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In vitro and in vivo antiviral properties of sulfated galactomannans against yellow fever virus (BeH111 strain) and dengue 1 virus (Hawaii strain)

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Abstract

Two galactomannans, one extracted from seeds of *Mimosa scabrella*, having a mannose to galactose ratio of 1.1, and another with a 1.4 ratio from seeds of *Leucaena leucocephala*, were sulfated. The products from *M. scabrella* (BRS) and *L. leucocephala* (LLS) had a degree of sulfation of 0.62 and 0.50, and an average molecular weight of 620×10^3 and 574×10^3 g mol^{-1}, respectively. Their activities against yellow fever virus (YFV; BeH111 strain) and dengue 1 virus (DEN-1; Hawaii strain) were evaluated. This was carried out in young mice following intraperitoneal infection with YFV. At a dose of 49 mg kg^{-1}, BRS and LLS gave protection against death in 87.7 and 96.5% of the mice, respectively. When challenged with 37.5 LD50 of YFV, mice previously inoculated with BRS + virus or LLS + virus, showed 93.3 and 100% resistance, respectively, with neutralization titers similar to mice injected with 25 LD50 of formaldehyde-inactivated YFV. In vitro experiments with YFV and DEN-1 in C6/36 cell culture assays in 24-well microplates showed that concentrations that produced a 100-fold decrease in virus titer of YFV were 586 and 385 mg l^{-1} for BRS and LLS, respectively. For DEN-1 they were 347 and 37 mg l^{-1}, respectively. Sulfated galactomannans, thus demonstrate in vitro and in vivo activity against flaviviruses.

Keywords: Sulfated galactomannan; Yellow fever; Dengue; Flaviviruses

1. Introduction

Among storage polysaccharides of higher plants, galactomannans constitute the second most abundant group. They are found especially in legume seeds, and have different degrees of side-chain substitution, molecular masses, and fine structures (Shcherbukhin, 1993; Whistler and Smart, 1953; Dea and Morrison, 1975; Soni and Bose, 1985; Daas et al., 2000). Their structure consists of a main chain of (1 → 4)-linked β-D-mannopyranosyl units substituted by α-D-galactopyranosyl units (Dey, 1978), and are distinguished by the content of D-mannosyl units which ranges from 60 to 80% (Whistler and Smart, 1953; Dea and Morrison, 1975).

The in vitro antiviral activity of sulfated polysaccharides has been explored and seems to be related to the presence of the negatively charged sulfate groups, which inhibit adsorption of the virus to the host cells (Herold et al., 1995). Studies have also been carried out to find rational correlations between the antiviral property and chemical structure/degree of sulfation. These studies were done with sulfated xylogalactans (Damonte et al., 1996), β-(1 → 3)-linked D-galactan (Amornrut et al., 1999), carrageenans (Carlucci et al., 1999), sulfated dextrans (Neyts et al., 1995; Witvrouw and De Clercq, 1997) and α-(1 → 3)-linked D-mannans (Kolender et al., 1997).

Dengue and yellow fever are important flavivirus infections in tropical countries (Figueiredo, 2000). Dengue epidemics have recently occurred in several Brazilian States, the most important being Rio de Janeiro (1986/1987) in which an estimated 1 million were infected by the dengue

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1 serotype (Brasil, 2002a). In the 1990s, Northern and Central-West States reported few cases of yellow fever (2–66 per year), due to widespread immunization with the attenuated 17D strain, but with a 50–100% mortality rate depending on the year (Brasil, 2002b). There is a high risk for an explosive outbreak in an unimmunized population, especially where coverage rates for yellow fever vaccine are not high enough. WHO estimated 200,000 yellow fever cases occurring each year, with an estimated 30,000 deaths in 34 at-risk countries, almost all of them in sub-Saharan Africa (WHO, 1998).

Yellow fever and dengue are mosquito-borne diseases, due to positive-strand RNA flaviviruses. Most dengue infections are relatively mild, characterized by a sudden onset of fever and non-specific symptoms, including frontal headache, retro-orbital pain, body aches, nausea and vomiting, joint pains, weakness and rash (Gubler, 1998). With the increased co-circulation of different serotypes, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) have a rising incidence with 1–5% mortality rates (Holmes and Burch, 2000). In humans, yellow fever is principally characterized by hepatic pathology. Differences observed between the infection processes of yellow fever and dengue viruses in a human hepatoma cell line could explain variations in liver pathologies (Marianneau et al., 1998). The clinical picture of yellow fever varies from a non-specific febrile illness to a fulminating fatal disease (Monath, 1996).

