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Effect of storage xyloglucans on peritoneal macrophages

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Abstract

Xyloglucans from seeds of *Copaifera langsdorffii* (XGC), *Hymenaea courbaril* (XGJ) and *Mucuna sloanei* (XGM) were obtained from milled and defatted cotyledons by aqueous extraction at 25 °C. The resulting fractions contained Glc, Xyl and Gal in molar ratios of 2.5: 1.5: 1.0 (XGC), 3.8: 2.6: 1.0 (XGJ) and 2.5: 1.6: 1.0 (XGM). HPSEC-MALLS/RI analysis showed that each polysaccharide fraction was homogeneous; M_w values were 1.6×10^5, 2.0×10^5 and 1.5×10^5 g/mol, respectively. The effect of the xyloglucans on the production of $O_2^{\cdot-}$ and $NO^{\cdot}$ and on the recruitment of macrophages to the mouse peritoneum was evaluated. All polysaccharides promoted an increase in the number of peritoneal macrophages in a dose-dependent manner. The largest increase, of 576% in comparison to the control group, was elicited by XGJ at 200 mg/kg. The effect of XGC, XGJ and XGM on $O_2^{\cdot-}$ production, in the presence or absence of phorbol 12-myristate 13-acetate (PMA), was not statistically significant. For $NO^{\cdot}$ production, the lowest concentration of XGC (10 μg/ml) gave rise to an increase of 262% when compared to the control group; the effect was dose-dependent, reaching 307% at 50 μg/ml. On the other hand, XGJ at a concentration of 50 μg/ml enhanced $NO^{\cdot}$ production by 92%. XGM did not affect $NO^{\cdot}$ production significantly. The results indicate that xyloglucans from *C. langsdorffii*, *H. courbaril* and *M. sloanei* have immunomodulatory activity.

Keywords: *Copaifera langsdorffii*; *Hymenaea courbaril*; *Mucuna sloanei*; Leguminosae; Macrophages; $O_2^{\cdot-}$; $NO^{\cdot}$; Cell-eliciting activity; Xyloglucans

1. Introduction

Xyloglucans are the major hemicellulosic polysaccharides in the primary cell walls of dicotyledons and non-graminaceus monocotyledons. They are also present as a massive deposit in the cell wall of cotyledons of some leguminous seeds, where they have a storage function (Carpita and McCann, 2000; O'Neill and York, 2003; Petkowicz et al., 2006). Xyloglucans consist of a cellulose-like backbone carrying single α-D-xylopyranosyl units attached to *O*-6 (the disaccharide formed by glucosyl units of the main chain and the glycosyl residue of the lateral chain being designated X). Some xylosyl residues are further substituted at O-2 by β-D-galactopyranosyl units (the trisaccharide formed by glucosyl units of the main chain and the glycosyl residues of the lateral chain being designated L). In structural xyloglucans, some of these galactosyl residues are further substituted at O-2 by α-L-fucosyl units (the tetrasaccharide formed by glucosyl units of the main chain and the glycosyl residues of the lateral chain being designated F). Non-substituted glucosyl units in the backbone are designated as G (Fry, 1989; Fry et al., 1993; Hayashi, 1989).

Although many plants contain structural xyloglucans, storage xyloglucans are relatively rare. At present, only few tree species have been shown to produce storage xyloglucans in their seeds. These storage xyloglucans differ in their molecular mass, distribution and levels of substituted main-chain residues (Hayashi, 1989). The storage xyloglucan from *Tamarindus indica* (tamarind) is the most studied and is currently sold commercially. A comparative study of this xyloglucan and that of *Copaifera langsdorffii* has shown that they are similar. The polysaccharides are composed of the same four basic structural groups, namely XXXG, XLXG, XXLG and XLLG. These are combined

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in different proportions to give a fine structure that varies according to the species and even within the same species (York et al., 1990; Buckeridge et al., 1992).

Polysaccharides isolated from various botanical sources, such as mushrooms, algae, lichens and higher plants, have attracted attention due their therapeutic properties and relatively low toxicity (Schepetkin and Quinn, 2006). For example, β-glucans, galactomannans, mannans and arabinogalactans have been shown to stimulate the production of oxygen radicals, interleukines and tumor necrose factor. On the other hand, the literature contains relatively little reference to the biological activity of xyloglucans. Modified xyloglucans from tamarind seeds, with different degrees of sulphation, exhibited activity against rubeola virus (Mastromarino et al., 1997). Hensel and Meier (1999) showed that the xyloglucan from *Tropaeolum majus* seeds had antimutagenic activity against 1-nitropyrene and suggested that this polysaccharide could be added in food as an antimutagenic agent. Oligosaccharides from partially hydrolyzed xyloglucans from tamarind seeds lead to a reduction of plasma lipids (Yamatoya et al., 1996) and an inhibition of absorption of D-glucose in rats (Sone et al., 1992). Kato et al. (2001) described the anti-tumoral effects of xyloglucans isolated from the cell wall of commercial fruits.

