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Development of a water-soluble, sulfated (1 → 3)-β-D-glucan biological response modifier derived from *Saccharomyces cerevisiae*

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ABSTRACT

This report describes a method for the solubilization of micro-particulate (1 → 3)-β-D-glucan. Insoluble glucan is dissolved in methyl sulfoxide and urea (8 M) and partially sulfated at 100°. The resulting water-soluble product is called glucan sulfate. The conversion rate is 98%, and the preparation is endotoxin free as determined by the *Limulus* lysate procedure. Glucan sulfate is composed of 34.06% C, 6.15% H, 50.30% O, 5.69% S and 3.23% N, and has a repeating unit empirical formula of $(C_6H_{10}O_5)_8 \cdot 3\ SO_3NH_4^+ \cdot 4\ H_2O$, suggesting that, on the average, a sulfate group is substituted on every third glucose subunit along the polymer. Molecular weight averages, polydispersity, and intrinsic viscosity were determined by aqueous high-performance size-exclusion chromatography (HPSEC). Two polymer peaks were resolved. Peak 1 ($M_w = 1.25 \times 10^6$ g/mol) represents < 1% of the total polymer mass. Peak 2 ($M_w = 1.45 \times 10^4$ g/mol) comprises > 99% of polymers. ^{13}C NMR spectroscopy confirmed the β-(1 → 3) interchain linkage. In solution, glucan sulfate polymers self-associate in a triple helix. Glucan sulfate stimulates murine bone marrow proliferation following intravenous administration. The ability to prepare a immunologically active, water-soluble (1 → 3)-β-D-glucan preparation will greatly enhance the clinical utility of this class of compounds.

INTRODUCTION

Glucan is a (1 → 3)-β-linked polyglucose immune stimulant that is isolated from the inner cell wall of *Saccharomyces cerevisiae*[1,2]. Glucan exerts a beneficial effect on a variety of experimental disease states of bacterial[3,4], viral[5,6], fungal[7], and parasitic[8] origin. Glucan has also been shown to ameliorate immunosuppression[9]

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and modify the course of experimental neoplastic disease[10]. These observations have stimulated research into the potential biomedical applications of polymeric β-D-glucans[11,12]. Glucans belong to the class of drugs known as biological response modifiers (BRMs). A major obstacle to the clinical utilization of β-glucan BRMs is their relative lack of solubility in aqueous media. Specifically, (1 → 3)-β-D-glucan is an insoluble microparticulate (~ 1–2 μm), upon initial isolation from *S. cerevisiae*[13]. While topical or intralesional administration of insoluble microparticulate (1 → 3)-β-D-glucan induces no toxicity[14,15], systemic (i.e., intravenous) administration of the microparticulate form is associated with hepatosplenomegaly[16], granuloma formation[10], microembolization and enhanced endotoxin sensitivity[17]. Previously, we have demonstrated the feasibility of preparing water-soluble, immunologically active, (1 → 3)-β-glucans from insoluble *S. cerevisiae* glucan[13]. This was accomplished by phosphorylation[13] or sulfation[18] of the glucan polymer. While the phosphorylation of yeast glucan resulted in an adequate conversion to the water-soluble form (~ 70%), sulfation of yeast glucan was associated with a low (~ 37%) yield of water-soluble, immunologically active sulfated glucan[18]. We reasoned that the low yield obtained from the initial sulfation method was due to excessive hydrolysis of glucan polymers by sulfuric acid during the solubilization process. If this were true, then it might be possible to reduce the hydrolytic effect of sulfuric acid by dilution with methyl sulfoxide (Me_2SO). To test this hypothesis an alternative method for producing glucan sulfate was developed.

This report describes: (*i*) an alternative method for the solubilization of yeast-derived (1 → 3)-β-D-glucan that results in a high yield of a water-soluble sulfated preparation, which we have termed glucan sulfate; (*ii*) preliminary data on the physicochemical characterization of glucan sulfate, and (*iii*) preclinical efficacy data examining the ability of glucan sulfate to stimulate hemopoiesis.