There are to date no specific antiviral therapies for dengue and yellow fever and many aspects of the mechanisms involved with the antiviral activity of sulfated polysaccharides remain to be explored and perhaps exploited.

2. Materials and methods

2.1. Isolation of the galactomannans from Mimosa scabrella (BR) and Leucaena leucocephala (LL)

M. scabrella and *L. leucocephala* seeds were each treated with water at 100 °C for 30 min, crushed and submitted to exhaustive aqueous extractions at 25 °C with stirring. The extracts were clarified by centrifugation (13,000 rpm, 20 min) and then through cellulose acetate membranes with a 3 μm pore diameter. The concentrated extracts (under reduced pressure at 50 °C) were then treated with 0.1 M NaCl and the polysaccharides precipitated with 2 volumes of ethanol. The monosaccharide composition of the polysaccharides was determined by complete hydrolysis with 2 M TFA for 6 h at 100 °C, the resulting mannose and galactose being converted into their alditol acetates (Wolfrom and Thompson, 1963). The mixtures were examined by gas–liquid chromatography using a model 5890 HP chromatograph, incorporating a DB-225 capillary column with N_2 as carrier gas. Oven: 220 °C, injector: 250 °C, detector: FID, 250 °C.

2.2. Sulfation of BR and LL and purification of the derivatives

According to O'Neill (1955), the galactomannans (1.0 g) were allowed to swell in pyridine:formamide (160 ml:30 ml) with stirring at 25 °C (12 h) until finely dispersed suspensions were obtained. These were cooled to 4 °C and chlorosulfonic acid (13 ml) was slowly added to the mixtures with stirring over 24 h at 4 °C. The resulting solutions were neutralized with saturated aq. $NaHCO_3$ and dialyzed against water for 120 h, centrifuged (8800 rpm, 25 min) and filtered through acetate membranes with a 0.22 μm pore diameter. The solutions were treated with sodium chloride to a final concentration of 0.1 M and submitted to precipitation with 2 volumes of ethanol. In the case of the *L. leucocephala* polysaccharide, two consecutive sulfations were carried out.

Aqueous solutions of the sulfated derivatives were treated with 3% aq. cetyltrimethylamonium bromide (Scott, 1965) and the resulting precipitates were isolated following centrifugation (3000 rpm, 20 min). After solubilization of the complexes in 4 M NaCl, HOAc was added to pH 7.0. The sulfated polysaccharides were precipitated with excess ethanol, re-dissolved in water and dialyzed against water. NaCl was then added to 0.1 M and the derivatives were again precipitated with ethanol.

2.3. Carbohydrate, protein and sulfate analyses

Carbohydrate contents were determined according to Dubois et al. (1956). Total protein was measured according to Hartree (1972). The degree of sulfation was determined by a turbidimetric method using the barium chloride-gelatin reagent (Dodgson and Price, 1962).

2.4. Molecular weight determination

Determination of the absolute molecular weight and size distribution was performed using an on-line coupled high performance size exclusion chromatograph-multi-angle laser light scattering photometer (model Dawn DSP-F-Wyatt Technology, λ: 633 nm) and a differential refractive index detector (model Waters 410), according to Wyatt (1993). The derivatives were solubilized in 0.1 M $NaNO_2$ and filtered through a 0.22 μm membrane filter unit. The injection line consisted from 2000, 500, 250 and 120 Ultragel columns in series, with 0.1 M $NaNO_2$ as eluent. An Astra V-45 software package was used to calculate the weight-averaged molecular weight (M_w) of the derivatives.

2.5. Viruses

Yellow fever virus (BeH111 strain) was provided by the Instituto Evandro Chagas, Belém, State of Pará, Brazil, and dengue virus type 1 (Hawaii strain) was donated by the Dengue Branch, Division of Vector-Born Viral Diseases, Center for Disease Control, San Juan, Puerto Rico. For the

animal studies, the wild strain BeH111 of yellow fever virus (YFV) was used at its 24th passage, obtained by intracerebral inoculation of 2–3 days old suckling mice and storage at −70 °C. Stock viruses were prepared as 10% brain suspensions in 0.85% aq. NaCl containing 100 units penicillin ml^{-1} and 100 μg streptomycin ml^{-1} and clarified by centrifugation (1876 × *g* for 20 min at 4 °C). For in vitro studies, YFV—BeH111 strain and dengue 1 virus (DEN-1)—Hawaii strain were used at their 2nd passage on the *Aedes albopictus* C6/36 cell line. Viral suspensions were obtained by freezing (−70 °C) and thawing the contents of bottles with C6/36 cell cultures infected with the virus 8 days after inoculation, followed by clarification by centrifugation (469 × *g*, 20 min at 4 °C).

