteristics of Curdlan and the synthesized curdlan derivatives

Curdlan / curdlan derivatives	Molecular weight of stuctural unit	Ion exchange capacity (meq/g)		DS*) with anionic groups		DS with palmitoyl hydrophobic groups
		COOH	SP	COOH	SP	
Curdlan	162	-	-	-	-	-
SP	212	-	1.65	-	0.35	-
Palm CM/SP	250	1.43	1.39	0.26	0.30	0.1

*)*Degree of substitution (the number of hydroxylic glucopyranosic groups substituted with carboxymethyl, sulfopropyl or palmitoyl units)*

The synthesized curdlan derivatives (Palm CM/SP and SP) were soluble in phosphate buffer saline and stock solutions of 1 mg/ml (pH 7.2 – 7.4) were prepared to be used in all experimental models.

Curdlan derivatives scavenging capacity

It is a known fact fact that there are two types of antioxidant products: scavengers and inhibitors of ROS production and release; in our non-cellular experimental models, the scavenging capacity of Curdlan and curdlan derivatives (Palm CM/SP and SP) has been tested (Table 2).

Table 2. Scavenging capacity of Curdlan and curdlan derivatives; tests were performed by both ORAC and TAC assays (non-cellular systems). Values are means ± SD of three independent experiments

SAMPLE	ANTIOXIDANT CAPACITY - ORAC (TE*)/mg)	TOTAL ANTIOXIDANT CAPACITY - TAC (μM ascorbic acid/mg)
Curdlan	<0.01	<0.01
Palm CM/SP	5.527 +/- 0.511	<0.01
SP	<0.01	<0.01

**)*Trolox equivalents (1 TE = 1μM Trolox)*

The results presented in Table 2 showed that the tested Curdlan or curdlan derivatives had not scavenging capacity, either for peroxyl radicals (ORAC), or for reactive oxygen species (TAC).

Modulation of human PMNs oxidative stress

Cells normally respond to various external stimuli and toxicants by developing oxidative burst. The oxidative burst developed by granulocytes in response to an infectious agent is an effective non-specific mechanism for eradicating the invading pathogen.

We focused on the capacity of the Curdlan and curdlan derivatives to modulate human PMNs to release ROS.

Curdlan derivatives have been used as 1 mg/ml solutions in phosphate buffer saline (20μg/system). Due to the fact that Curdlan was soluble only in alkaline solutions, we had to use a Curdlan solution of only 0.01 mg/ml; it was the solution with the highest amount of Curdlan possessing a physiologic pH (7.2-7.4). Curdlan, 0.1μg/system, was used in our chemiluminometric assay.

ROS production was quantified by luminol-enhanced chemiluminometry and superoxide radicals production was measured by lucigenin-enhanced chemiluminometry. The

values obtained, expressed as Relative Luminescence Units (RLU), were used to quantify final results as percent of ROS release or superoxide anion production respectively in two experimental models (Fig. 3 and Fig. 4).