On the other hand, one important biological activity, namely macrophage activation, has received almost no attention in the case of xyloglucans. Macrophages are distributed throughout the body (e.g. heart, brain, lung and liver) and have a critical role, since they act as a link between the innate and acquired immune systems (Beutler, 2004). They are involved in tissue remodeling during embryogenesis, wound repair, removal of damaged or senescent cells subsequent to injury or infection, haemopoiesis and homeostasis (Klimp et al., 2002). They also respond to microbial invasion and recognize and kill tumor cells through the release of products such as oxygen radicals and tumor necrose factor (Klimp et al., 2002; Beutler, 2004).

In their review, Pauly et al. (1999) claimed that the xyloglucan of *T. indica* had immunostimulating effects on monocytes, cells that later differentiate into macrophages. However, they provide little information and do not cite any source for their claims. The present study therefore aimed to evaluate the response of macrophages to xyloglucans. We selected three Brazilian species that are currently known to contain XGs in their seeds, namely *Copaifera langsdorffii* (Kuntze), *Hymenaea courbaril* (M. Gómez) and *Mucuna sloanei* (Fawcett & Rendle). In Brazil, these species are known as Copaiba, Jatobá and Mucuna, respectively. The chemical structures of these polymers have already been well characterized (Buckeridge et al., 1992; Lima et al., 1995; Teixeira, 2005; Teixeira et al., 2003). We evaluated the macrophage response by studying the effect of the xyloglucans on the migration of macrophages to the peritoneum and on O_2^- and $NO^{\cdot}$ production by peritoneal macrophages.

2. Results and discussion

2.1. Isolation and characterization of polysaccharides

Milled and defatted cotyledons of *C. langsdorffii, H. courbaril* and *M. sloanei* were subjected to aqueous extraction at room temperature, furnishing viscous xyloglucans named XGC, XGJ and XGM, respectively. Table 1 shows the yield, total sugar and protein content of each fraction.

The monosaccharide composition of the isolated xyloglucans is shown in Table 2. For XGC the Glc:Xyl:Gal ratio was 2.5:1.5:1.0. Buckeridge et al. (1992) obtained for xyloglucans from *C. langsdorffi* from savanna and forest populations Glc:Xyl:Gal ratios of 2.7:1.9:1.0 and 2.4:1.6:1.0, respectively. XGJ contained Glc:Xyl:Gal in a molar ratio of 3.8:2.4:1.0. Lima et al. (1995) determined for the xyloglucan isolated from the same species Glc:Xyl:Gal ratios of 3.6:2.6:1.0 and Buckeridge et al. (1997) obtained a ratio of 3.3:2.8:1.0. The results obtained herein for XGC and XGJ suggest some degree of monosaccharide degradation during hydrolysis. The monosaccharide composition of XGM was in a Glc:Xyl:Gal ratio of 2.4:1.6:1.0. This result was similar to that obtained with *Mucuna flagellipes*, which has a molar ratio Glc: Xyl: Gal of 2.8:1.5:1.0 and traces of arabinose (Onweluzo et al., 2002). Arabinose is usually found in storage or structural xyloglucans (Lima et al., 1995; York et al., 1996; Vincken et al., 1997; Watt et al., 1999; Busato et al., 2001; Jia et al., 2003; Freitas et al., 2005).

The polysaccharides XGC, XGJ and XGM were analyzed by high performance size exclusion chromatography (HPSEC) with multi-angle laser light scattering (MALLS) and refractive index (RI) detection. The elution profiles of xyloglucans XGC, XGJ and XGM (Fig. 1) indicate their homogeneity. The average molar masses (M_w) of the

Table 1
Yield and total sugar and protein contents of fractions XGC, XGJ, and XGM

Fraction	Yield[a] (%)	Total sugar[b] (%)	Protein[c] (%)
XGC	8.0	97.0	0.0
XGJ	16.6	99.4	0.0
XGM	3.8	79.1	4.3

[a] Based on defatted cotyledons.
[b] Dubois et al. (1956).
[c] Hartree (1972).

Table 2
Monosaccharide composition of XGC, XGJ and XGM

Fraction	Monosaccharides [a] Mol (%)			
	Ara	Xyl	Gal	Glc
XGC	1.2	29.2	19.9	49.6
XGJ	–	32.4	14.0	52.7
XGM	0.7	31.3	19.6	48.1

[a] Determined by GC.

