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The cytokine stimulating activity of (1→3)-β-D-glucans is dependent on the triple helix conformation

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Abstract

The immunomodulating properties of comb-like branched (1→3)-β-D-glucans scleroglucan, schizophyllan and lentinan depend on branching pattern, molecular weight and higher-order structure. The effect of weight average molecular weight M_w and higher order structure of scleroglucan, on stimulation of human monocytes cultured in vitro to secrete tumor necrosis factor-α (TNF-α) was investigated. The higher order structures of the scleroglucan samples were determined by electron microscopy. The data showed that the samples with a linear wormlike, triple helical structure with M_w less than 50×10^4 g/mol or larger than 110×10^4 g/mol stimulated the monocytes more efficiently than samples with M_w in the range $(67–110) \times 10^4$ g/mol. The denaturation of the linear triple helices by NaOH (>0.25 M), followed by neutralization yielded blends of linear and macrocyclic topologies with concomitant irreversible reduction of the cytokine inducing activity compared with the untreated scleroglucans. The dose-dependent ability to activate monocytes to cytokine production was not restored following annealing of the denatured–renatured samples, despite the fact that electron micrographs revealed similar structures of these annealed samples to the starting material. Pre-incubation of monocytes with antibodies against cluster of differentiation antigens CD14 or CD11b reduced the scleroglucan potency to stimulate TNF-α secretion mainly for mAb against CD14 in the presence of serum.

Keywords: Scleroglucan; Molecular weight; Conformation; Monocytes; TNF-α

1. Introduction

Scleroglucan is a high-molecular-weight, comb-like branched polymer of β-D-glucose. The chemical structure consists of a (1→3)-β-D-glucose backbone substituted with single (1→6)-β-D-glucopyranosyl residues at every third residue (Fig. 1) [1], yielding a degree of branching (DB) equal to 0.33 [2,3]. This polymer, extruded from the fungus *Sclerotium*, dissolves in aqueous solution with a triple helical conformation stabilized by hydrogen bonds internally in the triple helical structure

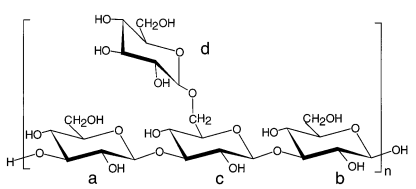

Fig. 1. Scleroglucan repeating unit. The lower case letters (a–d) denote the various glucose residues in the NMR assignments (Fig. 2).

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[2–4]. Schizophyllan and lentinan share the same chemical structure [5,6] and this family of polysaccharides is often referred to as (1→3)-β-D-glucans [7].

The branched (1→3)-β-D-glucans have shown immune stimulatory effects both in vitro and in vivo. They have been reported to possess anti-tumor [8–10], anti-bacterial and anti-viral effects [11] and they stimulated coagulation [12] and accelerated healing of wounds [11,13]. Molecular weight, DB, conformation and intermolecular associations of β-glucans have been shown to influence the immune stimulating effect to various extents [9,12,14–17]. The fraction of side-chains are reported to be important for their antitumor effect [18]. Samples with DB in the range 0.20–0.33 were found to be optimal for the stimulation [19]. The triple helix structure of these (1→3)-β-D-glucans appeared crucial for suppression of growth of implanted sarcoma S180 ascites in mice [15]. The tumor-inhibition ratio diminished as the portion of triple helixes decreased. On the other hand, single helixes (and polycarboxylated) (1→3)-β-D-glucans showed stronger activation of the limulus coagulation factor G than the triple helical conformation [12,13]. It has been reported that immunopharmacological activities of triple and single helical schizophyllan in mice might have a conformation dependence, which are probably assay dependent [20]. Another study suggested that triple helixes activated the alternative pathway of complement stronger than single helixes, while the single helixes were stronger activators of the classical pathway of complement [21].

