In vitro and *in vivo* immunomodulatory activity of sulfated polysaccharides from red seaweed *Nemalion helminthoides*

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A B S T R A C T

Water-soluble sulfated polysaccharides from the red seaweed *Nemalion helminthoides*: two xylomannan fractions (N3 and N4) and a mannan fraction (N6) were investigated to determine their *in vitro* and *in vivo* immunomodulatory activities. N3 and N4 induced *in vitro* proliferation of macrophages of the murine cell line RAW 264.7 and significantly stimulated the production of nitric oxide (NO) and cytokines (IL-6 and TNF-α) in the same cells, whereas this response was not observed with the mannan N6. The cytokine production was also stimulated by sulfated xylomannans *in vivo* in BALB/c mice inoculated intravenously with these polysaccharides. Remarkably, when mice were treated with N3 and N4 for 1 h before being infected with Herpes simplex virus type 2, they remained asymptomatic with no signs of disease. The *in vitro* and *in vivo* results suggest that sulfated xylomannans could be strong immunomodulators.

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1. Introduction

Sulfated polysaccharides are bioactive macromolecules in which some of the hydroxyl groups of the sugar residues are substituted by sulfate groups. These anionic polysaccharides are ubiquitous in nature and occur in a wide variety of organisms, including mammals, invertebrates and flora [1,2]. In algae, polysaccharides are the major component of matrix cell wall. Within this group, sulfated galactans such as agar and carrageenans are present in most red algae. However, other types of polysaccharides are synthesized by some of these macroalgae; the presence of neutral β-D-(1 → 4), β-D-(1 → 3) "mixed linkage" xylans and sulfated mannans and xylomannans has been reported for the Nemaliales [3].

Currently, research on natural compounds that can modulate the immune system are gaining attention in experimental fields such as immunopharmacology and oncotherapy [4].

The effects of polysaccharides from seaweeds on the immune system are, to date, much less studied than those of higher plants and mushrooms [5–7]. Immunomodulatory studies on sulfated polysaccharides from marine algae have been mainly carried out on fucoidans from brown algae [8], heteropolysaccharides from green

algae and cyanobacteria [4,9], and in lesser extent on carrageenans from red seaweeds [10–12].

Only a few systems of sulfated mannans and xylomannans were studied in detail. In previous studies, we carried out the isolation and chemical characterization of a sulfated mannan (N6) and two xylomannans (N3 and N4) from *Nemalion helminthoides* [13,14]. These mannans have a linear backbone of (1 → 3)-linked α-D-mannopyranose units with sulfate groups at the 4- or 6-position; in the xylomannans D-xylose side-chains are also linked at 2-position of the chain. Only the xylomannans showed some activity *in vitro* against Herpes simplex virus, but no anticoagulant nor cytotoxic effects on Vero cells were observed [13].

Given the background with other kind of polysaccharides, the purpose of this study was to inquire into the immunomodulatory potential of sulfated mannans and xylomannans of *N. helminthoides*. To the best of our knowledge, this is the first report on this kind of research.

2. Materials and methods

2.1. Extraction and fractionation of the polysaccharides

Fractions N3, N4 and N6 were obtained as described in our preceding paper [13]. Briefly, the product of the first extraction with hot water of the red alga *N. helminthoides* was precipitated with cetrimide and subjected to fractional solubilization with solutions

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of increasing concentrations of NaCl to obtain N3, N4 and N6, soluble at 1.50, 2.00 and 4.00 M NaCl, respectively.

2.2. Cells and viruses

The macrophage-like cell line RAW 264.7 derived from tumors induced in male BALB/c mice by the Abelson murine leukemia virus (ATCC, Rockville, MD, USA) was cultured for no more than 15 passages in Dulbecco' Modified Eagle' Medium (DMEM-GIBCO) supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin (mix GIBCO 15140-148), in buffer HEPES.

HSV-2 strain MS was obtained from the American Type Culture Collection and was propagated and titrated by plaque formation on Vero cells (kidney of a normal adult African green monkey *Cercopithecus aethiops*).