2.6. In vivo studies

Our protocol was approved by the Research Ethics Committee, Adolfo Lutz Institute—Brazil and carried out according to Resolution 196/96 concerning research involving human beings (Brasil, 1996), National Health Council of Brazil. To check the toxicity of the polysaccharides on female mice (Swiss) weighing 17–21 g ($n = 6$), each polymer was inoculated intraperitoneally once at 1–7 days and the effects observed for 14 days. The control group received injections of 0.85% NaCl. The native (BR and LL) and sulfated (BRS and LLS) polysaccharides were applied in 0.85% aq. NaCl with a volume of 1 ml per 20.5 g of animal weight, containing five different concentrations between 0.6 and 5.0 g l^{-1} (29–244 mg kg^{-1} of animal weight). Each mouse was weighed before the first and 24 h after the last administration. Significant weight loss or failure to gain weight was indicative of toxicity, as was death. Results were evaluated using the *t*-test (Bolton, 1990).

To determine antiviral activity, female mice weighing 17–21 g (three groups of 19 animals) were inoculated intraperitoneally with 0.85% NaCl containing 1 g l^{-1} sulfated polysaccharide (49 mg kg^{-1} of animal weight) simultaneously with a 25 LD50 of YFV suspension also in 0.85% NaCl. The negative control groups were inoculated with a 1 g l^{-1} sulfated polysaccharide solution in 0.85% NaCl and the positive control group with a 25 LD50 viral suspension. Another group of mice were inoculated with a 0.36 % (w/v) formaldehyde-inactivated 25 LD50 viral suspension. The above 1 g l^{-1}, polysaccharide solutions (10 g l^{-1}) were sterilized by autoclavation (121 °C, 20 min), and, at lower concentrations, by 0.22 μm membrane filtration. The animals were observed daily for 14 days for sickness symptoms or death. At 25 days after the first inoculation of each group, mice were challenged by an intraperitoneal injection of the 37.5 LD50 viral suspension (3 groups of 5 animals). The different groups were: (1) negative control group first inoculated with BRS, (2) negative control group first inoculated with LLS, (3) negative control group first inoculated with 0.85% NaCl, (4) test group first inoculated with viral suspension + BRS, (5) test group first inoculated with viral suspension + LLS, and (6) control group first inoculated with a formaldehyde-inactivated viral suspension. After the challenge, the mice were observed for 50 days. A neutralization test (Shope and Sather, 1979) with suckling mice was performed with a pre-challenge serum pool of the groups ($n = 5$), to determine the antibody neutralization titers against YFV. Undiluted serum samples were incubated with increasing dilutions (10^{-1} to 10^{-11}) of YFV suspension for 1 h at 37 °C. Groups of six suckling mice (2–3 days old) were then intracerebrally inoculated with the mixtures. Mortality was monitored daily for 14 days.

In a second experiment, female mice (17–21 g, $n = 6$) were intraperitoneally inoculated initially with a 25 LD50 YFV suspension following treatment with 1 g l^{-1} BRS or LLS (49 mg kg^{-1}), given in two administrations in a single dose at the 3rd and the 4th day after virus inoculation. 0.85% NaCl was administered to the positive control group after virus inoculation. The negative control group was not inoculated with the virus suspension, but was similarly treated with 0.85% NaCl. The animals were observed for 14 days.

2.7. In vitro studies

Determination of the in vitro toxicity of the polysaccharides was carried out according to Denizot and Lang (1986), by quantitating the viable cells using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide. The C6/36 clone of *A. albopictus* cells (Singh, 1967), developed by Igarashi (1978), was cultured in 96-well microplates and maintained using no. 15 Leibovitz medium supplemented with 4% fetal bovine serum, 2% non-essential amino acids, 3% tryptose phosphate broth, pH 6.8, and polysaccharides (BR, BRS, LL, LLS) at six different concentrations of 0.25–8.0 g l^{-1} (38–1200 μg per well). The plates were incubated at 28 °C under a 5% CO_2 atmosphere for 8 days. The cells were then incubated with MTT and the resulting formazan was solubilized in dimethyl sulfoxide. The absorbance was read in a microplate reader (Titertek Multiskan) at 492 nm and the non-specific absorbance at 620 nm was subtracted. The 50% cytotoxic concentration (CC_{50}) was defined as the polysaccharide concentration that reduced by 50% the number of viable cells when compared with a control group without addition of polysaccharides.