The triple helical structure of high-molecular-weight (1→3)-β-D-glucans can be dissociated (denatured) either by increasing the temperature in aqueous solutions above the transition temperature (135 °C) [22], or dissolving the molecules in either dimethyl sulfoxide, with water weight fraction (W_H) less than 0.13 at 25 °C [23,24], or in aqueous hydroxide (>0.18–0.25 M NaOH/KOH) [2,5,25]. Renaturation of the (1→3)-β-D-glucans by either cooling, dialysis to remove dimethyl sulfoxide or neutralization, has been reported to yield a labile single-stranded conformer that slowly re-associates to the triple helical structure or coexisting linear and cyclic topologies formed by at least part regeneration of the triple helical structure [26–29].

The aim of this work was to determine the influence of M_w and higher order structure of scleroglucan on their ability to stimulate in vitro cultured human monocytes to produce TNF-α. The cellular and molecular mechanisms of cell activation by (1→3)-β-D-glucans are not understood in detail and different groups have suggested different molecules being involved in the signal transduction [30–34]. In this study, we included monoclonal antibodies (mAb) against CD11b and CD14 to test their possible involvement in the molecular signaling pathway leading to TNF-α expression from monocytes stimulated with scleroglucan.

2. Results

Scleroglucan samples.—The weight average molecular weights of the samples depolymerized from the purified scleroglucan $SC0_0$ ($M_w = 220 \times 10^4$ g/mol), were in the range 110×10^4–25×10^4 g/mol, $SC3_0$–$SC30_0$ (Table 1, [35]). The schizophyllan sample SPG was determined to have $M_w = 44 \times 10^4$ g/mol, which is close to the previously determined value of 45.5×10^4 g/mol [27] and this was slightly below the value of the $SC15_0$ sample. The DB of the alkali denatured sample, $^{nmr}SC15_{0.35}$, was determined to be 0.34 ± 0.01 from the integration of the shifted signals of C-2, C-3 and C-5 of the various residues in the NMR spectrum (Fig. 2). Scleroglucan treated with 0.1 M aqueous sodium hydroxide, $^{nmr}SC15_{0.1}$, or 2 h thermal annealing at 133 °C, $^{nmr}SC8_0A_{133}$, were found to have DB in the same range (Table 1). These values equaled the one previously determined for a non-heat treated scleroglucan from the same fermentation broth, SC (Table 1, [36]), and in agreement with the repeating unit (Fig. 1).

The electron micrographs of the $SC0_0$ to $SC30_0$ samples revealed linear dispersed wormlike polymers with average chain lengths reflecting the M_w of the samples (data shown only for $SC15_0$, Fig. 3(B)). Heat treatment at 100 °C at pH 7 for 2 h without subjecting the

Table 1
Properties of scleroglucan samples

Sample	M_W (10^4 g/mol)	DB	Topology	
			% Linear	% Circular
$SC0_0$	220		>90	0
$SC3_0$	110		>90	0
$SC8_0$	67		>90	0
$SC15_0$	49		>90	0
$SC30_0$	25		>90	0
SC		0.33[a]		
$^{nmr}SC8_0A_{133}$		0.35 ± 0.02	>80	0
$^{nmr}SC15_{0.1}$		0.33 ± 0.01	77 ± 3	6 ± 1
$^{nmr}SC15_{0.35}$		0.34 ± 0.01	30 ± 5	38 ± 5
$SC15_{0.1}$			70–80	5–10
$SC15_{0.35}$			53 ± 6	8 ± 4
$SC3_{0.35}A_{100}$			64 ± 5	8 ± 3
$SC3_{0.1}$			70–80	5–10
$SC3_{0.35}$			50 ± 4	15 ± 1
SPG	44/(45.5)[b]		>90	0

[a] Data obtained on the scleroglucan from the same batch by Hjerde et al. [36].
[b] Data reported by Kitamura et al. [16].