2.3. Cell proliferation

For the proliferation assays, 50 μL of RAW 264.7 murine macrophages suspension (1×10^4 cells/well), were incubated in 96-wells plates with DMEM supplemented with 0.5% of fetal bovine serum (minimum concentration required to maintain cell viability) in the presence of 50 μL of polysaccharides at concentrations of 0.5, 5 and 10 μg/mL, in triplicate. Cells were incubated 48 h, at 37 °C with 4% CO_2. After the incubation period elapsed, 20 μL of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) reagent (Promega Cell Titer *96 Aqueous Non-Radioactive Cell Proliferation*) were added per well, and after 2 h more of incubation, the absorbance was determined in a microplate reader (Biotek ELx808) at 490 nm ref. 630 nm. The number of cells was calculated using a standard curve, performed by adding different number of cells (from 1×10^3 to 1×10^6) per well in quadruplicate and recording the optical density at 490 nm ref. 630 nm. The correlation coefficient of the line was 0.998, indicating a linear response between cell number and absorbance. LPS (5 μg/mL) was used as positive proliferation control.

2.4. Cytotoxicity

Cell viability was measured by the MTS method. Briefly, 24 h confluent cultures of RAW 264.7 cells (1×10^4 cells/well) in 96-well plates were exposed to four different concentrations of polysaccharides, in triplicate, and incubated 48 h at 37 °C. Then 20 μL of reagent was added per well, and after 2 h the absorbance was determined in a microplate reader (Biotek ELx808) at 490 nm ref. 630 nm. Absorbance of untreated cells was considered as 100% viability. The 50% cytotoxic concentration (CC_{50}) was calculated as the compound concentration required to reduce cell viability by 50%.

2.5. Cytokine determination and nitric oxide assay

Confluent monolayers of RAW 264.7 cells in 6-wells plate were exposed to 5 μg/mL of polysaccharides (in DMEM containing 1% fetal bovine serum) during 8, 16 and 24 h, each treatment in triplicate, in a total volume of 1 mL. LPS (5 μg/mL) was used as positive control for macrophage activation. Supernatants were collected after the corresponding times and IL-6, TNF-α and nitric oxide (NO) concentrations were determined. IL-6 and TNF-α secretion was measured by ELISA (BD Biosciences, USA), in triplicate. Absorbance at 450 nm was determined in a microplate reader (Biotek ELx808). Production of NO was estimated by measuring nitrite levels in cell supernatant with Griess reagent (1% sulfanilamide in 2.5% phosphoric acid, 0.1% napthyl ethyl diamine dihydrochloride in 2.5% phosphoric acid), in triplicate. Absorbance was read at 540 nm.

2.6. Experimental animals

Eight-weeks-old BALB/c mice (body weight, 18–20 g) were housed in polycarbonate cages and fed with a standard animal diet and water *ad libitum* under controlled temperature conditions 22 ± 2 °C with a 12 h light/dark cycle for a week before treatment. All procedures of animal care were performed according to national and international laws and policies (Facultad de Ciencias Exactas y Naturales, Buenos Aires, Argentina (CD 140/00) and Public Health Service, NIH, 2002).

2.7. In vivo effect of polysaccharides on serum cytokines levels

Groups of 6 animals each received 0.2 mL of different polysaccharide solutions (30 mg/kg) intravenously (iv). At 30, 60 and 90 min after inoculation, blood samples were collected and centrifuged 10 min at 1000 rpm. Serums cytokines (TNF-α and IL-6) concentrations were determined by ELISA. Control group received 0.2 mL of physiological solution iv.

2.8. Immunoprotection of mice against viral infection

Groups of 4 animals each were inoculated iv with 0.20 mL of polysaccharide solution (30 mg/kg). After 1 h, mice were infected with 20 μL of HSV-2 (3×10^4 PFU) by intranasal route. Animals morbidity and mortality were monitored during 3 weeks.

2.9. Statistical analysis

The data are expressed as means ± SD. Statistical analysis was done by analysis of variance (ANOVA) followed by Bonferroni's test. *P* values less than 0.05 or 0.01 were considered as significant.