The in vitro antiviral activity of BRS and LLS against YFV and DEN-1 was determined inoculating 100 times the tissue culture infection dose 50% ($CCID_{50}$) on C6/36 cells cultured on 24-well microplates, simultaneously with polysaccharides at six different concentrations from 5.1 to 500 mg l^{-1} (0.8–75 μg per well). After 8 days' incubation at 28 °C (5% CO_2), the medium was removed. On the 8th day after inoculation, a cell suspension of each well was added to a single spot on 12-spot glass microscope slides. After drying at room temperature, the slides were immersed in cold acetone for 10 min and the cells were then examined for the presence of YFV antigens, by means of an indirect fluorescent antibody test (Gubler et al., 1984) using polyclonal,

diluted anti-flavivirus serum, and a fluorescein-conjugated goat anti-mouse immunoglobulin. The 50% effective concentration (EC_{50}) was calculated as the drug concentration that reduced the number of immunofluorescent wells by 50% in the compound treated cultures, when compared with the untreated ones. The antiviral activities against YFV and DEN-1 were also determined by simultaneous infection of the viral suspension (100 $CCID_{50}$) and polysaccharide solution on C6/36 cells, cultured in T25 polystyrene bottles (Corning) for 8 days at 28 °C. The supernatant of each test bottle was re-titrated by a 24-well microplate infection; the detection of positive wells being carried out by indirect immunofluorescence, as with the positive-control wells infected without polysaccharide. The polysaccharide concentration that promoted a 100-fold decrease in the viral titer was determined.

3. Results

3.1. General analysis of sulfated galactomannans

The yields of crude galactomannans, obtained following aqueous extraction of seeds, centrifugation and filtration through 3 μm pore-diameter membranes were 13.0% (total anhydrous sugar: 77.5% and protein: 3.7%) for *L. leucocephala* seeds and 22.0% (total anhydrous sugar: 75.9% and protein: 4.3%) for *M. scabrella* seeds. The galactomannans had Man:Gal ratios of 1.1 and 1.4 for *M. scabrella* (BR) and *L. leucocephala* (LL) seeds, respectively. After sulfation, each respective derivative (BRS and LLS, Table 1) had similar carbohydrate content. The degree of sulfation of BRS was slightly higher (0.62) than that of LLS (0.50).

The distribution of light scattering (a function of both polymer concentration and weight-averaged molecular weight—M_w) and refractive index (or concentration signal) responses for BRS and LLS showed the predominance of single peaks for both derivatives (data not shown). These fractions had M_w of $\sim 620 \times 10^3$ g mol^{-1} for BRS and $\sim 574 \times 10^3$ g mol^{-1} for LLS. As suggested by the refractive index response, the small peaks seen after 40 ml of elution reflects only a little heterogeneity. These fractions did not scatter light, which indicates the absence of low molecular weight species.

Table 1
Total carbohydrate, protein and sulfate contents of sulfated galactomannans of *M. scabrella* (BRS) and *L. leucocephala* (LLS)

Derivative	Carbohydrate[a] (%)	Protein[b] (%)	Sulfate content[c] (%)	Degree of sulfation
BRS	58.4	1.3	15.3	0.62
LLS	61.1	4.1	14.3	0.50

[a] Dubois et al. (1956).
[b] Hartree (1972).
[c] Dodgson and Price (1962).

3.2. Determination of in vivo toxicity

Female young mice (Swiss) weighing 17–21 g ($n = 6$) were inoculated intraperitoneally with native (BR, LL) and sulfated (BRS, LLS) galactomannans at concentrations of 0.6–5.0 g l^{-1} (total of seven single administrations on different days). The animals were weighed daily and the percentage of gained weight compared with the initial weight was determined. After 14 days' observation, death did not occur with any of the animals at all concentrations of BR, BRS, LL or LLS. However, LLS gave rise to a lower weight gain and even gave rise to weight losses that were statistically significant in comparison to the saline control group, mainly during the first administrations. Even so, all the LLS animal groups had a weight recovery by the last administration. An analysis of the percentage of weight loss in relation to that gained by the saline control group on the 1st, 3rd, and 7th days of administration indicated a lower weight loss with increasing time (data not shown). On the first day, a calculated concentration of 8.9 mg kg^{-1} of animal weight would reduce the gained weight to 50% of the usual gain. On the second day, it was 43.6 mg kg^{-1} and on the 7th it was 168.1 mg kg^{-1}.