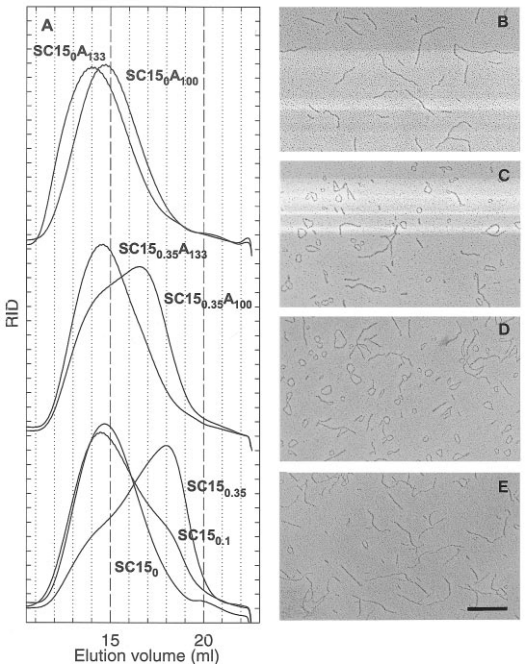

Fig. 3. The HPLC elution profiles (A) of 15 min sonicated scleroglucan samples $SC15_YA_Z$ and electron micrographs of samples $SC15_0$ (B), $SC15_{0.35}$ (C), $SC15_{0.35}A_{100}$ (D) and $SC15_{0.35M}A_{133}$ (E). The ordinate depicts signals from the refractive index detector (RID). All the seven curves, being separated in three vertical groups, start from the same y value. The scale bar (B–E) is 200 nm.

samples to alkaline denaturation/renaturation revealed no changes in the high-performance liquid chromatography (HPLC)-elution profiles ($SC15_0A_{100}$, Fig. 3(A)) or EM (data similar to the untreated sample, $SC15_0$, not shown). Increasing the annealing temperature (T_a) yielded a small shift of the elution profile of the $SC15_0A_{133}$ (Fig. 3(A)) to a larger elution volume (V_{el}). The lack of material for elution volumes larger than $V_{el} = 21$ mL indicated that depolymerization did not affect the samples to a large extent when annealing at 133 °C for 2 h. The electron micrographs of $SC15_0A_{133}$ (not shown) indicated mainly linear triplexes similar to that observed for $SC15_0$.

The HPLC profile of the sample exposed to 0.1 M NaOH, $SC15_{0.1}$ (Fig. 3(A)), showed a small shoulder eluting at larger V_{el} than the main peak of both $SC15_{0.1}$ and $SC15_0$. The

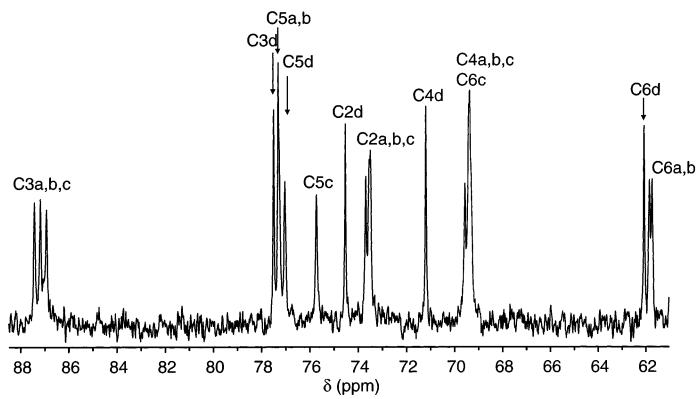

Fig. 2. ^{13}C NMR spectrum of scleroglucan sample $^{nmr}SC15_{0.35}$ in Me_2SO-d_6 at 90 °C.

electron micrographs (not shown) showed mainly linear molecules as in the untreated $SC15_0$ sample, but additionally contained a small fraction of rings and hairpins. Increasing the sodium hydroxide concentration to 0.35 M in the alkaline denaturation increased V_{el} of the main part of the renatured sample and circular topology appeared ($SC15_{0.35}$, Fig. 3(A) and (C)). The fractional content of the macrocyclic topology can be tuned by varying the temperature and duration of a subsequent thermal annealing procedure (Table 1) [35]. The denatured/renatured and annealed sample $SC15_{0.35}A_{100}$ (Fig. 3(A) and (D)), containing about 60% of the material in the form of circular topology, was included in the samples selected for immunological testing. Increasing T_a to 133 °C, yielded an HPLC profile ($SC15_{0.35}A_{133}$, Fig. 3(A)) almost identical to the non-treated $SC15_0$. $SC15_{0.35}A_{133}$ contained mainly linear triplexes (Fig. 3(E)) analogous to that observed for $SC15_0$ (Fig. 3(B)).