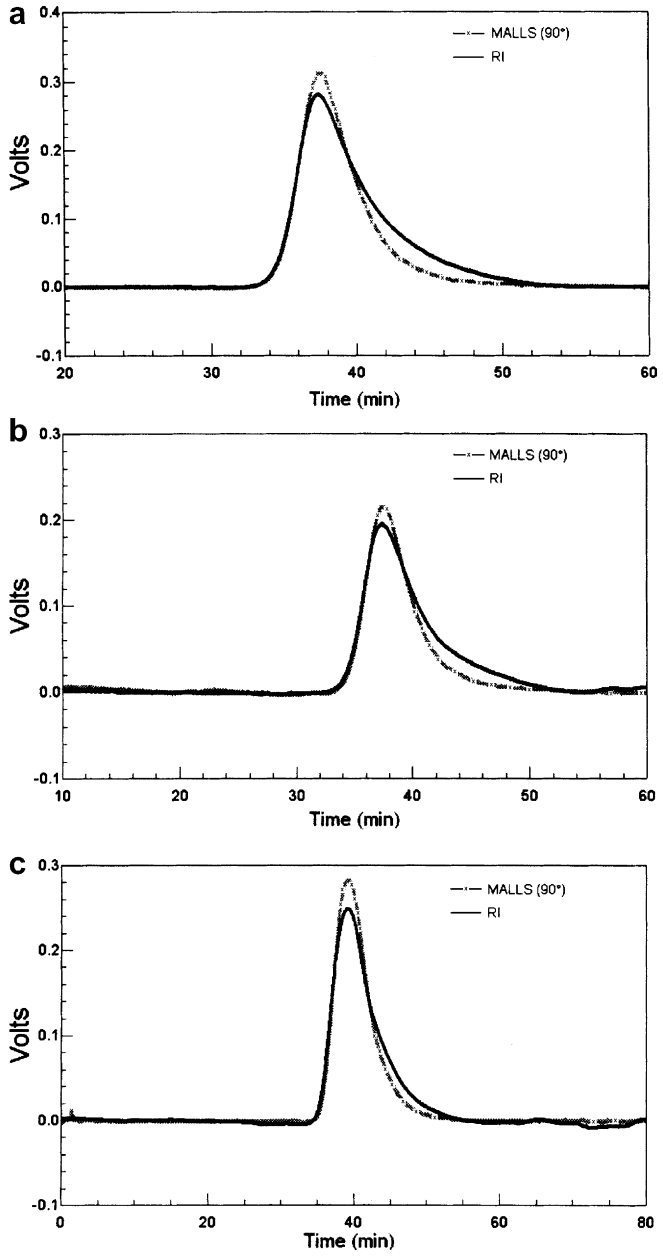

Fig. 1. High pressure size exclusion chromatography (HPSEC) of polysaccharides (a) XGC, (b) XGJ and (c) XGM.

xyloglucans were determined by light scattering (dn/dc of 0.303, 0.278 and 0.383, for XGJ, XGC and XGM, respectively). The M_w of polysaccharide XGJ was 2.0×10^5 g/mol. This result is similar to that determined by Martin et al. (2003). It is lower than that obtained by Freitas et al. (2005) for xyloglucan from the same species (1.5×10^6 g/mol). However, this difference is understandable because the different extraction conditions that (Freitas et al., 2005) used appear to have caused aggregation of their polysaccharide. After they heated the polysaccharide at 85 °C, the M_w was measured as 8.7×10^5 g/mol, which is much closer to our value. The possibility of aggregation with xyloglucans is a well-known phenomenon (Picout et al., 2003). M_w values obtained for polysaccharides XGC and XGM were 1.6×10^5 g/mol and 1.5×10^5 g/mol, respectively.

2.2. Effect of storage xyloglucans on recruitment of mouse peritoneal macrophages

Macrophages isolated from the mouse peritoneal cavity were used as a model to evaluate the capacity of the xyloglucans from *H. courbaril*, *C. langsdorffii* and *M. sloanei* to act as biologic response modifiers (BRM). Solutions of XGC, XGJ and XGM were injected intraperitoneally (doses of 100 and 200 mg/kg) and the total number of peritoneal exudate cells (PEC) was determined 24 h after injection. Fig. 2 shows that these xyloglucans stimulated an increase in peritoneal macrophages, in comparison to the control, in a dose-dependent manner. XGC promoted an increase of cell number by 146% at the dose of 100 mg/kg and by 388% at the dose of 200 mg/kg. XGJ promoted an increase as high as 576%, at 200 mg/kg. For XGM the increase was 207% at 200 mg/kg.

