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Carbohydrate Polymers 57 (2004) 113–122
Carbohydrate Polymers
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Yeast mannan-vanadium (IV) complexes and their effect on peritoneal macrophages

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Received 30 January 2003; accepted 28 November 2003
Available online 28 July 2004

Abstract

In order to evaluate the formation of complexes between the vanadyl ion (IV) and baker's yeast mannan (MPOLY), methyl α-D-mannopyranoside (MM) was used as a model. The interaction was examined using potentiometric titration and infrared spectroscopy. The potentiometric studies provided the binding constant for the complex and the resulting complexed species were a function of pH. FTIR spectra contained a sharp and strong band at 1381 and 1382 cm^{-1} for MM–VO and MPOLY–VO, respectively, absent in non-complexed spectra. In contrast to free carbohydrates, MPOLY–VO and MM–VO were highly toxic to macrophages. The mannan and its complexes with vanadyl ion lowered superoxide anion production in macrophages triggered with PMA. MPOLY enhanced nitric oxide production (55%), this effect not being observed for MPOLY–VO as well as MM and MM–VO.

Keywords: Yeast-mannan; Metal complexes; Saccharides; Vanadyl ion

1. Introduction

Vanadium in its tri-, tetra-, and pentavalent oxidation states have the ability to interact with biomolecules, which may be responsible for important biological effects (Baran, 2000). This distinct behaviour is, at least in part, due to its capacity to change its coordination and oxidation states, assuming anionic or cationic forms. Different aspects of the coordination chemistry of vanadium, which are relevant for its presence and activity in biological systems, have been reviewed (Morinville, Maysinger, & Shaver, 1998; Sigel & Sigel, 1995). A variety of effects have been ascribed to vanadium compounds. The most studied is insulin mimetic activity, which has stimulated the search for their possible therapeutic value as an orally active agent against diabetes (Crans, 2000; Godwaser, Gefel, Gerhonov, Fridkin, & Shechter, 2000; Morinville et al., 1998; Yasui, Takechi, & Sakurai, 2000). Vanadium also has the ability of modulate an immune response in humans and experimental animals, macrophages and lymphocytes representing important targets (Cohen et al., 1996; Grabowski, Paulauski, & Godleski, 1999; Olivier et al., 1998; Tsuji & Sakurai, 1996; Yamaguchi et al., 1995; Zellikof & Cohen, 1995). The vanadyl ion (IV, VO) is less toxic than vanadate (V) (Tsuji & Sakurai, 1996) and is the main chemical form in tissues and organs (Yasui et al., 2000). Toxicity effects related to the vanadyl ion (VO) showed a relationship with the inhibition of several enzymatic activities such as ATP phosphohydrolases, ribonuclease, adenylate kinase, the glycolytic enzymes phosphofructokinase, and glyceraldehyde-3-phosphate dehydrogenase among others (Macara, 1980). The strong preference for oxygenated environments enables the interaction of vanadium species with carbohydrates and other polyhydroxyl compounds (Baran, 2000; Sreedhara, Raghavan, & Rao, 1994; Verchère, Chapelle, Xin, & Crans, 1998). Sugars can interact with metal ions either as reducing agents or as chelators (Baran, 2000; Whitfield, 1993). It has been reported that low molecular weight saccharide complexes of the vanadyl ion introduce nicks in pUC18 DNA and causes lipid peroxidation in isolated rat hepatocytes (Sreedhara, Nobuyuki, Patwardhan, & Rao, 1996a).

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0144-8617/$ - see front matter © 2004 Published by Elsevier Ltd.
doi:10.1016/j.carbpol.2003.11.017

Polysaccharides isolated from microorganisms (Konopski, Smedsrød, Seljelid, & Eskeland, 1994; Kulicke, Lettau, & Thielking, 1997) as well as other sources have been recognized as biological response modifiers (BRM) (Bohn & BeMiller, 1995). Besides native polysaccharides, derivatives arising from partial hydrolysis (Kulicke et al., 1997) or chemical modifications such as carboxymethylation (Bohn & BeMiller, 1995; Sasaki, Abiko, Nitta, Takasuka, & Sugino, 1979), oxidation and/or introduction of new groups, have also been successfully studied as BRM (Cross et al., 2001). Among these polysaccharides (1 → 3)-linked β-glucans, isolated from microbial cell walls, represent one of the most frequently studied classes. Both particulate and soluble (1 → 3)-β-glucans, derived from a variety of plant, fungal, and bacterial sources, have been shown to activate the immune system (Bohn & BeMiller, 1995; Demleitner, Kraus, & Franz, 1992). Several researchers have reported that some polysaccharides can interfere with the respiratory burst of macrophages (Badway & Karnovski, 1980; Berton & Gordon, 1983; Williams, Topley, Alobaidi, & Harber, 1986). Certain (1 → 3)-β-D-glucans, were subclassified according to their activity as inducers of IFN-γ and/or NO production (Hashimoto, Ohno, & Yadomae, 1997). An α-glucan (Stuelp et al., 2002) and a galactomannan (Noleto et al., 2002) both isolated from the lichen *Ramalina celastri* were shown to modify the macrophage response in terms of release of hydrogen peroxide and NO production, respectively. Ramesh, Yamaki, and Tsushida (2002) demonstrated that the galactomannan from fenugreek (*Trigonella foenum-graecun* L.) interferes with phagocytic ability of rat macrophages and both proliferation and IgM secretion in HB4C5 cells. Metal–carbohydrate complexes are described as an important tool in the pharmacology, since carbohydrates can act as a metal carrier (Cantos, Barbieri, Iacomini, Gorin, & Travassos, 1993; Roberts, Hariprashad, Rainey, & Murray, 1996). However, the effect of polymers complexed with vanadium on macrophage functions has been poorly studied. In spite of the existence of reports on the complexation of vanadyl ion with monosaccharides (Baran, 2000; Etcheverry, Williams, & Baran, 1997; Geraldes & Castro, 1989; Sreedhara et al., 1994; Sreedhara, Rao, & Rao, 1996b; Verchère et al., 1998), the formation of vanadyl complexes with polysaccharides has been poorly studied. The complexation of a galactomannan isolated from the lichen *R. celastri* with vanadyl (VO), was demonstrated to drastically modify the effect of the polymer on peritoneal macrophages and *Leishmania* parasite, the effect of the polymer–vanadyl complex occurring at a 100-fold lower concentration than with the native polymer (Noleto et al., 2002). We now, evaluate the ability of mannan (MPOLY; Fig. 1A) to form complex with vanadyl ion. Methyl α-D-mannopyranoside (MM; Fig. 1B) was also used as a model molecule and MPOLY was isolated from baker's yeast (MPOLY; Fig. 1A). Its structure consists of a main chain of (1 → 6)-linked α-Man*p* units, substituted at