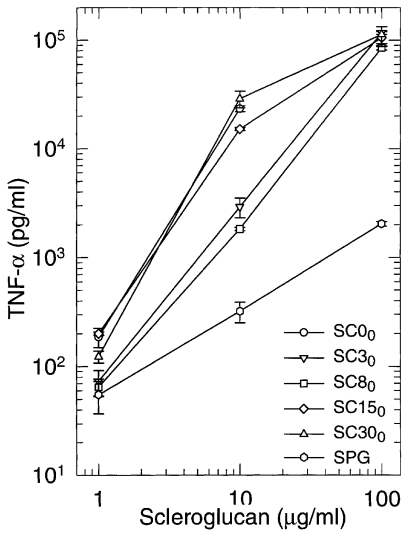

Fig. 4. TNF-α concentration in the growth media of human monocytes following cultivation for 6–9 h in RPMI with 20% A+ medium versus concentration of added scleroglucan. Results from one of six independent experimental series using linear triplex scleroglucan samples with various molar mass are shown. The TNF-α concentration in the media of monocytes incubated in the absence of scleroglucan was 115 (± 68). LPS (*E. Coli*, 1.0 mg/mL) stimulation of monocytes was used as a positive control and the TNF value was 143,700 (± 17,100) pg/mL.

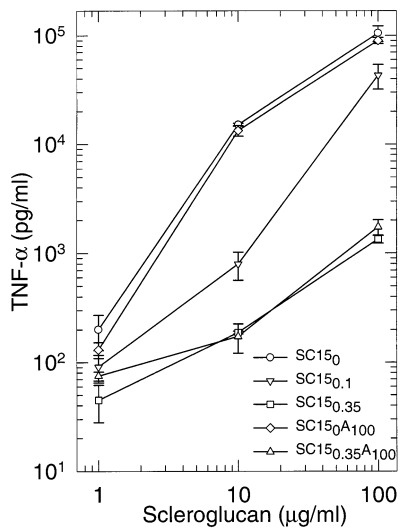

Fig. 5. Production of TNF-α from monocytes stimulated with different scleroglucans, $SC15_YA_Z$, subjected to various alkaline denaturation and annealing conditions; non-treated, or denatured/renatured and/or annealed scleroglucan. The amount of TNF-α produced from unstimulated or LPS (1.0 μg/mL, positive control) stimulated monocytes was 115 (± 68) pg/mL and 143,700 (± 17100) pg/mL, respectively (as in Fig. 4).

Activation of monocytes by scleroglucan with various M_w.—Fig. 4 shows the amounts of TNF-α released by human monocytes versus the scleroglucan $SC0_0$–$SC30_0$ concentration (c_p) in the medium. Generally, c_p below 1–2 μg/mL did not induce excess TNF-α secretion from the cultured monocytes.

Scleroglucan with the highest and lowest M_w had the most potent TNF-α inducing effect. The samples with intermediate M_w (67–110) × 10^4 g/mol ($SC8_0$–$SC3_0$, Table 1) were the least effective samples per weight concentration units. This trend was found for c_p in the range 5–70 μg/mL, either with or without 20% serum added to the medium. One of the series revealed a less pronounced dependence on M_w. At $c_p = 100$ μg/mL the dose response curves approached a common plateau level, and hence, there was no dependence on M_w in this concentration range.