EXPERIMENTAL

Preparation of water-insoluble, microparticulate glucan.—Water-insoluble, microparticulate (1 → 3)-β-D-glucan was isolated from *S. cerevisiae* as previously described[13,18].

Preparation of glucan sulfate.—Soluble glucan sulfate was prepared as outlined in Fig. 1. Water-insoluble, microparticulate glucan (4 g) was dissolved in 100 mL of Me_2SO containing 72 g of urea (8 M). In a separate flask, Me_2SO (100 mL) and concd H_2SO_4 (10 mL) were thoroughly mixed, and then added dropwise to the glucan–Me_2SO–urea solution with stirring. The solution was heated to 100° in a water bath with stirring, and the reaction was carried out for 4 h. Following heating, the solution was cooled to ambient temperature and diluted in 4 L of ultrapure, pyrogen-free, deionized water obtained from a water purification system (Millipore, Bedford, MA). The glucan sulfate solution was passed through a Millipore prefilter (1.2 μ) to remove unreacted microparticulate glucan. The glucan sulfate solution was dialyzed with a Pellicon tangential flow dialyzer using a

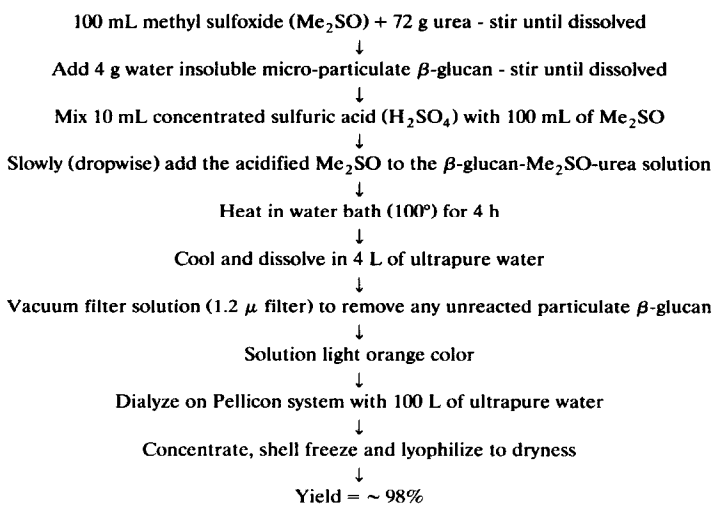

Fig. 1. Flow chart describing the preparation of glucan sulfate from insoluble β-glucan.

10000 MW cutoff filter (Millipore, Bedford, MA) against 100 L of ultrapure, pyrogen-free water, concentrated to 500 mL, shell frozen and lyophilized to dryness (Virtis, Gardiner, NJ). The yield was 98%, and the lyophilized product was endotoxin free as determined by the *Limulus* lysate procedure. The ultrapure water employed in the dilution and dialyzing of glucan sulfate was determined to be endotoxin free by the *Limulus* lysate procedure (Sigma Chemical Co., St. Louis, MO). All chemicals were analytical reagent grade. Several other combinations of Me_2SO and H_2SO_4 were examined, but provided inferior results with regard to solubilization.

Elemental analysis of glucan sulfate.—Elemental analysis of C, H, O, P, N, and S were conducted by a Galbraith Laboratories, Knoxville, TN.

High-performance size-exclusion chromatography (HPSEC) of glucan sulfate.—To evaluate the polymer distribution, glucan sulfate was analyzed by aqueous HPSEC. The basic HPSEC system consisted of a Waters 600E pump, a U6K manual injector and a column heating chamber (Waters Chromatrography Division, Millipore Corp. Milford, MA). The mobile phase, 0.05 M sodium nitrite, was stored in a sterile reservoir (Kontes, Vineland, NJ) and was thoroughly degassed by sparging and blanketing with He prior to use. The mobile phase was delivered at a flow rate of 0.5 mL/min. Three Ultrahydrogel (Waters Chromatography Division, Milford, MA) aqueous HPSEC columns having exclusion limits of 2×10^6, 5×10^5, and 1.2×10^5 daltons were connected in series along with an Ultrahydrogel guard column. The columns were maintained at 30°. Flow rate, column temperature and pump operating conditions were controlled by Maxima 825 GPC software (Dynamic Solutions, Ventura, CA). The system was calibrated using narrow-band pullulan standards (Showdex P-82 series, J.R. Science, NY) and broad-band

dextran standards (Pharmacia, Piscataway, NJ). For analysis, glucan sulfate was dissolved in the mobile phase at a concentration of 2–3 mg/mL by gentle rocking until completely hydrated (~ 2–3 h). A 200-μL injection volume was used for all analyses.