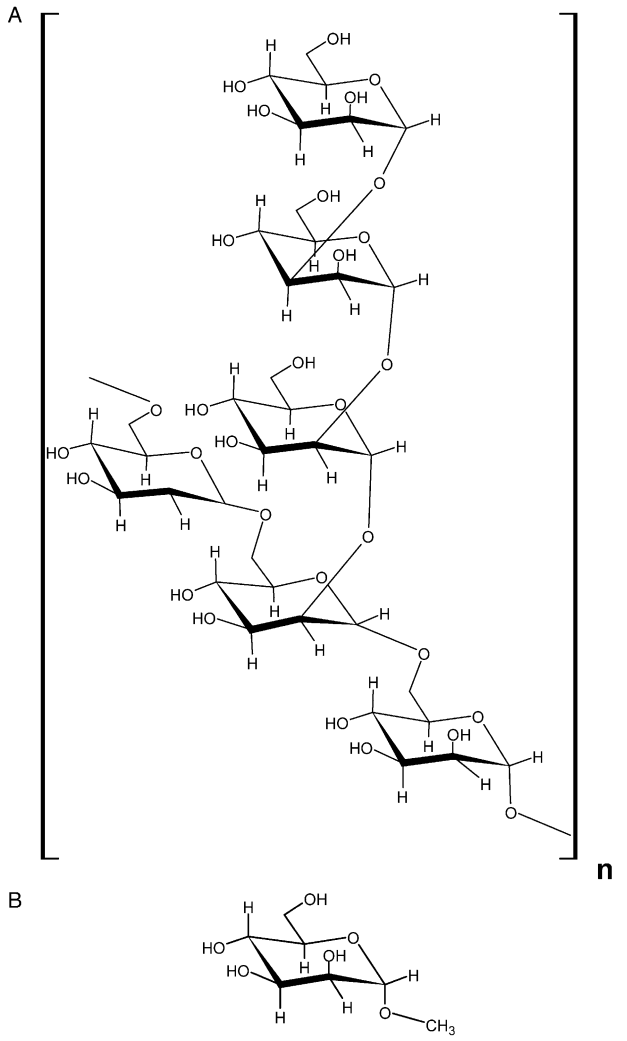

Fig. 1. (A) Partial structure of baker's yeast mannan (MPOLY). (B) Structure of methyl α-D-mannopyranoside (MM).

O-2 with side chains of α-Man*p* units linked (1 → 2) with a lower proportion of (1 → 3)-links (Lee & Ballou, 1965).

2. Materials and methods

2.1. Materials

Phorbol 12-myristate 13-acetate (PMA), lipopolysaccharide (LPS), *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES), ferricytochrome *c*, superoxide dismutase (SOD) (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) (MTT), sulfanilamide, naphthylethylene–diamine were obtained from Sigma Chemical Co. (St Louis, MO). Vanadyl sulfate was obtained from Sigma-Aldrich. Polymyxin was from Inlab (Brazil). All other reagents used were commercial products of the highest available purity. Tissue culture materials were provided by Corning or Nunc. Eagle (MEM) was supplied by Flow Laboratories. Fetal bovine serum was obtained from

Cultilab (Brazil). PMA was dissolved in Me_2SO and stored at −20 °C as a 1 mg/ml stock solution. Other chemical reagents were obtained from Merck. Yeast mannan (MPOLY) was prepared and donated by Cantos et al. (1993) and methyl α-D-mannopyranoside (MM) was prepared by one of us (Dr Philip A.J. Gorin).

2.2. Animals

Swiss normal mice (8 week old) that received a standard laboratory diet (Purine) were used. All recommendations of the national law (no. 6638, 05/11/1979) for scientific management of animals were respected.

2.3. Potentiometric titration experiments

Following early described procedures (Martell & Motekaitis, 1992; Mercê, Fernandes, Mangrich, & Sierakowski, 2000; Mercê, Fernandes, Mangrich, Sierakowski, & Szpoganicz, 2001a; Mercê, Landaluze, Mangrich, Szpoganicz, & Sierakowski, 2001b; Mercê, Lombardi, Mangrich, Reicher, & Szpoganicz, 1998; Mercê, Spir, Salmón, Giannoni, & Mangrich, 1999; Noleto et al., 2002) a standardized pH meter (Orion, USA) fitted with glass and Ag/AgCl reference electrodes was used to read $-\log [H^+]$ (pH) directly. A standard 0.1 M KOH solution using potassium hydrogen phthalate was the titrant. The solution of vanadyl ion (VO) 0.1 M was prepared from vanadyl sulphate in 0.4 M HNO_3 as previously described (Mercê et al., 1999) and standardised for metal concentration by $KMnO_4$ titration (Furman, 1962) and by Gran's Plot (Martell & Motekaitis, 1992) for hydrogen ion concentration. All solutions were prepared with doubly distilled, deionised and CO_2-free water. All potentiometric titrations were performed under a stream of pure N_2 controlled ionic strength ($\mu = 0.100$ M KNO_3) and constant temperature (25.0 ± 0.1 °C, thermostated bath, Microquímica, MQBTC, 99-20, Brazil).