The increase of the total number of peritoneal exudate macrophages observed was greater than those previously reported for other polysaccharides. For the pectic polysaccharide isolated from the fruit of *Feronia limonia* (Linn.), an increase of 56% was observed 24 h after inoculation at 100 mg/kg (Saima et al., 2000). An α-D-glucan from the lichen *Ramalina celastri* elicited 130% more macrophages in the peritoneal cavity than the control group, 7 days after inoculation with 200 mg/kg (Stuelp-Campelo et al., 2002). An arabinogalactan isolated from gum exudate of *Anadenathera columbrina* caused increases of 18%, 44% and 88%, 24 h after inoculation with 50, 100 and 200 mg/kg, respectively (Moretão et al., 2004). The increase in the PEC number suggests that XGC, XGJ and XGM

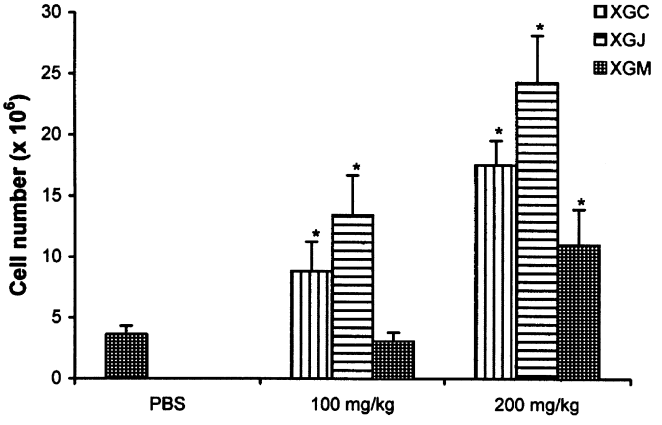

Fig. 2. Number of macrophages found following intraperitoneal administration of XGC, XGJ or XGM. Mice ($n = 4$, for each group) were i.p. injected with 100 or 200 mg/kg of XGC, XGJ or XGM, or with sterile PBS (control group). PEC was collected 24 h after inoculation. Cells in the intraperitoneal fluid from animals of each group were counted using a Neubauer hemocytometer and the results are expressed as mean ± s.d. (*) Significant difference from the control group by the Tukey test ($p < 0.05$).

had an irritating action similar to that of thioglycollate (Cohen et al., 1981).

2.3. Effect of storage xyloglucans on the viability of mouse peritoneal macrophages

Fig. 3 shows macrophage viability determined by the MTT method, using XGC, XGJ and XGM at different concentrations (10–500 μg/ml). There was no significant loss in viability following 2 h of exposure to the polysaccharides (Fig. 3a). However, after 48 h (Fig. 3b), the viability decreased by over 30%, for concentrations of 100 μg/ml or greater of XGC and XGJ. XGM did not show any cytotoxic effect under the concentrations and conditions used.

2.4. Effect of storage xyloglucans on the production of $O_2^{\cdot-}$ by mouse peritoneal macrophages

The macrophage activation process enhances uptake of glucose and oxygen and the production of $O_2^{\cdot-}$ and stimulates specific protein secretion, in a process known as the "respiratory burst" (Halliwell and Gutteridge, 2003; Schepetkin and Quinn, 2006). In order to evaluate if XGC, XGJ and XGM could affect the respiratory burst in macrophages, these cells were incubated in solution with varying concentrations of xyloglucans, in the presence or absence of PMA, an agent that triggers an *in vitro* respiratory burst. In experiments carried out in the absence of PMA, no interference in superoxide anion production by xyloglucans XGC, XGJ and XGM was found at any of the concentrations tested, as shown in Fig. 4a. Other polysaccharides from plants have been found to cause a respiratory burst. For example, an arabinogalactan isolated from *Phyllanthus niruri* induced the production of $O_2^{\cdot-}$ and this effect was three times greater when compared to a control group (Mellinger et al., 2005).

Fig. 4b shows that the presence of xyloglucans did not interfere with PMA induced effects, indicating neither synergic effect, nor scavenging properties. In contrast, an arabinogalactan from *A. columbrina* acted synergistically with PMA (Moretão et al., 2003). For other polysaccharides, such as mannan and galactomannan, $O_2^{\cdot-}$ production by PMA-stimulated macrophages decreased when the polysaccharides were present (Noleto et al., 2002; 2004), indicating a possible scavenger effect of these polymers. Tsiapali et al. (2000) suggested that the antioxidant effect of a polysaccharide does not involve the type of intrachain linkages, molecular weight, or degree of polymer branching, but depends on the monosaccharide composition of the polymer.