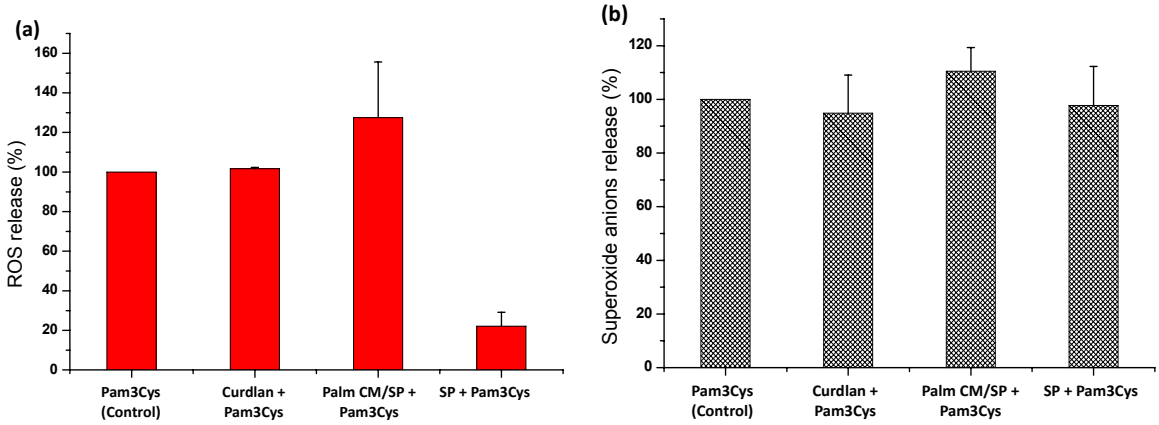


Figure 3. Effects of Curdlan (0.1µg/system) and Curdlan derivatives (20µg/system) on luminol (a) and lucigenin-amplified chemiluminescence (b) of human PMNs. Cells were treated with Curdlan/cudlan derivatives and then activated with Pam3Cys (0.6 µM).
Results are expressed as percent of ROS release (a) or superoxide anion production (b).
Data represent the means ± SD of five independent experiments

As seen in Figure 3, Curdlan did not significantly modify ROS release (a) and superoxide anion production (b) in PMNs. Palm CM/SP slowly up-regulated both ROS (27%) and superoxide anion (10%) release (a, b) in PMNs. Despite the fact that SP was able to strongly down-regulate (78%) ROS production (a), it had no influence on superoxide anions release (b).

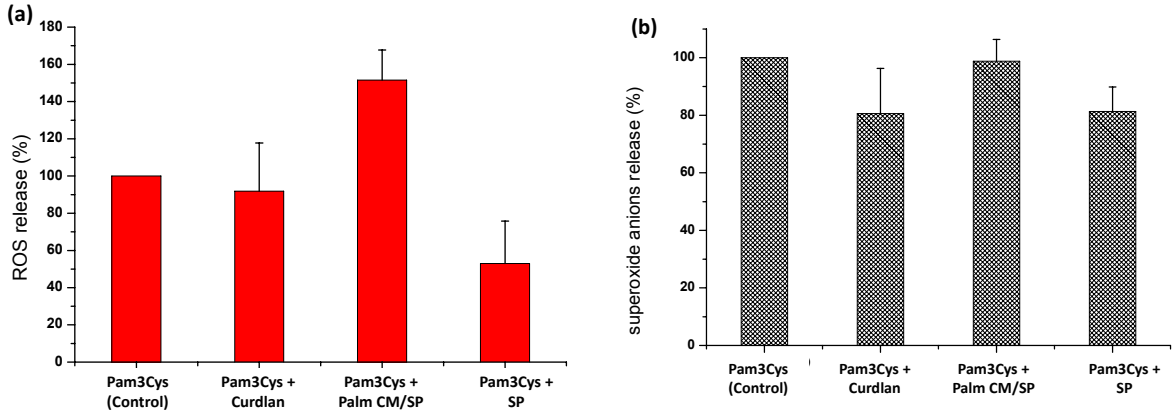


Figure 4. Effects of Curdlan (0.1µg/system) and Curdlan derivatives (20µg/system) on luminol (a) and lucigenin-amplified chemiluminescence (b) of human PMNs. Cells were activated with Pam3Cys (0.6 µM) and then treated with Curdlan/curdlan derivatives.
Results are expressed as percent of ROS release (a) or superoxide anion production (b).
Data represent the means ± SD of five independent experiments

As it can be observed in Figure 4, Curdlan had a bearing to down-regulate (8%) ROS (a) and (19%) superoxide anions (b) release in Pam3Cys activated PMNs. Palm CM/SP up-regulated (52%) ROS release (a) but did not influence superoxide production (b) in human PMNs. SP showed inhibitory activity (47%) on ROS release (a) and it was able to slowly down-regulate (19%) superoxide production (b) in activated PMNs.

Discussions

It is known that the immunomodulatory effects of β-glucans are influenced by their degree of branching, polymer lengths and tertiary structure, but there is still no consensus on the basic structural requirements for biological activity. There are also evidences that macrophages respond to β-glucans, possibly released from fungal cell walls, via receptors such as Dectin-1 and Toll-like receptors, to induce inflammatory reactions. As the major β-glucan receptor in mammals, Dectin-1 is required for control of fungal infections [44] and has been shown to play a role in the immune response to bacterial infections as well. Moreover, a collaborative role for Dectin-1 in TLR signaling has been demonstrated [45].