Aqueous MPOLY solutions (0.34 mmol in respect to the anhydrohexose units), alone and in the presence of the metal ion VO, in varying MPOLY to metal ratios were titrated in triplicate in the reaction vessel using a Sigma Techware (USA) manual piston burette.

The reported binding constants as well as the last figure deviation are the average of the three performed potentiometric titration experiments and were calculated with the aid of the microcomputer BEST7 program (Martell & Motekaitis, 1992). All mathematical algorithms as well as the chemical and mathematical model employed in the calculations are described elsewhere (Martell & Motekaitis, 1992; Mercê et al., 1998).

2.4. Preparation of MPOLY–VO and MM–VO

Solid MPOLY–VO (2:1) and MM–VO (2:1 or 1:1) samples were prepared by dissolving MPOLY (0.33 mmol as anhydrohexose units) and 0.33 mmol of the MM in water (5 ml), following addition of the vanadyl solution (0.165 or 0.33 mmol). The pH was adjusted to 9.0 with 0.1 M KOH solution and absolute ethanol (3v) was then added to the preparation. The precipitate was dried at 60 °C (Etcheverry et al., 1997).

2.5. FTIR analysis

Infrared spectra of MPOLY, MM and MPOLY–VO and MM–VO were processed in a KBr matrix containing 1% of sample and recorded from 500 to 5000 cm^{-1}, using a Hartmann and Braun, Model DA-8-vacuum spectrophotometer (Canada).

2.6. Preparation of macrophages

Mice peritoneal macrophages were colleted by infusing their peritoneal cavity with ice-cold PBS. The cells were plated in a culture medium (MEM, 5% fetal bovine serum and antibiotics) or HBSS to give (5×10^6 cells) in 5 cm diameter polystyrene tissue culture Petri dishes or 4×10^5 cells/well in 96-well dishes. After incubation for 2 h at 37 °C under 5% CO_2 humidified incubator, non-adherent cells were removed by washing with PBS at 37 °C (Sasada, Pabt, & Johnston, 1983).

2.7. MPOLY and MPOLY–VO toxicity on macrophages

Adherent macrophages were incubated for 2 and 48 h with varying concentrations of MPOLY (0.1–2 mg/ml), MM (0.5–2 mg/ml) or MPOLY–VO and MM–VO (0.5–100 μg/ml). Evaluation of toxicity was performed using a MTT reagent according to the description of Reilly, Belleveue, Worster, and Svesson (1998).

2.8. Determination of superoxide anion

Adherent macrophages were incubated in a standard reaction mixture consisting of HBSS containing ferricytochrome *c* (80 μM) in the presence or absence of PMA (1 μg/ml). To the standard reaction mixture was added MPOLY (0.25–2 mg/ml), MPOLY–VO (1–10 μg/ml), MM (0.5–2 mg/ml) or MM–VO (1–10 μg/ml). Controls in the absence of the polysaccharide or MM and MM–VO and in the presence of adequate amounts of Me_2SO (solvent of PMA) or vanadyl sulfate were performed. The extinction molar coefficient $\varepsilon = 2.1 \times 10^4$ M^{-1} cm^{-1} was used for reduced cytochrome *c* (Sasada et al., 1983).

2.9. Nitric oxide production

For measurements of nitric oxide production, adherent macrophages (4×10^5/well) were incubated with MPOLY (10–250 μg/ml) in the presence or absence of either LPS (50 ng/ml) plus interferon-γ (26 U/ml) or only in the presence of interferon-γ (26 U/ml). The cells were also

incubated with MM (0.25–1 mg/ml) in the absence or in the presence of interferon-γ (26 U/ml). Experiments with MPOLY–VO and MM–VO (0.5 and 1 μg/ml) were carried out in the presence or absence of interferon-γ (26 U/ml). After 48 h, NO production was assessed by measuring nitrite in the culture medium using the Griess reaction (Cortizo, Caporossi, Lettieri, & Etcheverry, 2000b; Green et al., 1982). For a control of an eventual LPS contamination of MPOLY, macrophages were incubated for 48 h with MPOLY (0.5 and 1 mg/ml), which was previously treated with (50 μg/ml) of polymyxin B for 1 h before use (Ramamoothy, Kemp, & Tizard, 1996). Control experiments using vanadyl sulfate were also performed.

2.10. Protein determination

After removal of the reaction mixture, the cells were washed with PBS 37 °C and the cells processed according to Sasada et al. (1983). The protein content was determined by the Bradford (1976) method, using bovine serum albumin as standard.

2.11. Statistical analysis

Statistical analysis of data was carried out using analysis of variance and the test of Tukey for mean comparison. Mean values ± SD were used.

3. Results and discussion

3.1. Interaction between MPOLY and vanadyl ion

3.1.1. Potentiometric titration

MPOLY from baker's yeast (Fig. 1A) and MM (Fig. 1B) are carbohydrates possessing structures with various configurations of hydroxyl groups as potential sites for metal complexation. The potentiometric titration method, which is recognized as a useful tool to study the interaction between metals and saccharides (Mercê et al., 1998, 2001a; Shahgholi, Callahan, Rapolli, & Rowley, 1997), was used to determine the interaction between vanadyl ion and MPOLY and MM. The potentiometric pH profiles of the ligands (L) MPOLY and MM in the presence or absence of VO as metal (M), are shown in Fig. 2A and B, respectively. The ligand to metal molar ratios was for MPOLY 1:1 or 4:1 and for MM 1:1 or 2:1. The free ligands exhibit intermolecular hydrogen bonds when in aqueous solutions (Etcheverry et al., 1997). These hydrogen bonds are steadily disrupted as the metal ion VO displaces the H in the hydroxyl groups of the mannosyl units in MPOLY and in MM, as indicated by buffer regions in the potentiometric titration profiles (Fig. 2A and B).