Determination of molecular weight, root-mean-square (rms) radius, and polydispersity of glucan sulfate by multi-angle laser light scattering (MALLS) photometry.—To determine the absolute molecular weight, glucan sulfate was analyzed by HPSEC with on-line MALLS photometry employing a Dawn F MALLS photometer fitted with a K5 flow cell (Wyatt Technology Corp, Santa Barbara, CA). Absolute molecular weight (MW) distribution, number average (M_n), weight average (M_w), z average (M_z), molecular weights rms radius, and polydispersity (I) were established with ASTRA software (version 2.0). A differential index of refraction (dn/dc) of 0.146 cm^3/g was assumed.

Determination of intrinsic viscosity ([η]) by differential viscometry (dv).—For the determination of [η], the column eluent was analyzed by on-line dv employing a Viscotek Model 200 differential refractometer/viscometer (Viscotek, Porter, TX). Molecular weight determinations of standards using this technique show good agreement with MALLS data. Intrinsic viscosity of pullulan standards was in agreement with previous data[19].

^{13}C Nuclear magnetic resonance (^{13}C NMR) spectroscopy.—To investigate the type of interchain linkages and to elucidate the polymer backbone, microparticulate glucan and glucan sulfate were dissolved in Me_2SO-d_6 and analyzed by ^{13}C NMR spectroscopy[13,18,20]. Analyses were performed on a Bruker 200-MHz instrument (Bruker Instruments, Inc., Billerica, MA) operating in the pulsed Fourier-transform mode. Broadband, proton-decoupled spectra were obtained for solutions in Me_2SO-d_6, with the central resonance of the solvent peak (δ 39.7) serving as the internal references. Parameters were as follows: frequency, 50 mHz; relaxation delay, 1 s; pulse window, 15–20°, with approximately 15 000–75 000 scans being taken.

In addition, glucan sulfate was dissolved in D_2O or varying concentrations of NaOD and analyzed by ^{13}C NMR spectroscopy. All samples were prepared at 50 mg/mL. Laminarin, derived from *Laminaria* species (K&K Laboratories, Plainville, NY), in Me_2SO-d_6 was employed as a (1 → 3)-β-linked triple-helical glucopyranose standard[13,18,20].

Helix–coil transition analysis.—The conformational structure of glucan sulfate in aqueous solution was established by helix–coil transition analysis according to a modification of the Ogawa and Hatano procedure[21]. Briefly, Congo Red (Sigma Chemical Co., St. Louis, MO) was dissolved in 0.001 M NaOH to a final concentration of 88 μM. Glucan sulfate was dissolved in 0.001 M NaOH. Laminarin was employed as a (1 → 3)-β-linked triple-helical control. Dextran (40 000 daltons) was employed as a random-coil control. Glucan sulfate or polysaccharide standards (250 μL) were added to 10 mM microcuvettes containing 750 μL of either Congo Red–NaOH or water–NaOH. Absorbance (λ_{max}) was determined using an LKB

Ultrospec II spectrophotometer (LKB Instruments, Gaithersburg, MD). Polysaccharides existing in an ordered conformation form a complex with Congo Red in dilute aqueous NaOH solution. To assess the order–disorder transition, the λ_{max} for solutions of Congo Red–β-D-glucan sulfate or Congo Red–polysaccharide standards were determined at NaOH concentrations ranging from 0.001 to 1.0 M.