3.3. Determination of in vivo antiviral activity

The antiviral activity experiments were carried out with female young adult mice. YFV (BeH111 strain) was injected intraperitoneally at a dilution capable of killing all animals, as shown by the saline control group (Fig. 1). The sulfated derivatives BRS and LLS protected against paralysis symptoms and death by yellow fever, when they were inoculated simultaneously with the wild virus strain at a 1 g l^{-1} concentration (dose: 49 mg kg^{-1} of animal weight). LLS gave 96.5% protection, while that of BRS was 87.7%. Neither LLS nor BRS alone caused any animal death, as was also the case in the control group inoculated with 25 LD50 formaldehyde-inactivated YFV. Interestingly, in the test group inoculated with YFV + LLS, one animal developed a left rear paw paralysis. It was kept under observation for 3.5 months, after which it was sacrificed, although not

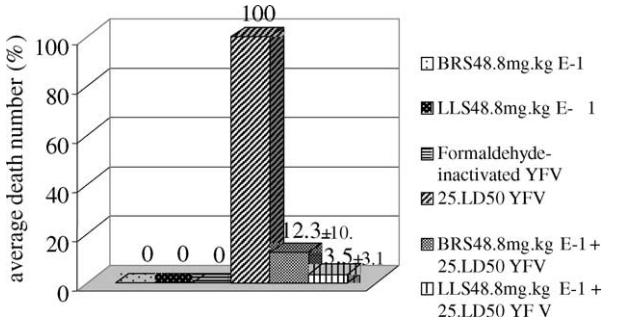

Fig. 1. In vivo antiviral activity response (mouse weight: 17–21 g, $n = 3$ groups of 19 animals) for intraperitoneal inoculation of BRS and LLS at 49 mg kg^{-1} dose.

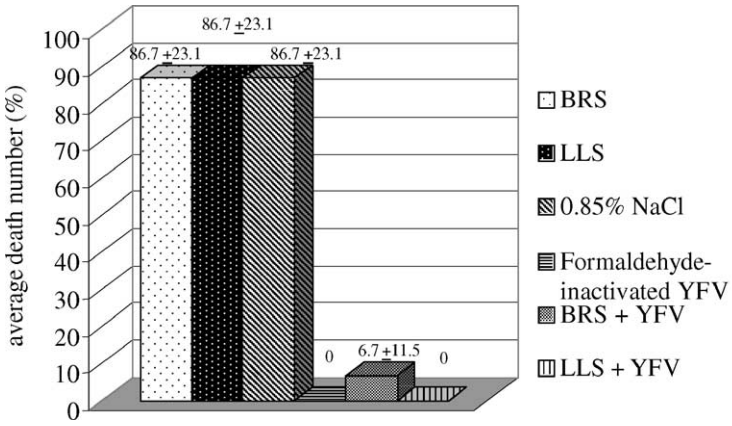

Fig. 2. YFV challenge experiment ($n = 3$ groups of five animals) upon intraperitoneal inoculation of mice of 37.5 LD50 YFV (BeH111).

suffering other sequels. The control group always gave rise to disease progression with eventual death.

When the sulfated galactomannans were administered as single doses on the 3rd and 4th days after inoculation of the viral suspension, no protection occurred at a dose of 49 mg kg^{-1}. Up to the 3rd day, the animals of the control group had not developed apparent disease symptoms. The positive control group (viral suspension alone), the BRS test group (treated with BRS), and the LLS test group (treated with LLS) suffered a 100% mortality, while all members of the negative control group treated with 0.85% NaCl, survived. Treatment using the sulfated polysaccharides did not retard the onset and normal progression of the disease.