The chemical model for the present investigation is similar to that reported for the interaction between the plant *Leucaena leucocephala* galactomannan with Cu^{2+}

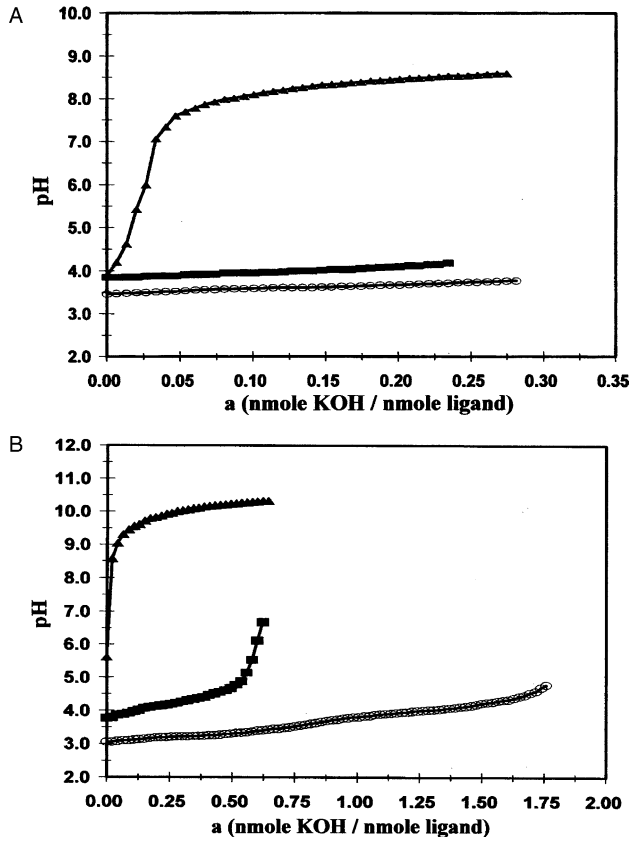

Fig. 2. Potentiometric pH profiles of MPOLY in the presence or absence of VO. (A) (▲) MPOLY (0.34 mmol); (■) MPOLY–VO (4:1); (○) MPOLY–VO (2.5:1). $T = 25.0$ °C, $\mu = 0.100$ M KNO_3. (B) (▲) MM (0.10 mmol); (■) MM–VO (1:1); (○) MM–VO (2:1). $T = 25.0$ °C, $\mu = 0.100$ M KNO_3.

(Mercê et al., 1998) and for the galactomannan from the lichen *R. celastri* with the vanadyl ion (Noleto et al., 2002). The model involved deprotonation on one site of the ligand, the more acidic –OH group being at C-6 in the monomeric mannosyl unit.

The calculated protonation constant was $K_a = 2.60 \pm 0.04 \times 10^{10}$ to MM and $K_a = 1.17 \pm 0.04 \times 10^{9}$ to MPOLY as defined in Eq. (1) or (1′). The hydrolysis constants for the metal ion reported by Baes and Mesmer (1976) were used in the calculations and the dissociation constant of water (pK_w) at $T = 25.0$ °C and $\mu = 0.100$ M was 13.78 (Furman, 1962). The equilibria of the complexed species are represented in Eqs. (2) and (3)

$$^{-}\mathrm{O{-}L} + \mathrm{H^+} \rightleftharpoons \mathrm{HO{-}L} \qquad (1)$$

$$K_a = [\mathrm{HO{-}L}]/[^{-}\mathrm{O} - \mathrm{L}][\mathrm{H}]$$

or

$$\mathrm{HL} \rightleftharpoons \mathrm{H} + \mathrm{L} \qquad K_a = [\mathrm{H}][\mathrm{L}]/[\mathrm{HL}] \qquad (1')$$

$$\mathrm{VO} + \mathrm{H\,L} \rightleftharpoons \mathrm{LVO} + \mathrm{H^+} \qquad (2)$$

$$K_{LM} = [\mathrm{LVO}][\mathrm{H^+}]/[\mathrm{VO}][\mathrm{HL}]$$

$$LVO + H\,L \rightleftharpoons L_2VO + H^+ \quad (3)$$

$$K_{L2VO} = [L_2VO][H^+]/[LVO][HL]$$

Computer calculations provided the binding constants for the complexed species LM and L_2M (Table 1). Only the species present in the equilibria in a percentage higher than 10% were taken into account in the calculations (Martell & Motekaitis, 1992).

Species distribution diagrams of the resulting complexes for MM as a function of pH are shown in Fig. 3. With the metal concentration set at 100%, the species LM and L_2M were predominant at pH 3.5–5.5 and pH > 7, respectively. For MM–VO, the maximum content of the LM species was 65% at pH 4.6 and for the L_2M species it was 99.9% at pH 9.0. For MPOLY–VO (data not shown), the maximum content of the LM species was 60% at pH 4.3 and for the L_2M species it was 99.9% at pH 8.6. It is meaningful that the complexed species LM and L_2M were present in significant percentages at pH close to physiological values.

As the binding constant determined for MM was higher than that of MPOLY, for both LM and L_2M species (Table 1), it can be suggested that the OH groups in MM are in a more favourable configuration to bind with the metal ion. This is probably due to the availability of the all OH groups in MM, which are not involved in glycoside linkages, contrasting with the monosaccharide units of MPOLY (Fig. 1A). MPOLY, being a structurally complex polymer, suffers the interference of steric factors, which could affect the binding of the vanadyl ion with the mannosyl unit of the polysaccharide. Accordingly, such high binding constant values have also been reported for the interaction between VO and a lichen galactomannan (Noleto et al., 2002). Quite similar values for binding constants were observed for Cu^{2+}-binding in a plant galactomannan (Mercê et al., 1998), when it was postulated that the polysaccharide in aqueous solution had a certain mobility and that Cu^{2+} can bind with one or more monomeric sugar units from its various chains.