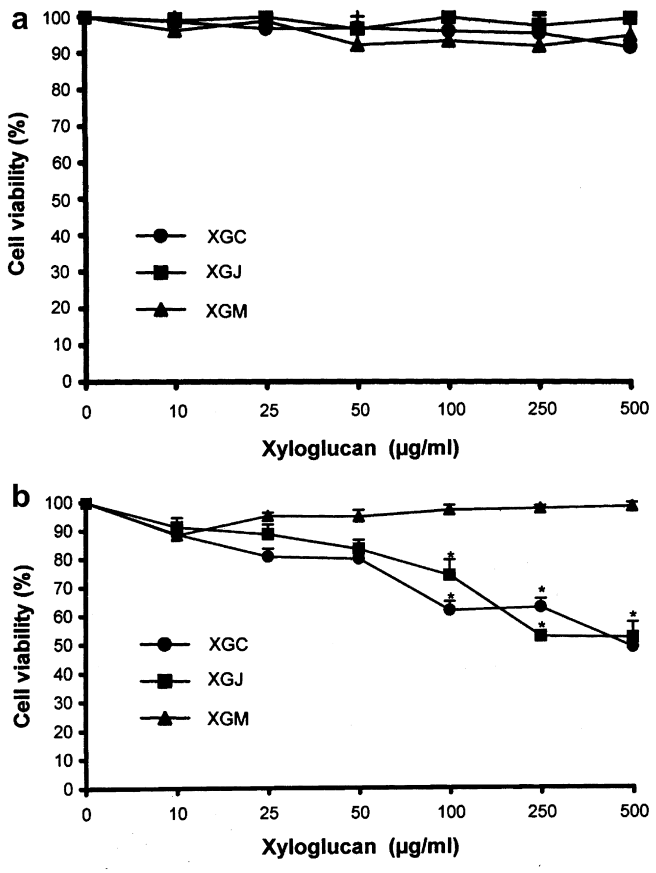

Fig. 3. Cellular viability determined by the MTT method. a: macrophage incubated with XGC, XGJ or XGM for 2 h, these results being expressed as mean ± s.d., three independent experiments in triplicate. b: macrophages incubated with XGC, XGJ or XGM for 48 h, these results are expressed as mean ± s.d., three independent experiments in quadruplicate. (*) Significant difference from the control group by the Tukey test ($p < 0.05$).

2.5. Effect of storage xyloglucans on the production of $NO^{\cdot}$ by mouse peritoneal macrophages

Fig. 5 shows the action of XGC, XGJ and XGM on $NO^{\cdot}$ production by peritoneal macrophages. Concentrations of 10, 25 and 50 μg/ml were used for XGC and XGJ, while for XGM concentrations were determined in accordance with the cellular viability of macrophages at 48 h (Fig. 3). At the lowest concentration, XGC caused an increase of 262% in $NO^{\cdot}$ production when compared to the control group. The effect was dose-dependent, with the $NO^{\cdot}$ production reaching 307% at 50 μg/ml. In the case of XGJ, $NO^{\cdot}$ production was not enhanced at 10 μg/ml. However, as the concentration of XGJ was increased from 25 μg/ml to 50 μg/ml, the enhancement of $NO^{\cdot}$ production increased from 68% to 92%, in relation to the control. Unlike XGC and XGJ, XGM did not show a statistically significant effect. With respect to other plant polysaccharides, those cited above do not stimulate $NO^{\cdot}$ production, although polysaccharides isolated from *Salicornia herbacea* did induce the production of $NO^{\cdot}$ by the monocyte cell line, RAW 264.7, in a dose-dependent manner (Im et al., 2006). Schepetkin et al. (2005) evaluated the induction of iNOS protein expression and $NO^{\cdot}$ production, by increasing amounts of polysaccharide fractions from *Juniperus scopolorum*, using murine peritoneal macrophages. The increase in $NO^{\cdot}$ production was time- and dose-dependent.