3. Results and discussion

The chemical composition and the molecular structure of the polysaccharides obtained from *N. helminthoides* have been previously reported [13,14]. Some chemical features of mannan N6 and xylomannans N3 and N4 are shown in Table 1. The chemical structure of these fractions was determined by methylation and desulfation–methylation analyses, ^{1}H and ^{13}C NMR spectroscopy [13]. N6 consisted of a linear backbone of $(1 \rightarrow 3)$-linked α-D-mannopyranose units with sulfate groups at the 4- or 6-position; in xylomannans N3 and N4, single stubs of β-$(1 \rightarrow 2)$-linked D-xylose were also found.

Fractions F3 and F6 also obtained in our laboratory from *Nothogenia fastigiata* (another Nemalial red seaweed considered as a rich source of mannans and xylomannans) were used for comparison (Table 1). The structural analysis of the control fractions was carried out as described before by methylation and desulfation-methylation analyses, ^{1}H and ^{13}C NMR spectroscopy [15]. F6 was also a $(1 \rightarrow 3)$-linked α-D-mannan but 2- and 6-sulfated; xylomannan F3 contained, in addition, β-$(1 \rightarrow 2)$-linked xylopyranosyl side chains.

3.1. Cell cytotoxicity

In order to study if the polysaccharide fractions had any effect on cell viability, confluent monolayer of RAW 264.7 were analyzed by the MTS method.

As shown in Fig. 1, N6 reduced significantly the cell viability (80% with respect to control cells) even at the lowest concentration assayed (8 μg/mL), similarly to the fraction F6 used as reference substance. In the case of the xylomannans N3 and N4, the cell viability was moderately reduced at 8 μg/mL (28 and 32%, respectively)

Table 1
Chemical features of the fractions N3, N4 and N6 from *N. helminthoides*. Fractions F3 and F6 from *Nothogenia fastigiata* are included for comparison.

Fraction	Range of precipitation (M, NaCl)	Yield (%)	Molecular weight (kDa)	Man:Xyl:Sulfate (molar ratio)
N3	1.0–1.5	0.8	13.6	1.00:0.37:0.45
N4	1.0–2.0	0.5	11.7	1.00:0.22:0.84
N6	3.0–4.0	13.8	43.8	1.00:0.01:0.64
F3	1.0–1.5	5.7	39.1	1.00:0.28:0.84
F6	3.0–4.0	3.1	30.0	1.00:0.02:0.82

Table 2
Plasma levels of IL-6 and TNF-α in mice injected i.v. with N4 (30 mg/kg) after 30, 60 and 90 min. PBS and F3 were administered in the same way and used as control and reference fraction, respectively. The results are presented as means $\pm$ SD obtained by ELISA ($n = 3$). Significant differences ($p < 0.01$) between N4 and PBS at 60 and 90 min.

Treatment	IL-6, pg/mL			TNF-α, pg/mL		
	30 min	60 min	90 min	30 min	60 min	90 min
N4	60 $\pm$ 10	1850 $\pm$ 97	2100 $\pm$ 121	52 $\pm$ 10	317 $\pm$ 20	915 $\pm$ 51
F3	55 $\pm$ 3	750 $\pm$ 59	1100 $\pm$ 67	58 $\pm$ 5	690 $\pm$ 35	1300 $\pm$ 45
PBS	10 $\pm$ 2	5 $\pm$ 1	18 $\pm$ 2	62 $\pm$ 1	68 $\pm$ 2	70 $\pm$ 9

while F3, the other fraction used as reference, decreased 50% cell viability.

These results suggest that fractions with xylose side chains (N3, N4 and F3) are less cytotoxic than mannans N6 and F6; moreover, the influence on cell viability of the sulfation pattern (C-4 and C-6 in fractions N3 and N4, C-2 and C-6 in fraction F3) could be taken into account. Due to the high cytotoxicity of N6 and F6 observed on RAW 264.7 cells, these fractions were not tested in the cell proliferation assay.