The schizophyllan sample SPG included in one of the experimental series has earlier been tested for its antitumor activity against Sarcoma 180 ascites cell [16]. All scleroglucan samples $SC0_0$–$SC30_0$ isolated from the fermentation broth stimulated the monocytes more efficiently than the SPG sample both with (Fig. 4) and without (not shown) serum added to the medium.

Effect of scleroglucan structure on in vitro stimulation of human monocytes.—These series of experiments explored whether alkaline (0.10 and 0.35 M NaOH) and/or higher temperature (100 and 133 °C for 2 h) treatments of the

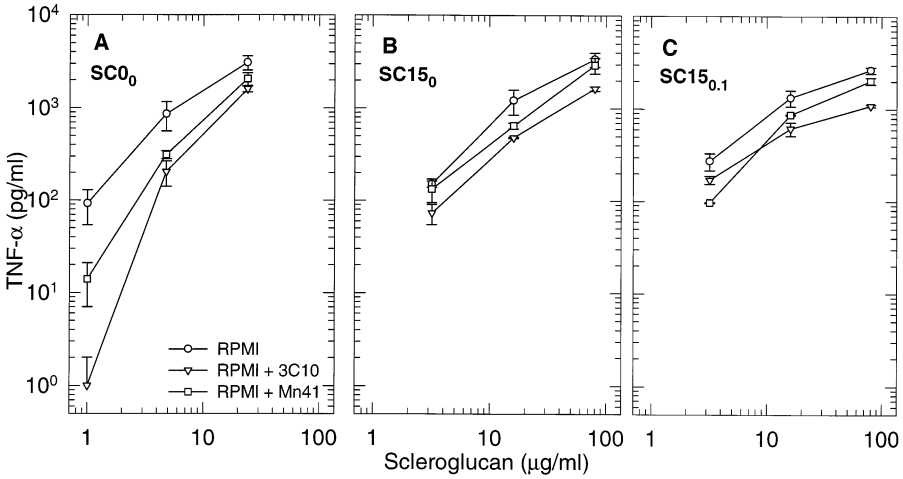

Fig. 6. TNF-α in the growth media from scleroglucan stimulated monocytes in the presence of monoclonal antibodies against CD11b (Mn41) or CD14 (3C10) and using serum free RPMI-medium; $SC0_0$ (A), $SC15_0$ (B) and $SC15_{0.1}$ (C). The TNF-α production from unstimulated monocytes was <16 (± 15) pg/mL, while LPS (0.1 μg/mL) stimulated monocytes in medium, medium with 3C10 or medium with Mn41 yielded 11,500 (± 3300), 1200 (± 250) and 8600 (± 800) pg/mL of TNF-α, respectively.

scleroglucans influenced their ability to stimulate monocytes to induce TNF-α production.

Fig. 5 shows the TNF-α production from stimulated monocytes versus medium concentration of the scleroglucan sample $SC15_0$ and some of its respective alkaline ($SC15_Y$) and/or annealed treated samples ($SC15_YA_Z$/$SC15_0A_Z$). The untreated sample $SC15_0$ and the annealed $SC15_0A_{100}$ samples yielded the highest amounts of TNF-α released by the monocytes. Increase of T_a to 133 °C, just below the conformational transition temperature of the triple helix (135 °C) [24], yielded a loss in the TNF-α inducing effect of scleroglucan ($SC15_0A_{133}$, data not shown) similar to that for treatment with 0.35 M sodium hydroxide (see below).

The $SC15_{0.1}$ sample had less cytokine stimulating effect on monocytes (Fig. 5) than its untreated analogue. Increasing the alkali concentration during the denaturation of the scleroglucans to 0.35 M sodium hydroxide yielded further reduction in the potency to induce TNF-α from the monocytes ($SC15_{0.35}$; Fig. 5). Annealing of the alkali treated sample after neutralization, either at 100 or 133 °C did not restore any of the scleroglucan's ability to stimulate monocytes to produce TNF-α ($SC15_{0.35}A_{100}$, Fig. 5). The amount of TNF-α produced by the monocytes subjected to $SC15_{0.35}A_{133}$ stimulation (not shown) was similar to the $SC15_{0.35}A_{100}$ stimulated monocytes. These alkaline or heat induced effects on scleroglucan were independent of molecular weight ($SC0_YA_Z$–$SC15_YA_Z$), influencing their cytokine stimulating activity.