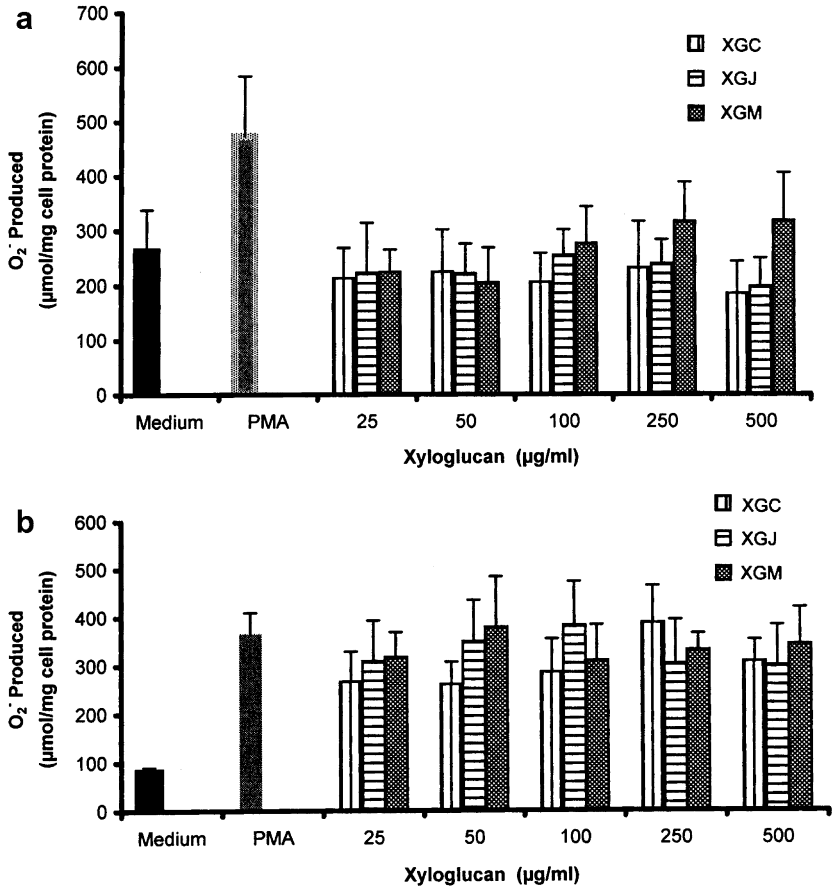

Fig. 4. Superoxide anion production. Control cells were incubated with PMA. Absorbance was measured at 550 nm. These results are expressed as mean ± s.d., two independent experiments in quadruplicate. a: Adherent macrophages were incubated in a mixture consisting of HBSS containing ferricytochrome *c* (80 µM) and one of the polysaccharides XGC, XGJ or XJM. b: Adherent macrophages were incubated in a mixture consisting of HBSS containing ferricytochrome *c* (80 µM), PMA and one of the polysaccharides XGC, XGJ or XJM.

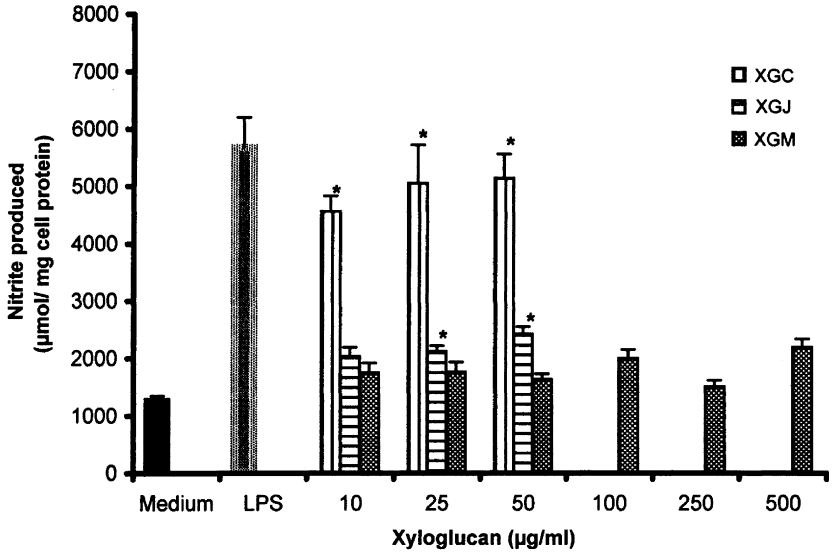

Fig. 5. Adherent macrophages were incubated with supplemented MEM medium and with polysaccharides XGC, XGJ and XJM. Control cells were incubated with LPS. Absorbance was measured at 550 nm. Results are expressed as mean ± s.e. (standard error), with three independent experiments in quadruplicate and one in triplicate. (*) Significant difference from the control (medium) group by the Tukey test ($p < 0.05$).

The stimulation of NO· production by XGC and XGJ was greater than that observed for a galactomannan from the lichen *R. celastri*, which enhanced NO· production by 40% when compared to untreated cells (Noleto et al., 2002).

To exclude the possibility of contamination by LPS, a known immunomodulator and often a contaminant in biological preparations, control assays were examined using polymyxin B, an antibiotic recognized for its LPS-neutralizing effect (Lasfargues et al., 1989). No difference was observed in NO· production in the presence of polymyxin B in fractions XGC and XGM. However, a difference of 32% was observed for XGJ at 25 μg/ml. Meanwhile, as already shown, XGJ gave a lower increase in NO· production, when compared with XGC and the reduction in the presence of polymyxin B was not dose-dependent (data not shown), suggesting that the studied polysaccharides were LPS-free. However, since polymyxin B has been reported to cause inhibition of iNOS gene expression and cytokine production in mouse macrophage cells and could interact with sites on the plasma membrane that are functionally related to the plant polysaccharide targets (Schepetkin and Quinn, 2006), it can be difficult to interpret the results when polymyxin B is added to neutralize LPS. Therefore we used the limulus amoebocyte lysate (LAL) test (The United-States Phamacopeia, USP XXVI, 2003. Method 85 – Bacterial endotoxins) to verify the presence or not of LPS in the XGJ. This test did not detect the presence of LPS in the polysaccharide sample (data not shown).