Fig. 4 shows the FTIR spectra of solid complexes and free MM. The bands in the 'fingerprint' or 'anomeric' region (950–750 cm^{-1} and below of 750 cm^{-1}; Malthlouthi & Koening, 1986), were different for MM–VO, in which the well-resolved bands appearing with free MM are lost. In this region, the band for V=O stretching

Table 1
Binding constants for MM–VO and MPOLY–VO

	K_{MM-VO}	$K_{MPOLY-VO}$
[LM]/[M][L]	$(1.6 \pm 0.1) \times 10^9$	$(5.0 \pm 0.3) \times 10^7$
$[L_2M]/[ML][L]$	$(1.6 \pm 0.1) \times 10^8$	$(3.2 \pm 0.3) \times 10^6$

Binding constants were calculated using a Best-7 program. L = ligand MPOLY or MM; M = (metal,VO); LM and L_2M represent complexed species detected in proportions of 1:1 and 2:1, respectively; $T = 25.0$ °C and $\mu = 0.100$ M (KNO_3). Values are means ± SD of three independent experiments.

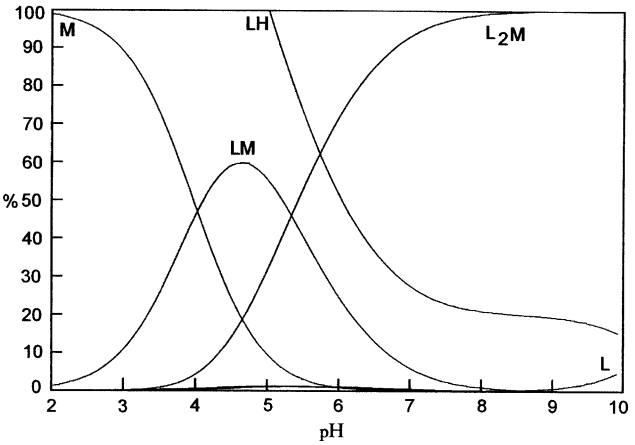

Fig. 3. Species distribution for pH values from 2.0 to 10.0. Complexed species were obtained with the metal concentration set at 100%. M = metal (VO); L = ligand (MM).

vibration (950 cm^{-1}; Etcheverry et al., 1997; Sreedhara et al., 1994, 1996b) was also detected. It probably overlaps other bands in the anomeric region of the MM–VO spectrum. Spectra of MM–VO exhibited profiles with an increase of amplitude and broadening of bands centered around 3400 cm^{-1} (–CH_2OH region) and 1200 cm^{-1} (primary alcohol), when compared with the free ligand spectra. On complexation, a cleavage of the H-bonds could occur, so that the well-resolved bands at 3400 cm^{-1} of the free ligand disappear (Etcheverry et al., 1997). A similar profile was reported for monosaccharide–Ce complexes (Mukhopadhyay, Kolehmainen, & Rao, 2000). The most significant difference between free MM and MM–VO was a sharp and strong band at 1381 cm^{-1}. Similar spectra were obtained for MPOLY–VO (not shown), this strong band being at 1382 cm^{-1}, being present only in spectra of complexes. It can be proposed that this band is probably due to CH_2 and COH deformations (Malthlouthi & Koening, 1986). Considering that the C-6 position is the most reactive (primary OH), when not masked as a participant of

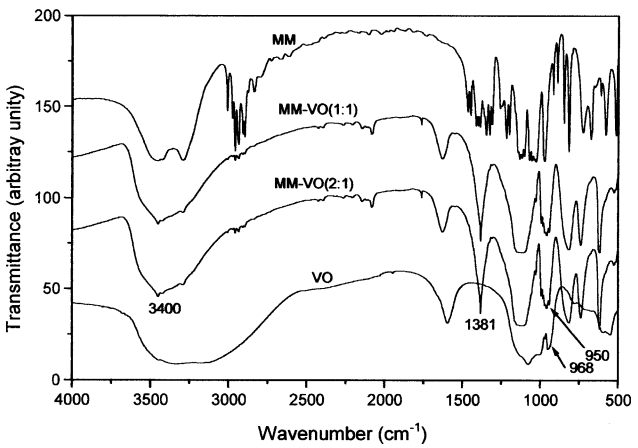

Fig. 4. FTIR spectra of free MM and MM–VO. Infrared spectra of MM and MM–VO were processed in a KBr matrix containing 1% of sample and recorded from 500 to 5000 cm^{-1}.

the glycoside linkages in MPOLY, this could be a preferential position for binding of the metal ion.

3.2. Effects of saccharides and their complexes on macrophages

3.2.1. Cell viability

Fig. 5A and B, shows that MPOLY–VO and MM–VO caused loss of cell viability at low concentrations, the effect being significant after 2 h exposure (Fig. 5A). The effect was also time and dose dependent since 10 μg/ml was sufficient to decreased macrophage viability after 48 h by 90% (Fig. 5B). Parallel experiments performed with free carbohydrates did not affect the cell viability up to 2 mg/ml (data not shown). These results show that the complexation gives rise to highly cytotoxic compounds, since the effect occurred at a 100-fold lower concentration than uncomplexed carbohydrates.