3.2. Cell proliferation

Proliferation of RAW 264.7 cells induced by the xylomannans (N3 and N4) at concentrations between 0.5 and 10 μg/mL is shown in Fig. 2. Fraction N3 excerted a moderate proliferative effect throughout all the range of concentrations tested, similar to the comparative fraction F3. Xylomannan N4, meanwhile, showed a marked cellular proliferation in a concentration dependent manner; remarkably, this effect was even higher than the observed in control cells stimulated with LPS.
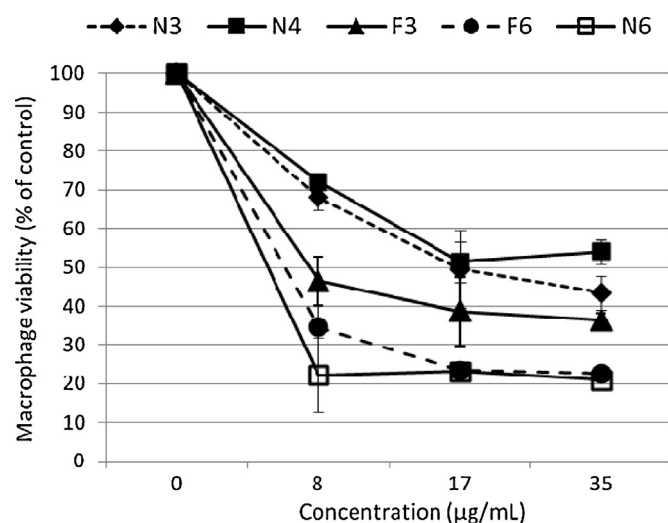
3.3. Production of IL-6, TNF-α and NO by RAW 264.7 cells

Macrophage cells are essential for maintaining homeostasis regardless of varying external conditions and play a main role in host defense against pathogens and invading cells, including cancer cells [16]. To achieve their task, activated macrophages release some immunomodulatory factors such as NO, IL-6, TNF-α, among others. Generally, RAW 264.7 cells are used to determine the immunomodulatory activities by investigating their cytokines secretion.

The production of IL-6 by RAW 264.7 cells stimulated by the different polysaccharide fractions (5 μg/mL concentration) is shown in Fig. 3a. Cellular control (CC) corresponds to incubation with DMEM only. During the first 8 h of treatment, the level of cytokines in stimulated cultures was similar to unstimulated control cells (data not shown). Nevertheless, the xylomannan fractions N3, N4 and the reference fraction F3 increased the production of IL-6 at 16 h and at 24 h with respect to CC. A smaller increase in IL-6 production was also seen when the mannan fraction N6 and the reference fraction F6 were added to the cells. LPS, used as a positive control, strongly increased the production of IL-6.

In the same way, the concentration of TNF-α was determined. No difference with respect to control cells in the level of this