2.6. General considerations

Differences in the fine structure of xyloglucans can generate distinct macromolecular behavior and conformations. Although the three polysaccharides used in our biological activity experiments have classical structures of storage xyloglucans, they show differences in their fine structure. Xyloglucans from cell wall are classified as XXXG-type or XXGG-type based on the number of backbone glucosyl residues that are branched (Vincken et al., 1997). Most storage xyloglucans are typically classified as XXXG-type. In xyloglucan from *C. langsdorffii* seeds, the pattern of Glc-substitution is composed of repeating XXXG groups, with variable galactosyl substitution (York et al., 1990; Buckeridge et al., 1992; Vincken et al., 1997). Teixeira (2005) used β-glucanase to show that xyloglucan from *M. sloanei* also possesses a repeating pattern based on an XXXG repeating unit. However, when compared to *C. langsdorffii*, differences in distribution of galactosyl residues along the main chain were observed.

Buckeridge et al. (1997) examined the fine structure of *H. courbaril* xyloglucan, using a sequential enzymatic digestion and detected the presence of a new family of oligosaccharides, named XXXXG. The data available to date indicate that this structural feature is unique among storage xyloglucans (Freitas et al., 2005; Tiné et al., 2006). Recently, Tiné et al. (2006) showed that the xyloglucan from *H. courbaril* seeds has a structure composed of XXXXG and XXXXXG backbone units in addition to XXXG. These units exist in the polysaccharide in a ratio of about 2:1:0.2, respectively. The galactosylation profiles deduced by these authors suggest that the polymer from *H. courbaril* seeds has a much less uniform distribution of galactose units within the backbone.

Since the macrophage activation by polysaccharides is thought to be mediated primarily through the recognition of polymers by specific receptors, differences in conformational features could explain the distinct imunnomodulatory responses obtained with storage xyloglucans from different species.

3. Conclusions

The homogeneous xyloglucans from *Copaifera langsdorffii* (XGC), *Hymenaea courbaril* (XGJ) and *Mucuna sloanei* (XGM) stimulated the migration of macrophages into the peritoneum of mice. The increase of the total number of peritoneal exudate cells observed was greater than has been previously reported for other polysaccharides. The activation of macrophages *in vitro* by XGC and XGJ was evidenced by NO· production, while XGM did not have any effect. Storage xyloglucans obtained from different seeds, although having similar chemical structures, elicited immunomodulatory responses of different intensities, which can be attributed to differences in the molar mass and fine structure.

4. Experimental

4.1. General experimental procedures

Total carbohydrate was estimated by the phenol-sulfuric acid method (Dubois et al., 1956) and protein by the Hartree method (1972). Total acid hydrolysis was performed with a sample dissolved in 72% (w/w) H_2SO_4 for 1 h at 0 °C and then diluted to 8% for 6 h at 100 °C (Bierman, 1989). The hydrolysate was neutralized with $BaCO_3$ and the resulting insoluble material removed by filtration. Monosaccharides were reduced with $NaBH_4$ and acetylated with Ac_2O-pyridine (1:1 v/v, 16 h, at 25 °C). The resulting alditol acetates were analyzed by GC, using a 5890 A II HP gas chromatograph at 220 °C (FID and injector temperature, 250 °C) with DB-210 capillary column (0.25 mm i.d × 30 m), film thickness 0.25 μm, the carrier gas being N_2 (2.0 ml/min)

High pressure size exclusion chromatography (HPSEC) was carried out on 1 mg/ml XGC, XGJ and XGM solutions, using a multidetection equipment in which a Waters 2410 differential refractometer (RI) and a Wyatt Technology Dawn F multi-angle laser light scattering (MALLS) detector were adapted on-line. Four Waters Ultrahydrogel

2000/500/250/120 columns were connected in series and coupled to the multidetection equipment. A 0.1 M $NaNO_2$ solution, containing NaN_3 (0.5 g/l), was used as eluent. The refractive index increment was determined by using a refractive index detector. The value of d*n*/d*c* (differential refractive index to change in solute concentration) was determined using five concentrations, between 1 and 0.2 mg/ml (filtered by Millipore filter 0.22 μm). HPSEC data were collected and analyzed by a Wyatt Technology ASTRA program.

4.2. Plant material

Seeds from *Hymenaea courbaril* and *Copaifera langsdorffii* were acquired from IPEF (Instituto de Pesquisas e Estudos Florestais, São Paulo) -Brazil. Seeds from *Mucuna sloanei* were kindly supplied by Professor Dr Renato Azevedo Moreira from the Universidade Federal do Ceará (UFC), Brazil, where a voucher specimen is deposited in the Herbarium Prisco Bezerra EAC (UFC), under the number 000024482.