In our study we investigated two synthesized curdlan derivatives as possible antioxidants and/or modulators of human PMN cells respiratory burst. We demonstrated that either Curdlan or the curdlan derivatives were able to act on PMN cells but not as oxygen radicals scavengers (they did not show antioxidant properties by both ORAC and TAC non-cellular tests). Curdlan derivatives demonstrated different modulatory activities on PMNs respiratory burst.

The data obtained using the ORAC fluorimetric method sustained the results provided by the TAC colorimetric technique proving that all the tested compounds were not able to block the oxygen radicals.

Aiming that the tested compounds were polymers with high molecular weights, their transfer across cellular membrane is less probable. In this case, it was possible that their capacity to modulate cellular activity was due to the binding of the compounds to specific receptors (able to detent signaling pathways leading to ROS production and release).

Cross-talk between different classes of PAMPs is considered an important mechanism for external signals integration and specific innate immune responses induction. It has been demonstrated that engagement of the Dectin-1 (β-glucan receptor) amplified cytokine production induced by TLR2 stimulation [46].

The first chemiluminescence system (PMNs treated with Curdlan/Curdlan derivatives and then activated with Pam3Cys) has been used to investigate if the tested compounds could have a protective role against infectious agents.

Our results showed that Curdlan did not significantly modify superoxide anion and ROS release in PMNs. Regarding the action of curdlan derivatives, our data indicated that they presented different actions as follows: Palm CM/SP up-regulated both superoxide anion and ROS release, probably as an agonist of TLR2 or Dectin-1 receptors. SP strongly down-regulated ROS production and had no effect on superoxide anion release in PMNs, being a possible inhibitor of TLR2 (either directly or by Dectin-1 receptor).

By activating PMNs with Pam3Cys (as TLR2 agonist) and then treating the cells with the tested compounds (system 2), we investigated their possible use in the infections treatment. The data obtained demonstrated that Curdlan slowly down-regulated ROS and superoxide release in activated PMNs. Palm CM/SP significantly up-regulated ROS production but it did not present any influence on superoxide anion release. SP strongly down-regulated ROS release partially due to the inhibition of superoxide anion production.

Altogether, the results obtained in both chemiluminometry models showed that Curdlan did not significantly influence the ROS and superoxide anion production and release in human PMNs. Palm CM/SP, a possible agonist of TLR2 or Dectin-1 receptor, strongly increased the ROS production. Knowing that Dectin-1 is the specific receptor for β-glucans (the backbone of Palm CM/SP), we could conclude that this curdlan derivative directly acted on Dectin-1 receptor and its effect on TLR2 signaling pathway was due to Dectin-1 – TLR2 co-operation. One could presume that the presence of the grafted groups (palmitoyl and/or

carboxymethyl) were responsible for increasing of ROS release. Palm CM/SP could be useful in immune functions stimulation, before the admission of an infection and it could not be indicated after the infection detent.

SP significantly down-regulated ROS release in both experimental systems, suggesting that it could be a TLR2 or Dectin-1 inhibitor. Taking into account the fact that in the second chemiluminometry test TLR2 receptors had been activated before the adding of SP, we could conclude that SP was a possible Dectin-1 antagonist. In our previous study [47] we demonstrated that SP alone cannot induce ROS production (luminol-enhanced chemiluminometry) in human PMNs; moreover, when the cells were treated with SP in the presence of the TLR-2 specific agonist (Pam3Cys), an inhibition of ROS production was observed. It was possible that sulfopropyl groups have been responsible for down-regulation of ROS production.

By comparing with Curdlan, we demonstrated that the synthesized curdlan derivatives could be more attractive and useful compounds, able to modulate human PMNs respiratory burst.

In summary, the data obtained in this study are important because they increase the knowledge on curdlan derivatives containing palmitoyl, carboxymethyl and sulfopropyl groups, the information gathered here having important implications for our understanding of how immune responses to fungal pathogens develop, as well as for the design of immunomodulators containing curdlan derivatives.

Acknowledgements

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