Fig. 1. Cytotoxicity of fractions derived from *N. helminthoides* on RAW 264.7 cells determined by MTS method after 48 h of incubation. F3 and F6 fractions derived from *N. fastigiata* are used as reference. Data are presented as means $\pm$ SD ($n = 3$).
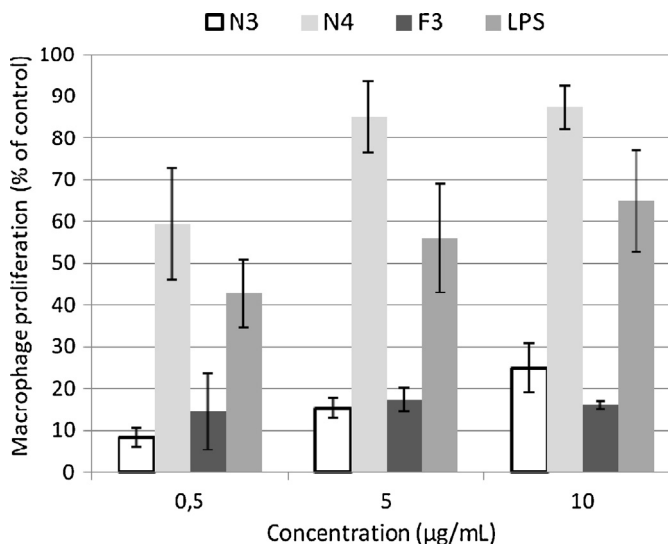
Fig. 2. Proliferation of RAW 264.7 cells induced by xylomannans. F3 and LPS were included as reference fraction and control, respectively. Data are presented as means $\pm$ SD ($n = 3$).
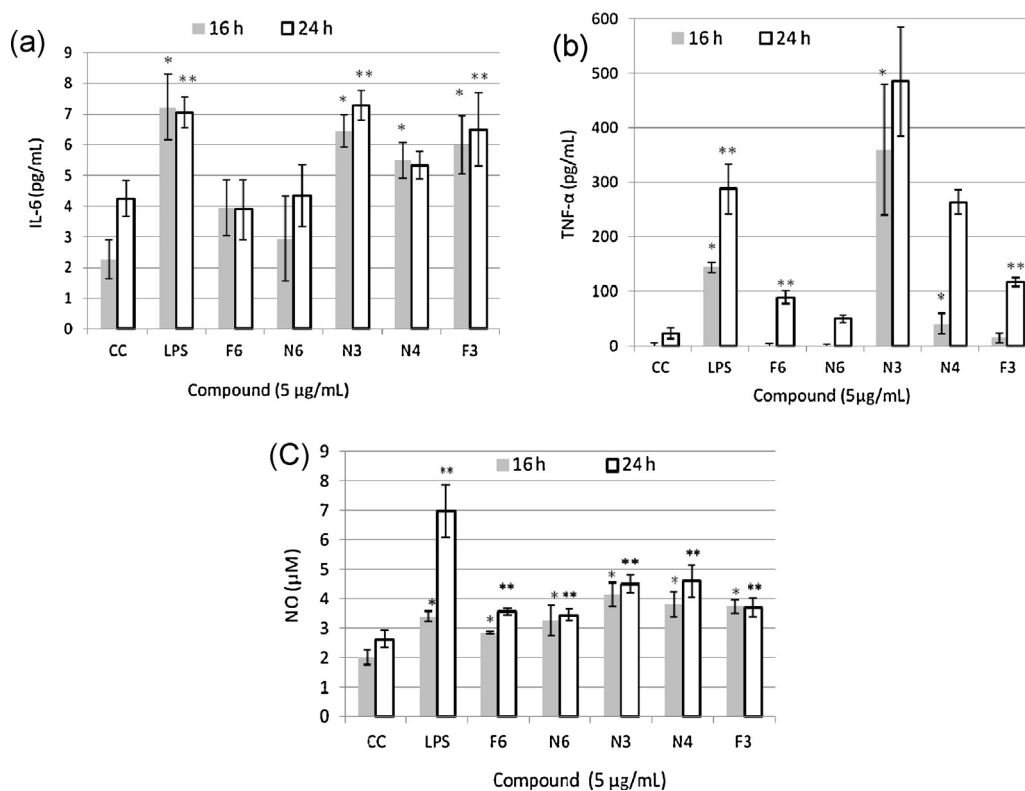
Fig. 3. Secretion levels of cytokines released from RAW 264.7 cells treated with various fractions of polysaccharides (a) IL-6, (b) TNF-α, and (c) NO. Data are presented as means $\pm$ SD ($n = 3$). Significant differences ($p < 0.05$) from the cellular control are indicated as: *(16 h) and **(24 h).

cytokine was observed within the first 8 h after the addition of the compounds (data not shown). However, a great increase in TNF-α production was obtained in cultures stimulated with fraction N3, even higher than that recorded in the control cells stimulated with LPS at 16 and 24 h post-treatment. For N4 and F3, activation was significant after 24 h of exposure, N4 levels being twice that for F3. A slight increase in the level of TNF-α was seen with N6 and F6 fractions only alter 24 h of treatment (Fig. 3b).