4.3. Isolation of polysaccharides

Seeds of *C. lagsdorffii, H. courbaril* and *M. sloanei* were submitted to enzymatic inactivation with boiling water for 15, 40 and 30 min, respectively. The seeds were kept at 4 °C until swelling took place. Thereafter, seeds were dehulled and the cotyledons milled and defatted with toluene:EtOH (2:1, v/v) in a Soxhlet apparatus. Milled and defatted cotyledons were submitted to extraction with MeOH:H_2O under reflux for 30 min. The materials were dried and submitted to aqueous extraction at 25 °C for 1 h. After centrifugation, the extracts were treated with 2 vol EtOH and the resulting precipitate washed twice with EtOH and dried in vacuo. The fractions were named XGC from *C. langsdorffi*, XGJ from *H. courbaril* and XGM from *M. sloanei*.

4.4. Experimental animals

Swiss mice (6–8 weeks old) received a standard laboratory diet (Purine®) and water ad libidum. All recommendations of the Brazilian National Law (no. 6.638,05 Nov. 1979) for scientific management of animals were respected.

4.5. Macrophage isolation

Peritoneal macrophages of mice were collected by infusing their peritoneal cavity with 8 ml of sterile phosphate-buffered saline (PBS). The cells were plated in culture medium (minimum essential medium-MEM, 5% fetal bovine serum and antibiotics) or Hank's balanced saline solution (HBSS) to give 5×10^5 cells/well in 96 well dishes. After incubation for 1 h at 37 °C under 5% CO_2 in a humidified incubator, non-adherent cells were removed by washing twice with PBS at 37 °C.

4.6. Cytotoxicity assays

Adherent macrophages were incubated for 2 and 48 h in the absence (control) or presence of XGC, XGJ and XGM at various concentrations (10 to 500 μg/ml). Toxicity was evaluated using 3- [4.5-dimethylthiazol-2-yl]- 2.5 diphenyltetrazolium bromide (MTT), according to Reilly et al. (1998).

4.7. Superoxide anion production

Adherent macrophages were incubated in a reaction mixture consisting of HBSS containing ferricytochrome *c* (80 μM) in the presence or absence of 0PMA (1 μg/ml). XGC, XGJ or XGM were added to the reaction mixture (at 10-500 μg/ml) and controls were prepared without the polysaccharides. Absorbance at 550 nm was measured after 2 h and the amount of superoxide anion released was calculated by dividing the difference in absorbance of the samples, with or without superoxide dismutase, by the extinction molar coefficient $C_{\text{oxid.red.}}\Delta\varepsilon = 2.1 \times 10^4\ \text{M}^{-1}\ \text{cm}^{-1}$ for reduced cytochrome *c* (Sasada et al., 1983; Johnston et al., 1978). Results are expressed as μmol/mg cell protein.

4.8. Nitrite quantification

Adherent macrophages (5×10^5 cells/well) were incubated with XGC, XGJ (10-50 μg/ml) and XGM (10–500 μg/ml) or LPS (50 ng/ml) as control. After 48 h, the isolated supernatants were mixed with an equal volume of Griess reagent and incubated at room temperature for 10 min (Green et al., 1982). Absorbance was measured at 550 nm and nitrite concentration was calculated from a $NaNO_2$ standard curve. Results are expressed as μmol/mg cell protein. For control of eventual LPS contamination of XGC, XGJ and XGM, samples were treated with polymyxin B (50 ng/ml) for 1 h before examination (Lasfargues et al., 1989).

4.9. Evaluation of xyloglucans effect in the peritoneal exudate cells count

Mice of the treated group received a single intraperitoneal dose of XGC, XGJ and XGM (100 and 200 mg/kg). In the control group, animals were inoculated with sterile PBS, each group being composed of 4 animals. After 24 h, the mice were killed by ether anesthesia and the peritoneal exudate cells were harvested by infusing their peritoneal cavity with 5 ml of PBS. After centrifugation at 1080*g* for 6 min, the cell pellet was resuspended in MEM medium and counted using a Neubauer hemocytometer.

4.10. Protein determination

After each determination ($O_2^{\cdot-}$ or $NO^{\cdot}$), the cells were washed twice with PBS at 37 °C and processed according to Sasada et al. (1983). The protein content was determined

according to the method of Bradford (1976), using bovine serum albumin as the standard.

4.11. Statistical analyses

Statistical analysis of data was carried out using an analysis of variance (ANOVA) test and the test of Tukey was used to determine the statistical significance ($*p < 0.05\%$).

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