Although BRS and LLS only showed antiviral activity when inoculated simultaneously with the viral suspension at the tested 1 g l^{-1} concentration, the animals of these test groups developed neutralizing antibodies against YFV. The ratio of the derivative test group serum titer to that of the derivative control group serum titer gave neutralizing indices of 3.3 for BRS and 3.7 for LLS. When the test groups of surviving animals, previously inoculated with BRS + YFV or LLS + YFV, were then challenged with 37.5 LD50 YFV alone, a 100% survival of the LLS group occurred (Fig. 2). There was a 93.3% survival with the BRS group. In the control groups, previously inoculated with BRS or LLS alone or with 0.85% saline alone, the virus challenge resulted in 86.7% mortality (not 100%, probably because of the animals' age), indicating that the immune response against YFV was directed to the virus and not due to an isolated polysaccharide response.

3.4. Determination of in vitro toxicity by MTT method

The MTT method is based in the conversion of the yellowish 3-[4,5-dimethylthiazol-3-yl]-2,5-diphenyltetrazolium bromide (thiazolyl blue) to a purple formazan by dehydro-

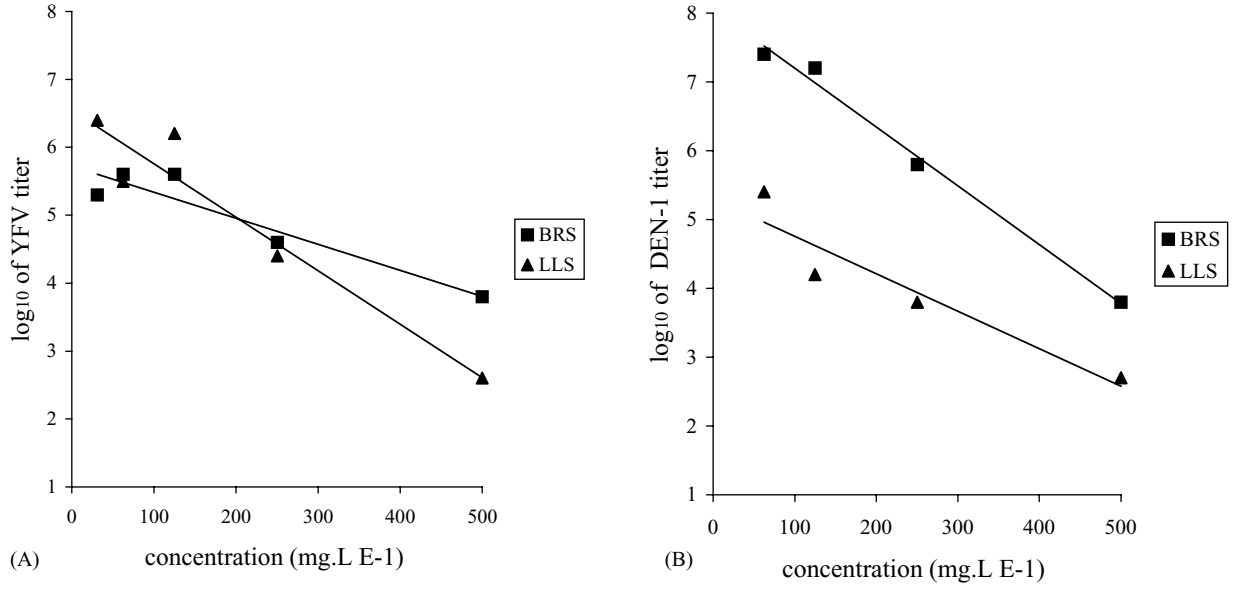

Fig. 3. YFV (A) and DEN-1 (B) titers in terms of concentration of derivatives, determined by titration of the supernatants of T25 bottles simultaneously treated with virus and sulfated galactomannans (BRS or LLS).

genases of living cells. C6/36 cells were incubated with polysaccharides at different concentrations for 8 days, identical to the period utilized in the in vitro antiviral experiments. The CC_{50} for native galactomannans was >8.0 g l^{-1}, and for derivative galactomannans it was 1.5 g l^{-1} for BRS and 2.0 g l^{-1} for LLS.

3.5. Determination of in vitro antiviral activity

The antiviral activity of the sulfated derivatives was first evaluated by simultaneous inoculation of 100 $CCID_{50}$ YFV and the polysaccharides at different concentrations. The concentration, capable of inhibition in 50% of the wells showing positive immunofluorescence (EC_{50}), was calculated by the Reed and Muench (1938) method. BR, LL, and BRS did not show activity against YFV, when tested with the former methodology. However, we observed that even with the positive immunofluorescence method, the proportion of fluorescent cells appeared to be less than that seen in positive control wells, when BRS was present at higher concentrations. The antiviral activity of BRS could not be quantified by this method. LLS had an EC_{50} of 200 mg l^{-1}.