The levels of NO were low for the times tested (Fig. 3c), except for the positive control of LPS at 24 h that was 7 μM. However, in supernatants of cell cultures stimulated with N3 and N4 for 24 h, the concentration of NO was 4.5 μM, which almost doubled the value of the control. Mannan N6 showed low levels of NO at 16 and 24 h compared with xylomannans. Then, the sulfated xylomannans differed markedly from the sulfated mannans in their immunomodulatory effects *in vitro*. The sulfated xylomannans, especially N4, promoted the proliferation and activation of macrophages causing the release of IL-6, TNF-α and NO. Although N3 and N4 showed some toxicity on macrophages, this effect was observed at concentrations significantly lower than their CC_{50}. Is important to mention that while N6 and F6 fractions showed a significant reduction in cell viability as observed after 48 h at a concentration of 8 μg/mL, they were included in this assay because in the conditions tested (5 μg/mL and up to 24 h incubation) they did not show any cytotoxic effect. We can therefore conclude that the observed variations in the production of IL-6, TNF-α and NO were due to an immunomodulatory effect of the fractions.

Taking into account its chemical structure, both sulfated mannans, N6 and F6, in all cases showed very similar performance, strongly suggesting that mannose residues but not sulfation pattern would be involved in the effects shown. Similarly, what was found for xylomannans N3, N4 and F3 seems more influenced by the single stubs of xylose than by the position of sulfate groups.

Even though mannans were obtained in much greater yield from both algae (Table 1), minor fractions appear as the more active.

3.4. In vivo cytokines secretion and NO production

Due to the considerable cytokine-releasing capacity of N4 in RAW 264.7 cells, it was selected to perform the *in vivo* experiments. F3 was used for comparison and PBS as control. Plasma levels of IL-6 and TNF-α increased significantly at 60 and 90 min in mice intravenously injected with the xylomannan N4 and the control fraction F3 ($P < 0.01$). Maximum levels were reached after 90 min post injection with concentrations of 2100 and 915 pg/mL of IL-6 and TNF-α, respectively (Table 2).

No variation in the level of NO was observed in the animals treated with N4 or F3 with respect to control animals inoculated with PBS over the range of time tested (data not shown).

3.5. Immunoprotection against HSV-2 infection

Knowing the stimulating effect on cytokine production caused by the xylomannans intravenously administered, we proceeded to test them as immunoprotective agents. For this purpose, mice were intranasally infected with a sublethal dose of HSV-2, 1 h after the administration of the xylomannans by intravenous route. This model appeared of interest because preliminary studies confirmed that HSV-2 does not induce activation of IL-6 and TNF-α in mucose at early times of infection, being the possible cause of illness and death [17].

As shown in Table 3, N3 and N4 were able to reduce morbidity by 75 and 100%, respectively. In the control group, where the xylomannans were replaced by PBS, a 75% of morbidity was registered. Surprisingly, no differences were observed with respect to the control group when F3 was employed.

Table 3
Protective effect of xylomannans in BALB/c mice infected intranasally with HSV-2.

Group	Morbidity (%)
PBS	75
N4	0
N3	25
F3	75

Therefore, these results confirmed the immunoprotective effect of the xylomannans from *N. helminthoides* in mice used as experimental model in a viral infection. Probably, a rapid increase in the levels of plasmatic cytokines produced by immunomodulating substances explain, at least in part, the efficacy of the treatment.

4. Conclusion

The sulfated xylomannans from *N. helminthoides* can stimulate macrophage cells and induce considerably production of cytokines both in RAW 264.7 cells and in mice. Besides, they can act as immunoprotectors against a herpetic infection.

The *in vitro* and *in vivo* results suggest that the sulfated xylomannans might be strong immunomodulators. Molecular mechanisms that allow the action of these compounds in immune cells could involve pattern recognition receptors (PRR; mannose receptor, DC-Sign, Dectin, C Lectin, among others) as well as was demonstrated with polysaccharides from other sources [18–21].

Since no information is at present available about the effect of these kind of structures on the immune system, our current data encourage more research for the potential application of these sulfated polysaccharides in the immunomodulation field, for example in immunopotentiators supplements or vaccine adjuvants. N4 from *N. helminthoides* could be a good model compound regarding its feature here described as well as the current research on natural products that can provide new prospects on the wide field of immunomodulation. Additionally, xylomannan is an infrequent structure in nature and in the world of red algae [3].

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