Stimulation of TNF-α production in the presence of 3C10 and Mn41.—The monoclonal antibodies (mAbs) 3C10 or Mn41 were used to assess the involvement of CD14 and CD11b, respectively, in the recognition of (1→3)-β-D-glucans by the monocytes leading to TNF-α production. This was carried out for some of the linear triplex samples and the $SC15_{0.1}$ sample. Fig. 6 shows that 3C10 and Mn41 to some extent reduced the TNF-α production when monocytes were stimulated by different scleroglucan samples in serum free medium.

Addition of mAb against CD11b, Mn41, to the serum-containing RPMI medium did not affect the stimulation of the monocytes by the (1→3)-β-D-glucans, whereas a reduction in TNF-α was observed when mAb 3C10 was added Fig. 7.

Stimulation of monocytes by scleroglucan gels.—The scleroglucan gels were found to stimulate the in vitro cultured monocytes to secrete TNF-α to the same level as 20–100 μg/mL scleroglucan in solution (RPMI medium) or 4–20 μg/mL scleroglucan in solution (RPMI with serum) (Table 2). The data showed that monocytes incubated on the surface of the scleroglucan gels retained their

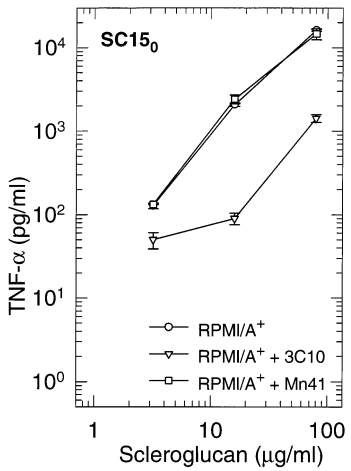

Fig. 7. TNF-α in the growth media from scleroglucan stimulated monocytes in the presence of monoclonal antibodies against CD11b (Mn41) or CD14 (3C10) and using RPMI-medium containing 20% A$^+$ serum. The TNF-α production from unstimulated monocytes was 20 (±7) pg/mL (3C10) and 81 (±21) (Mn41), while LPS (0.1 μg/mL) stimulated monocytes in medium, medium with 3C10 or medium with Mn41 produced 26,500 (±2100), 13,300 (±3200) and 24,100 (±1300) pg/mL of TNF-α, respectively.

ability to produce high levels of TNF-α. As for scleroglucan in solution, addition of serum enhanced the TNF-α inducing effect of the scleroglucan gels.

3. Discussion

Various laboratories have reported apparently conflicting results with respect to the relation between the structure of the (1→3)-β-D-glucans and their immunostimulating activity [19]. The main structural features that influence the action of (1→3)-β-D-glucans as a biological response modifier (BRM) appear to be the DB, molecular weight and higher order conformation. The latter should ideally include a description of structural features such as content of triple helix, various topologies and eventual cross-linking to control material properties.

The structural data of the untreated scleroglucans were in general agreement with a triple helical wormlike structure and resembled that previously described for (1→3)-β-D-glucans [37,38]. The average M_w values for the samples $SC0_0$–$SC30_0$ were all above 9×10^4 g/mol, where triple helixes and single chains were found to start coexisting in aqueous solution [15]. The differences in stimulation efficiency seen within the samples $SC0_0$–$SC30_0$ were therefore considered to be an effect only of molecular weight of the triple helical state and not to be an effect of different higher order structure. The present results indicated that the TNF-α inducing ability of the SCX_0 scleroglucans depended on their M_w, with the low M_w samples ($(25–49) \times 10^4$ g/mol) and the high M_w sample (220×10^4 g/mol) stimulating monocytes to produce TNF-α most efficiently. All the samples had a DB consistent with the repeating unit of scleroglucan (Fig. 1).