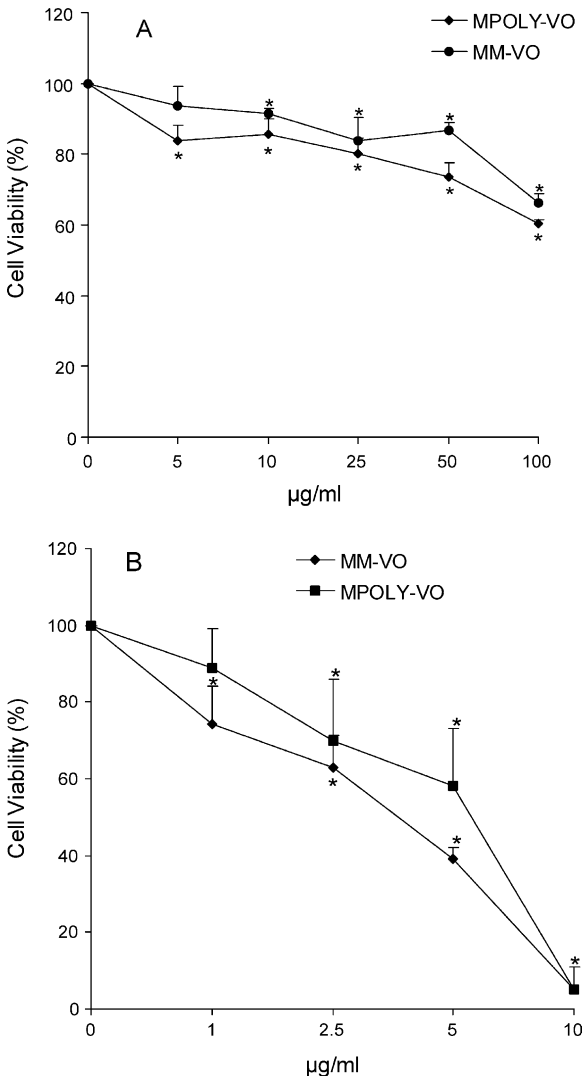

Fig. 5. Effects of MPOLY–VO and MM–VO on cell viability. Adherent macrophages were incubated with different concentrations of MPOLY–VO and MM–VO, as indicated. The medium was then removed and the MTT reagent was added, following incubation for 3 h. Excess of MTT was removed and formazan crystals were dissolved by addition of Me_2SO. The absorbance was measured at 550 nm. Values are means ± SD of three independent experiments each one in triplicate. *Significantly different from control, $p < 0.05$. Control (100%) corresponds to the medium in the absence of complexes. (A) 2 h exposure to complexes; (B) 48 h exposure to complexes.

3.2.2. Superoxide anion production

In order to evaluate whether MPOLY, MM, MPOLY–VO and MM–VO could affect the respiratory burst in macrophages, these cells were incubated in solutions with varying concentrations of the carbohydrates and their vanadyl complex (L_2M) and in the presence or absence of PMA, an agent that triggers an in vitro respiratory burst. In control experiments performed in the absence of PMA, MPOLY, MM as well as MPOLY–VO and MM–VO did not interfere with superoxide anion production. Fig. 6A shows that the cells responded to PMA with an accentuated production of superoxide anion. Addition of MPOLY, up to 2 mg/ml, promoted a time- and dose-dependent decrease of superoxide anion and at 75 min this reached 80% when 2 mg/ml of MPOLY was used. It must be emphasised that this result was not due to any loss of macrophage viability, since MPOLY, at concentrations used in the assays, did not affect this parameter (data not shown). In contrast, the monosaccharide derivative MM did not exhibit any inhibitory effects on superoxide anion production by macrophages (Fig. 6B). Fig. 6C shows that both MPOLY–VO and MM–VO lowered superoxide anion production, ~50% inhibition being observed at 2.5 μg/ml of MM–VO and ~35% inhibition at 10 μg/ml of MPOLY–VO. Thus, the modification produced by the complexed vanadyl ion, obviously led an exacerbation of effects of MPOLY and MM on macrophages. Higher concentrations than 10 μg/ml of MPOLY–VO and MM–VO could not be used, due to their drastic effect on cell viability (Fig. 5B). In terms of MPOLY–VO, the inhibitory effects occurred at a 100-fold lower concentration than with MPOLY, when MM that did not show any effect on superoxide anion production.

Polysaccharides as well as vanadium compounds have been described as modulators interfering in the respiratory burst of macrophages (Adachi, Olmo, & Yodamae, 1993; Berton & Gordon, 1983; Jun, Mei, & Yuan, 1993; Noleto et al., 2002; Williams, Topley, Alobaidi, & Harber, 1986; Yamaguchi et al., 1995). The respiratory burst, as measured by superoxide anion production, was affected by MPOLY in a similar manner to that observed for a lichen galactomannan (GMPOLY) (Noleto et al., 2002), but with a quite different behaviour when compared with other polysaccharides such as unpsonized zymosan (Berton & Gordon, 1983) and unpsonized yeast glucan, which increased superoxide anion production by macrophages (Williams et al., 1986).

The effect of vanadium compounds on cells varies according to the type of the compound and the cell system (Grabowski, Paulauski, & Goldleski, 1999).