With the intention of obtaining a more sensitive, quantitative response to antiviral activity, we carried out infection of C6/36 cells cultured in T25 polystyrene bottles with 100 $CCID_{50}$ YFV or DEN-1, in the presence of polysaccharides at different concentrations. The viral titer of each bottle was determined after 8 days' incubation, by inoculation on 24-well microplates and detection by immunofluorescence, and comparison with the viral titer of a positive control bottle. The plotted linear regression lines (Fig. 3) indicated the concentration able to reduce the viral titer 100-fold in comparison to that of the positive control bottle. These were 586 mg l^{-1} for BRS and 387 mg l^{-1} for LLS against YFV, and 347 mg l^{-1} for BRS and 37 mg l^{-1} for LLS against DEN-1 (the later being a theoretical value obtained extrapolating the regression line). For both viruses, the best response was obtained using LLS.

4. Discussion

A toxicity study on intraperitoneal injection in mice demonstrated that, below 5 g l^{-1} (244 mg kg^{-1}) of sulfated galactomannans of *M. scabrella* (BRS) and *L. leucocephala* (LLS), the doses were not lethally toxic. The injection of LLS was accompanied by a weight loss. Smee et al. (1996) observed a weight loss in mice treated intraperitoneally with a polysaccharide preparation from tragacanth gum at 200 mg kg^{-1} and suggested that it may have caused animal stress. Other studies with intravenous administrations of dextran sulfate (Flexner et al., 1991) and pentosan sulfate (xylan sulfate) (Lush et al., 1996) showed profound but reversible thrombocytopenia and liver abnormalities. Even observing a weight loss with LLS, we administered a 1 g l^{-1} solution (49 mg kg^{-1}) of the sulfated polysaccharides in these preliminary antiviral experiments. The in vivo toxicity data with LLS showed that the animals had less weight gain when compared to the saline control group at a 1 g l^{-1} concentration, but death did not occur.

The antiviral action of sulfated polysaccharides has been attributed to a mechanism of inhibition of the first step involved in viral replication, which is adsorption of the virus to the host cell, the same type of interaction that would occur between viral glycoproteins and glycosaminoglycans, particularly with heparan sulfate naturally present on the host cell surface (Herold et al., 1995). However, Ibrahim et al. (1999) questioned the role of heparan sulfate molecules at the cell surface as facilitators of viral infections. On treatment of peripheral blood lymphocytes with heparinase, which promoted depletion of heparan sulfate, they observed that there was no alteration in the sensitivity of the cells to infection by HIV strains. González and Carrasco (1987) and González et al. (1987) proposed a mechanism of antiviral activity of carrageenan apparently different from that of viral adsorption inhibition. They used labeled [^{35}S] HSV-1 and showed that virus replication was inhibited upon, entry of the polysaccharide–virus complex into the cells. Their results suggested that the inhibition step occurred after viral internalization, but before the onset of late viral protein synthesis.

The dissemination of YFV in the vertebrate host occurs after inoculation into the skin. The virus then replicates in local tissues and regional lymph nodes and spreads to other tissues. The hepatic parenchyma is the principal target organ and the hepatocellular damage is mediated by viral infection (Monath, 1996). In their role of prevention of disease progression BRS and LLS could interact with YFV particles and inhibit replication, but as mentioned above, there could even be internalization of the virus. If BRS or LLS are absent at the moment of virus entry, as in the experiment involving polysaccharide administration after 3 days of infection, no protective effect was observed. Simultaneous injection of YFV and BRS or LLS gave a high lethality protection (87.7 and 96.5%, respectively). Interestingly, mice inoculated with (YFV + BRS) or (YFV + LLS) developed resistance against the virus. Thus, in the presence of polysaccharides, there was apparently no severe disease development and the viral antigens were introduced to the immune system.

Several sulfated polysaccharides generally inhibit the replication of viruses such as HIV-1, HSV-1 and CMV, at EC_{50} values ranging from 0.1–13.7 mg l^{-1} (Witvrouw and De Clercq, 1997). The higher value of EC_{50} obtained for LLS seems not to be in accord with the explanation as to why that the observed in vivo antiviral activity is only due to the blockade of the early stages of the virus replication, although LLS and BRS did not protect the mice when administered 3 and 4 days after inoculation of the virus. On the other hand, Neyts et al. (1996) found that dextran sulfate (M_w 10,000) prevented YFV (17D strain) cytopathogenicity on Vero cells when it was present at the time of virus

infection. This gave an EC_{50} value of $27 \pm 3\,mg\,l^{-1}$ that may be estimated as comparable to an LLS EC_{50} of $200\,mg\,l^{-1}$, when taking into account that in the former study cells were infected with 10 $CCID_{50}$ instead of 100 $CCID_{50}$ presently employed (the methodology being identical).