The data show that 0.1 M sodium hydroxide-treated scleroglucan samples ($SCX_{0.1}$) reduced their efficiency to induce TNF-α production in monocytes, while both DB and EM data did not reveal any changes in structure. Undetected changes in the macromolecular structure may have occurred, affecting the

Table 2
Stimulation of in vitro cultures of human monocytes to secrete TNF-α by scleroglucan gels

	Scleroglucan concentration (mg/mL)	TNF-α, RPMI medium (pg/mL)	TNF-α, RPMI with 20% A$^+$ serum (pg/mL)
Scleroglucan gel	10	862 ± 141	2316 ± 438
	15	284 ± 33	2133 ± 368
	18	531 ± 26	2115 ± 176
	μg/mL		
Scleroglucan solution, $M_w = 500 \times 10^4$ g/mol	4	33 ± 1	2566 ± 288
	20	143 ± 12	2843 ± 288
	100	647 ± 60	6583 ± 581
Unstimulated monocytes	0	<10	<10

action as a BRM. Candidates for structural changes, not possible to resolve with the employed techniques, were partly unwound triplex segments with lengths less than the resolution limit of the EM technique (1–2 nm) and changes in the spatial localization of the side-chains. Furthermore, this reduction in the activity of the $SCX_{0.1}$ samples compared with the SCX_0 samples, makes it difficult to compare the present M_w dependence with the data reported by Kulicke and coworkers [14], because they were using 0.1 M alkali for the initial dissolution of the (1→3)-β-D-glucans.

Alkaline denaturation of scleroglucans using sodium hydroxide >0.25 M, followed by renaturation, yields linear triplex and cyclic topologies alongside clusters [27]. The present data show that there were losses of immunological activity concomitant with this structural change, independent of the initial M_w. Similarly treated (1→3)-β-D-glucans have been reported to yield a single-helix structure [12,20] and the immunological data reported here would have been interpreted as less stimulation efficiency by the single-helix state as compared with the triplex. However, structures resembling the original linear triplexes blended with macrocyclic and larger clusters and not that of a single-stranded state were observed. Fully carboxylated scleroglucan, sclerox, adopting a single-stranded conformation, revealed a much more flexible structure [39,40]. Thus, the structural data of the immunologically tested denatured samples here did not adopt a single-helical conformation, and hence, the present data cannot be used to assess the immunologically stimulation by single-stranded (1→3)-β-D-glucans.

The cytokine-inducing activity of the scleroglucans annealed at 100 and 133 °C for 2 h, pH 7, showed no changes after the annealing at 100 °C (SCX_0A_{100}), while increasing the annealing temperature to 133 °C inactivated the samples as effectively as denaturation using 0.35 M sodium hydroxide. Annealing at both of these temperatures did not yield a large change in the coarse-grained conformation similar to that seen for the 0.35 M sodium hydroxide denatured samples. The unchanged DB indicate that the observed change in the stimulation activity was not due to cleavage of (1→6)-β-linked side-chains. Possible mechanisms for the loss of immunological activity in the SCX_0A_{133} samples are therefore similar to that discussed above for the $SCX_{0.1}$, i.e., undetected structural changes (partial triplex unwinding). Annealing at 100 °C ($SCX_{0.35}A_{100}$) or 133 °C ($SCX_{0.35}A_{133}$) of alkaline treated samples (0.35 M sodium hydroxide) yielded an increase and decrease in the content of ring structures, respectively, but essentially no changes in the cytokine stimulating efficiency compared with the only alkaline denatured sample ($SCX_{0.35}$) were observed.

Thus, the native, linear triple helix appeared to be necessary for the most effective immunological stimulation by this (1→3)-β-D-glucan in an in vitro system of isolated human monocytes. This is in line with that reported previously [5,9]. Furthermore, immunological activity of these samples decreased following alkaline (0.1 M) or thermal treatments (133 °C) close to the strand-separation transitions. Determination of structure by EM or DB by NMR does not offer insight into possible molecular mechanisms for these effects. The results also showed that the level of stimulation following a denaturation was not regained. Although a loss of stimulation activity after alkaline or thermal treatment was observed, all of these samples stimulated human monocytes at a comparable level to schizophyllan sample SPG, which was previously reported to suppress tumor growth in mice [16].