Animals.—Male C57Bl/6J, mice (~ 18 g) were obtained from Jackson Laboratory, Bar Harbor, ME. Animals were housed in plastic cages and fed Purina Laboratory Chow and water *ad libitum*. Housing and veterinary care were provided by the Department of Vivarial Sciences, Tulane University School of Medicine. The experimental protocols described in this study conform to the National Institutes of Health guidelines for the care and use of laboratory animals.

Bone marrow proliferation.—In vitro bone marrow proliferation was evaluated as described by Williams et al.[18]. Briefly, femoral bone marrow cells obtained from C57Bl/6J mice, were washed (2 ×), resuspended in RPMI-1640 media containing 1% heat-inactivated fetal calf serum, 9% heat-inactivated newborn calf serum, amphotericin B (375 μg/mL), penicillin–streptomycin (50 U/mL), gentamycin (30 μg/mL), plated in Falcon 96-well microtiter plates (2×10^5 cells/well) and incubated for 48 h at 37° in 5% CO_2. Cells were pulse labeled with [^{3}H]thymidine (1 μCi/well) for the final 8 h of incubation. Following incubation, plates were washed (2 ×) with saline, 200 μL of distilled water was added to each well and the plates were freeze–thawed (2 ×). A 100-μL aliquot of the resultant suspension was transferred to scintillation vials, and the samples were counted in an LKB liquid scintillation counter. Thymidine incorporation was uses as an index of bone marrow proliferation.

Experimental protocol.—Bone marrow cells for in vitro proliferation were harvested from C57Bl/6J mice at 6 and 12 h on days 1, 3, 6, 9 and 12 following intravenous injection with 250 mg/kg of glucan sulfate at time zero. Isovolumetric dextrose served as control.

Statistics.—Statistical comparisons between groups were performed employing one-way analysis of variance followed by a Bonferroni multiple comparison test. A value of $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Herein we describe a method for the solubilization of β-D-glucan from *S. cerevisiae*. The resulting water-soluble preparation (glucan sulfate) is a biological response modifier. Solubilization is achieved by partially sulfating the β-glucan polymer in the presence of Me_2SO and a chaotropic agent (urea). Elemental analysis of lyophilized glucan sulfate revealed a chemical composition (mol%) of 34.06% C, 6.15% H, 50.30% O, 5.69% S, and 3.23% N. Phosphorus was < 0.05%. Based on the elemental analysis, the repeating unit empirical formula for glucan sulfate is $(C_6H_{10}O_5)_8 \cdot 3\ SO_3NH_4^+ \cdot 4\ H_2O$, suggesting that, on average, a sulfate group is substituted on every third glucose subunit along the polymer. It is

interesting to note that there is a significant amount of nitrogen present in glucan sulfate. The water-insoluble microparticulate glucan from which glucan sulfate was derived had no detectable nitrogen (unpublished observation). Therefore, microparticulate glucan was not the source of the nitrogen. We speculate that the nitrogen in glucan sulfate was derived from the urea employed as the chaotropic agent in the solubilization process. The incorporation of nitrogen into glucan sulfate may be unique to this process for solubilization. Previously, our laboratory has demonstrated that yeast β-glucan can be solubilized in the presence of Me_2SO and urea using phosphoric[13] or sulfuric[18] acid without the incorporation of nitrogen into the final product. Based on these observations we speculate that glucan sulfate may exist as an ammonium sulfate salt.

The decision to employ a sulfation procedure in the solubilization of microparticulate yeast glucan was carefully considered. The primary objective of sulfating the polymer was to increase its polarity, thereby increasing aqueous solubility, while maintaining immunobiological potency. This objective was achieved. Previously, we have shown that sulfated yeast β-glucan will exert significant antimicrobial[18] and antineoplastic[18] activity. Of potentially greater importance, the inhibitory effect of sulfated homopolysaccharides against human immunodeficiency virus (HIV) has been well documented[31,32]. We have confirmed the antiviral activity of sulfated β-glucan[18]. Whether sulfated β-glucans produced in our laboratory will inhibit HIV adsorption to susceptible cells is currently under investigation.