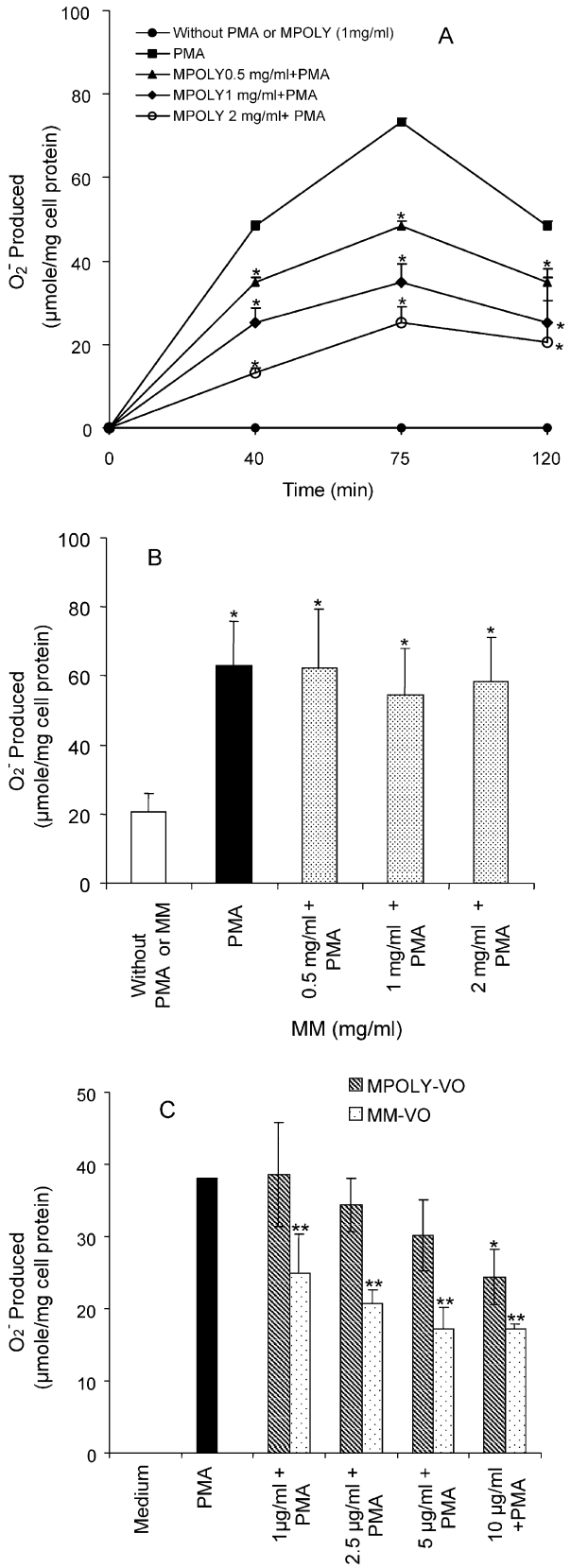

Fig. 6. Effects of MPOLY, MM, MPOLY–VO and MM–VO on superoxide anion production by macrophages. Adherent macrophages were incubated with the standard reaction mixture consisting of HBSS containing ferricytochrome *c* (80 μM), PMA (1 μg/ml). Aliquots were removed at indicated intervals and the absorbance measured at 550 nm. Results are expressed as μmol of superoxide anion/mg of cell protein. (A) MPOLY. Values are means ± SD of five different experiments, each one in triplicate. *Significantly different from experiments performed in the presence of PMA and absence of MPOLY; $p < 0.05$. (B) MM. Values are the means ± SD of four independent experiments each one in triplicate. *Significantly different from the superoxide anion production in the absence of PMA; $p < 0.05$. (C) MPOLY–VO and MM–VO. Values are the means ± SD of three independent experiments each one in triplicate. *Significantly different from the PMA-induced superoxide anion production in the absence of MPOLY–VO or MM–VO; $p < 0.05$. **Significantly different between MPOLY–VO and MM–VO.

Cortizo, Bruzzone, Molinuevo, and Etcheverry (2000a) using a model of osteoblastic-like cells showed different degrees of induction of oxidative stress, suggesting that the cytotoxicity of vanadium was partially dependent on oxidative stress. In our study, both MPOLY–VO and MM–VO gave rise to a significant cytotoxicity against peritoneal macrophages. However, inhibitory effects on superoxide anion were observed at concentrations where macrophage viability was not affected. The superoxide anion production triggered with PMA is a pathway dependent on protein kinase C (Yamaguchi et al., 1995), so that, the present results suggest that MPOLY and MPOLY–VO could interfere with this pathway. However, the possibility that MPOLY, MPOLY–VO and MM–VO are scavengers cannot be excluded. Tsiapali et al. (2001) demonstrated that mannan and other polysaccharides, with or without substituent groups, have an antioxidant effect in a dose-dependent manner, suggesting that this property is inherent to a variety of carbohydrates, besides not being responsible for their immunomodulative action. These authors also demonstrated that monosaccharide units have a lower scavenger activity than structurally related polysaccharides. In fact, our results seem to reinforce such a proposition since the monosaccharide derivative methyl α-D-mannopyranoside did not exhibit inhibitory effects on superoxide anion production by macrophages (Fig. 6B).

3.2.3. NO production

Polysaccharides, as well as vanadium compounds, are described as modulators interfering in macrophage functions such as NO production (Cortizo et al., 2000b; Deuk et al., 1998; Hashimoto et al., 1997; Jun, Yuan, Mei, & Wan, 1999; Kim, 1998; Matte et al., 2000; Ramamoorthy et al., 1996; Tsuji & Sakurai, 1996). The effect of MPOLY, MM, MPOLY–VO and MM–VO on NO production was determined in 48 h experiments in the absence or presence of IFN-γ an inducer of NO synthase; in such a system, LPS is recognised as co-signal (Cunha, Assreuy, Moncada, & Liew, 1993). The results are shown in Fig. 7(A–D). NO production was significantly increased (~55%) in MPOLY treated macrophages (Fig. 7A), 10 μg/ml being sufficient to promote maximum activation over that of the untreated control. This result was reproducible even in the presence