Some authors showed that some acidic polysaccharides can exert virucidal actions against HSV-1 and HSV-2 (Carlucci et al., 1999; Eo et al., 2000), but we have not evaluated such an activity using LLS and BRS.

We performed antiviral activity tests with LLS and BRS against YFV and DEN-1 (100 LD_{50}) in newborn mice, which did not prevent animal death at a concentration of $500\,mg\,l^{-1}$, namely $\sim 2\,mg\,kg^{-1}$ of animal weight (data not shown). Newborn mice allow a significant flavivirus replication after intracerebral infection, because of the virus tropism for these tissues. Two- to three-day-old mice have an immature immune system (Morein et al., 2002), so the possibility of an immunomodulatory action of BRS and LLS to prevent death of young adult mice after intraperitoneal infection cannot be discarded. Sidwell et al. (1994) showed that some fungal-origin polysaccharides were immunomodulators that significantly inhibited hepatotropic Punta Toro virus (PTV) infections in mice. Smee et al. (1996), in their study of antiviral activities of tragacanth gum polysaccharides on PTV infections in mice, also observed that they did not exhibit antiviral activities per se in cell culture, but were efficacious in vivo. Intraperitoneal treatments with 12.5–200 $mg\,kg^{-1}$ per day doses, applied 24 h before or after PTV inoculation, protected mice from mortality. These polysaccharides seemed to activate peritoneal macrophages, which protect the infected animals because mice pre-treated with fumed silica and infected with PTV were not protected by subsequent treatment. In our antiviral experiments, it also has to be taken into account that, together with the administration of a relatively high dose of polysaccharide ($49\,mg\,kg^{-1}$) in mice, they were infected with a viral suspension at 25 LD50 to assure 100% mortality in the positive control group. This corresponded to a 1 ml volume of a suspension prepared from a 10-fold dilution of brains (LD_{50}: $10^{-9.5}$) of newborn mice infected with YFV; so, a large quantity of viral suspension was also needed to promote death of young adult mice by intraperitoneal YFV inoculation. Other polysaccharides such as those isolated from *Angelica gigas* (Han et al., 1998), *Centrosema pubescens* (da Silva et al., 2000) and *Orbignya phalerata* (da Silva and Parente, 2001) have been shown to have immunomodulatory activities. The intraperitoneal administration of a pectic polysaccharide from *A. gigas* at a dose of $30\,mg\,kg^{-1}$ in mice, after immunization with sheep red blood cells, caused a 3.2-fold increase in the number of antibody-forming cells from the spleen. At a dose of $50\,mg\,kg^{-1}$, arabinogalactans from *C. pubescens* gave rise to enhancement of the phagocytic activity, when administered intraperitoneally.

Mice provide a good model of flavivirus encephalitis, but not other associated syndromes (Monath, 1996), and they are more sensitive to YFV than dengue virus infection (Karabatson, 1985). We did not find death of DEN-1-infected mice following the same scheme employed to evaluate antiviral activity against YFV.

Cell cultures provide a sensitive assay system for flaviviruses and to evaluate activity against DEN-1. Our sulfated galactomannans did not give rise to a statistically significant toxicity at tested concentrations. Simultaneous virus and BRS or LLS inoculation promoted a significant decrease of virus titer for DEN-1.

The in vitro observation of low values of EC_{50}, for instance that of sulfated dextrans against HIV-1 ($0.4\,mg\,l^{-1}$) and HIV-2 ($0.1\,mg\,l^{-1}$) (Neyts et al., 1995), do not assure an efficient response of in vivo antiviral activity. This was demonstrated by administration of dextran sulfate by intravenous infusion to subjects with symptomatic HIV infection for up to 14 days with a dose 200-fold greater than the EC_{50} value for in vitro HIV infectivity, which proved not to have efficacy in the treatment of symptomatic HIV infection (Flexner et al., 1991). This reinforces the concept that the antiviral activity of some polysaccharides cannot be explained only on the basis of inhibition of binding of viruses to host cell receptors, as appears to occur in vitro.

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