The molecular-weight averages, polydispersity, rms radius and intrinsic viscosity of glucan sulfate are presented in Table I. Two polymer peaks were resolved. Peak 1, which represents $< 1\%$ of the total polymer mass, has a weight-average M_w of 1.25×10^6 g/mol, rms radius of 28.7 nm and polydispersity (I) of 2.44. Peak 2, which comprises $> 99\%$ of the polymers, has a weight-average M_w 1.45×10^4

TABLE I

Molecular weight averages, rms values, polydispersity and intrinsic viscosity of glucan sulfate [a]

Parameter	Peak 1	Peak 2
M_n	5.10×10^5	1.35×10^4
M_w	1.25×10^6	1.45×10^4
M_z	4.72×10^6	1.55×10^4
M_w rms radius (nm)	28.7	25.4
I (polydispersity)	2.44	1.08
$[\eta]$ (intrinsic viscosity)	[b]	0.013 dL/g
% of total polymers	$< 1\%$	$> 99\%$

[a] The weight-average MW (M_w), expressed in g/mol, represents the average MW of the polymers in each peak. The number-average MW (M_n) is indicative of the proportion of low MW polymers. z-average MW (M_z) reflects the proportion of high molecular weight polymers. The polydispersity number (I) reflects polymer homogeneity. [b] Intrinsic viscosity could not be determined because of the low polymer concentration in peak 1.

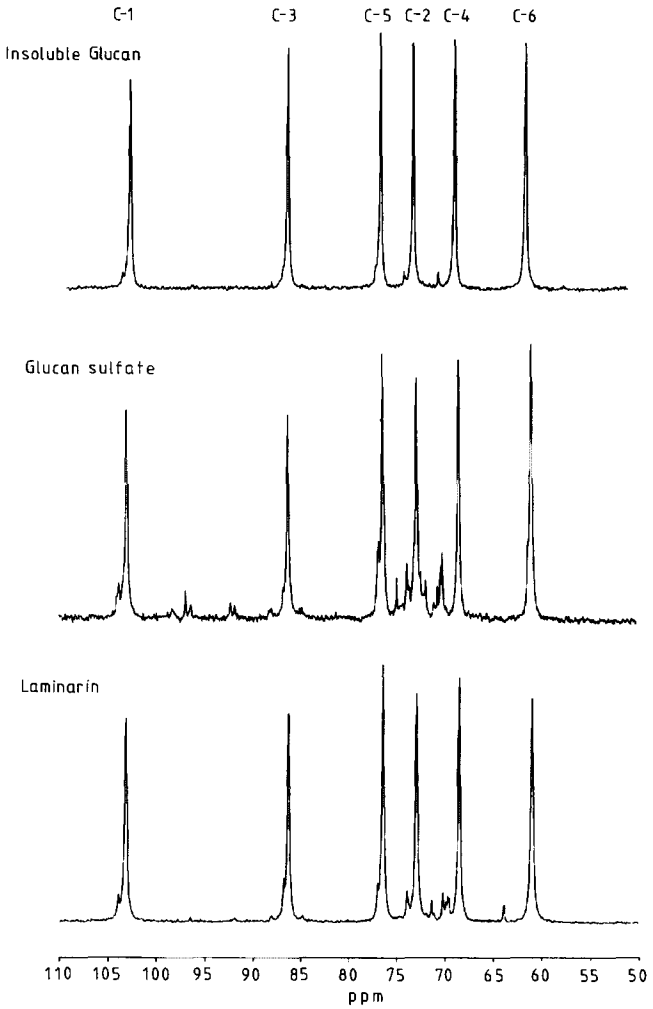

Fig. 2. ^{13}C NMR spectra of insoluble, microparticulate glucan (15433 scans), glucan sulfate (74862 scans) and laminarin (15685 scans) in Me_2SO-d_6. Laminarin served as the (1 → 3)-β-linked triple-helical polyglucose control. All samples were dissolved in Me_2SO-d_6 at 50 mg/mL. Spectra were obtained at 50 MHz.

g/mol, rms radius of 25.4 nm and polydispersity (I) of 1.08. The average intrinsic viscosity was 0.013 dL/g.