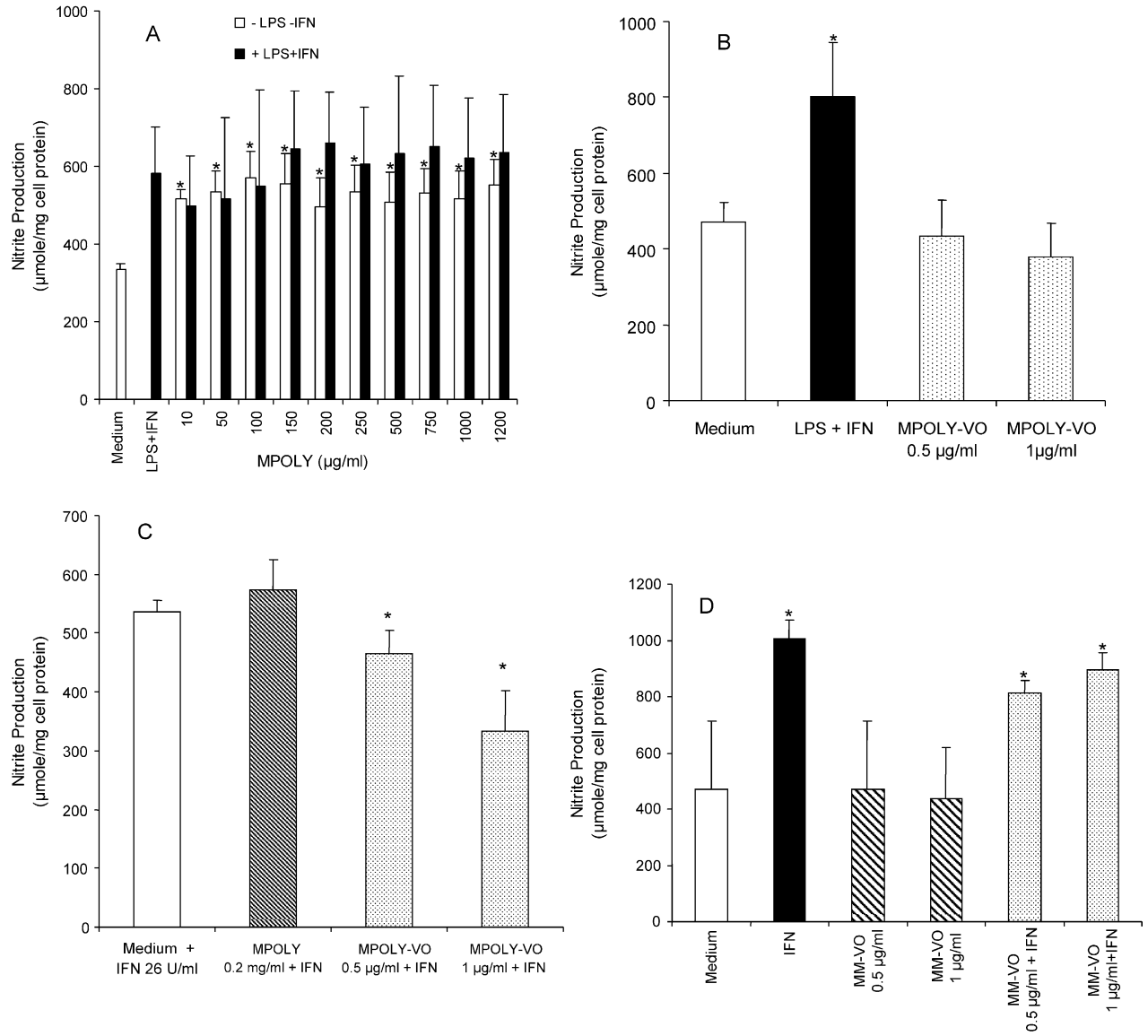

Fig. 7. Effect of MPOLY, MM, MPOLY–VO and MM–VO on nitric oxide production by macrophages. Adherent macrophages were incubated for 48 h in the absence or presence of substances. LPS (50 μg/ml) plus IFN-γ (26 U/ml) were used as a control for NO production. NO accumulation was measured in the supernatant using the Griess reaction and calculated as μmol nitrite/mg cell protein. Values are means ± SD of three or four independent experiments each one in triplicate. (A) MPOLY was incubated in the absence or presence of LPS (50 μg/ml) and IFN-γ (26 U/ml). *Significantly different from control (medium); $p < 0.05$. (B) MPOLY–VO was incubated in the absence of LPS (50 μg/ml) and IFN-γ (26 U/ml). *Significantly different from control (medium); $p < 0.05$. (C) MPOLY or MPOLY–VO were incubated with in a medium containing IFN-γ (26 U/ml). *Significantly different from control (medium + IFN-γ); $p < 0.05$. (D) MM or MM–VO at indicated concentrations in the absence or presence of IFN-γ (26 U/ml). *Significantly different from control (medium); $p < 0.05$.

of polymyxin B, an antibiotic recognised by its LPS neutralising effect (Mukhopadhyay et al., 2000). Fig. 7C shows that MPOLY did not modify the response of macrophages stimulated with IFN-γ. This result indicates that MPOLY cannot act as a co-signal for induction of NO synthase, in contrast with LPS. A similar observation has been described for a galactomannan isolated from *R. celastri* (Noleto et al., 2002). In contrast to MPOLY, MPOLY–VO had no stimulatory effect on this pathway (Fig. 7B), in the absence of LPS and IFN-γ as well as in the absence of IFN-γ, the vanadyl complex had no effect on this pathway. It is of interest that MPOLY–VO (0.5 and 1 μg/ml) showed a slight, dose-dependent inhibitory effect on IFN-γ-induced NO production (Fig. 7C). However, MPOLY did not affect NO production when LPS and IFN-γ were present (Fig. 7A).

For MM, the effect on NO production was different to that observed for MPOLY and MPOLY–VO. MM did not stimulates NO production in the absence of IFN-γ or LPS (data not shown), suggesting that the molecular size could be important in terms of pathway stimulation. A similar result was observed for MM–VO as demonstrated in Fig. 7D. In the presence of IFN-γ, MM–VO did not interfere with the NO production (Fig. 7D).

In conclusion, the chemical modification of MPOLY by the complexing vanadyl ion gives rise to a polymer (MPOLY–VO), which was effective at 100-fold lower concentration than with MPOLY. Considering the interference of the mannan on NO and superoxide anion production, it can be suggested that this polymer exhibited properties as a modulator of macrophage functions. Substances showing such characteristics are of interest because their potential use as biological response modulators.

Acknowledgements

This investigation was supported by the Brazilian Research Council (CNPq), CAPES, FINEP and (PRONEX-CARBOIDRATROS).

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