To confirm the type of interchain linkages associated with glucan sulfate, samples were analyzed by ^{13}C NMR spectroscopy in Me_2SO-d_6. This allows elucidation of the polymer backbone[13,18,22] and can also be employed to evaluate the type of side-chain branching, if any, along the backbone[22]. The ^{13}C NMR spectrum of water-insoluble, microparticulate β-D-glucan isolated from *S. cerevisiae* and water-soluble glucan sulfate prepared from the insoluble material are

TABLE II

^{13}C NMR chemical shifts of water insoluble, microparticulate glucan, glucan sulfate and laminarin in Me_2SO-d_6 [a]

C-atom	Insoluble glucan	Glucan sulfate	Laminarin
C-1	103.01	102.97	103.12
C-2	72.83	72.77	72.93
C-3	86.22	86.19	86.26
C-4	68.41	68.37	68.49
C-5	76.33	76.29	76.41
C-6	60.87	60.84	60.96

[a] Chemical shifts in ppm.

presented in Fig. 2. Laminarin in Me_2SO-d_6 served as the β-(1 → 3)-linked triple-helical control[13,18,20]. Comparison of the insoluble, microparticulate glucan and glucan sulfate peaks shows excellent correspondence with laminarin. In addition, the ^{13}C NMR spectrum of laminarin reported by Saito et al.[20] agrees well with the present laminarin spectrum. ^{13}C NMR chemical shifts in ppm for insoluble glucan, glucan sulfate and laminarin are presented in Table II. Comparison of the chemical shifts of insoluble glucan and glucan sulfate with laminarin confirms the β-(1 → 3) assignment. These data also indicate that the solubilization procedure does not substantially alter the basic molecule. The small peaks at 71.32, 73.87, 76.92, 86.72, and 103.87 ppm in the laminarin spectrum can be attributed to the presence of C-6 glucosyl side-chains which occur, on average, every 11th subunit along the polymer[23]. The three other small peaks in the laminarin spectrum at 63.86, 69.65, and 70.18 ppm are unassigned. The small peaks observed in the water-soluble microparticulate glucan spectrum correspond to the C-6 glucosyl side-chain peaks observed in the laminarin spectrum (Fig. 2). Comparison of the small peaks in the microparticulate glucan with those observed in laminarin suggests that the side-chain branching frequency of microparticulate glucan is approximately every 22nd glucose subunit. Subsequent ^{13}C NMR analysis of glucan sulfate dissolved in D_2O and varying concentrations of NaOD did not provide additional insights into the structure (data not shown).

Previous reports indicate that the immunologic and antitumor activity of certain (1 → 3)-β-D-glucan BRMs is related to the higher structure of the polymer[24,25]. Maeda et al.[25] have reported that the denaturation of Lentinan, a triple-helical β-linked glucan BRM, decreases antitumor activity. Renaturation of the polymer restored antitumor activity[25]. These data suggest that the higher structure, specifically the solution conformation, may be critically important with regard to induction of immunobiological activity. The solution conformation of glucan sulfate was determined by the technique of Ogawa and Hatano[21]. Glucan sulfate exhibits a triple-helical conformation as denoted by a shift in the absorption maxima between 0.1 and 0.3 M NaOH (Fig. 3). Laminarin, which served as the triple-helical control,

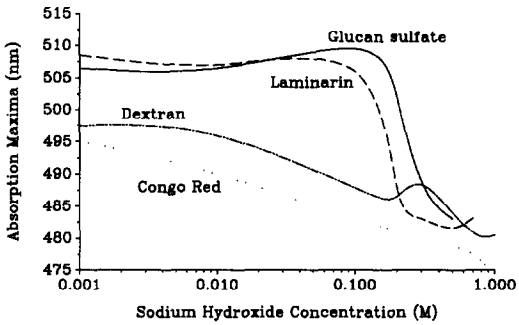

Fig. 3. Helix–coil transition of glucan sulfate in the presence of Congo Red and varying concentrations of NaOH. Glucan sulfate exhibits a shift to a lower λ_{max} between 0.1 and 0.3 M NaOH, indicating disruption of the ordered (triple helical) conformation. Laminarin served as the (1 → 3)-β-linked triple-helical control. Dextran (40000 MW) served as the random-coil control. Congo Red in NaOH served as the negative control.

exhibited a shift in absorption maxima between 0.1 and 0.2 M NaOH. Examination of a 40000-dalton dextran, which served as the random coil control, revealed no shift in absorption maxima. Congo Red in NaOH served as the negative control. The possibility exists that shifts in absorption maxima observed for glucan sulfate may be attributable to chain ionization effects. However, laminarin a (1 → 3)-β-linked water soluble, triple-helical polyglucose that has no group substitution showed a shift in absorption maxima similar to that observed with glucan sulfate. In addition, we have studied a branched, water-soluble (1 → 3)-β-linked polyglucose derived from *Sclerotium glucanicum* that has no charged groups[26]. The shift in absorption maximum observed with *S. glucanicum*-derived glucan is similar to that observed with glucan sulfate. These observations tend to argue against chain ionization effects in glucan sulfate polymers being solely responsible for the observed shifts in absorption maxima.

Numerous studies have shown that (1 → 3)-β-D-glucans will stimulate medullary and extramedullary hemopoiesis in rodents[27–29]. As demonstrated by Patchen and colleagues[27–29] the biological effectiveness of some β-D-glucans BRMs is intimately linked to their ability to stimulate proliferation of hemopoietic progenitors with a subsequent increase in the number of cells that become committed to the leukocyte lineage. The ability of β-D-glucan BRMs to increase the number of immunologically competent effector cells that can respond to infectious and/or neoplastic foci represents an important mechanism-of-action which is critical to their immunobiological activity. Based on these observations, studies were conducted to determine whether glucan sulfate would stimulate hemopoiesis in a well-established murine model[13,18,26,30]. The administration of glucan sulfate (250 mg/kg) on day zero to male C57Bl/6J mice resulted in a biphasic stimulatory effect on murine femoral bone marrow cells (Fig. 4). Murine bone marrow proliferation was significantly increased at 12 h (56%), 1 (46%) and 9 days (51%)

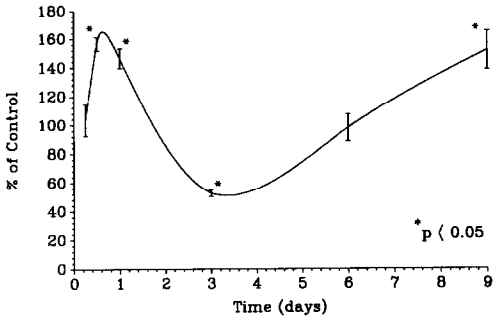

Fig. 4. Temporal effect of a single intravenous injection of glucan sulfate on murine bone marrow proliferation. Glucan sulfate (250 mg/kg) was injected intravenously into C57Bl/6J mice on day zero. Isovolumetric dextrose served as control. Femoral bone marrow cells were harvested at 6 and 12 h and on days 1, 3, 6 and 9. Bone marrow cells (1×10^5) were incubated for 48 h. The cells were labeled with [^{3}H]thymidine (1 μCi/well) for the final 8 h of incubation. [^{3}H]Thymidine incorporation as an index of cellular proliferation was assessed. Data are expressed as percentage of the time-matched dextrose control. Bars represent standard error of the mean. *$P < 0.05$; $N = 24$/group.

following glucan sulfate administration (Fig. 4). Bone marrow proliferation was significantly depressed on day 3 post-injection (47%), followed by a return to normal pretreatment baseline levels by day 6. The biphasic stimulatory effect of glucan sulfate on murine bone marrow proliferation is similar to that observed for other water-soluble β-D-glucan BRMs developed in our laboratory[26].

CONCLUSIONS

The results presented describe a new method for the solubilization of a yeast-derived (1 → 3)-β-linked glucan BRM. The water-soluble product, glucan sulfate, is a triple-helical (1 → 3)-β-D-glucan BRM. Glucan sulfate administration significantly enhanced murine femoral bone marrow proliferation. The ability to prepare an immunologically active, nontoxic, water-soluble (1 → 3)-β-D-glucan will greatly enhance the clinical application of this class of pharmaceuticals.

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