Scleroglucan gels also showed a stimulating effect on monocytes producing increased amount of TNF-α. The scleroglucan concentrations in these gels were chosen to yield mechanical stable gels with shear moduli in the range 0.4–2 kPa [41]. The starting concentrations of scleroglucan were much higher in the gels (10–18 mg/mL) than in the solutions (1–100 μg/mL) producing the same stimulating effect. One possible reason for this difference in the concentrations yielding the biological activity is the different fractions of scleroglucan in the gel and solution that are available to interact with the monocytes. We would anticipate that the majority of the scleroglucan chains in the interior of the gels are inaccessible to the cell surface receptors, while

scleroglucan chains in the solutions were mobile allowing them to diffuse to the receptors. Comparison of the dose–response curve within a given concentration range without considering the accessibility of the chains are therefore not possible.

The results of using the mAb against either CD11b (Mn41) or CD14 (3C10) are more difficult to assess. A clear inhibition of TNF-α production by antibodies was observed only in the case of mAb 3C10 used in a growth medium with added serum (Fig. 7). Experiments have shown that serum proteins, such as LBP and BPI, have modulating effects on binding of mannuronan to CD14 [42]. The GPI-anchored CD14 cell surface protein [43] is a well-known receptor for LPS [44], and mAbs against CD14 (3C10 and My-4) are reported to inhibit both LPS induced [45,46] mannuronan [46] and chitosan [47] induced TNF-α production. The results from the present mAbs studies and the finding that serum in most cases enhanced the scleroglucan induced TNF-α production from monocytes, analogous to modulation of serum on mannuronan binding to CD14 [42], therefore point in the direction of CD14 being involved in the signal transduction of (1→3)-β-D-glucans. The lack of a clear effect for both mAb Mn41 or 3C10 does not exclude the possibility that CD11b or CD14 are involved in the signal transduction provided that the epitope for the mAb interaction are different from glucan interaction site. The recent report of a toll-like receptor (TLR) involved in activation by zymosan [48] should encourage further investigations into the possible role of both CD14 and TLR in the signalling cascade for (1→3)-β-D-glucans.

4. Experimental

(1→3)-β-D-glucan samples.—Scleroglucan samples with various M_w and higher order conformation were obtained from a high M_w scleroglucan purified from a fermentation broth (kindly provided by Statoil/Norferm, Stavanger, Norway), as previously described [35]. Ultrasound induced depolymerization was carried out at standardized conditions (Braun Labsonic 1510, 50 W, aliquots of 4 mL with a concentration of 1.0 mg/mL) for 3 to 60 min. The samples were subsequently filtered using a sterile filter with pore size 0.22 μm, Millex-GV (Millipore), and kept hydrated for the immunological testing. These samples were depicted SCX_0, where X was substituted with the actual duration of the ultrasound irradiation in min and the subscript zero indicates no treatment. The M_w of the depolymerized samples, determined by HPLC using universal calibration, ranged from 25×10^4 g/mol ($SC30_0$) to 110×10^4 g/mol ($SC3_0$) (Table 1) [35]. Schizophyllan sample SPG was kindly provided by Dr S. Kitamura, Kyoto Prefectural University, Kyoto, Japan.

Scleroglucan samples with different higher order structures were prepared by alkaline denaturation–renaturation [35]. The triple helical structure was first dissociated by dialyzing 4 mL of aq scleroglucan at a concentration $c_p = 1.0$ mg/mL against 200 mL of 0.1 or 0.35 M aq NaOH, and the temperature was increased to 55 °C for 20 min at the end of the dialysis. These denatured samples were neutralized by transferring the dialyzing tubes to a 0.1 M aq NaOH at 55 °C and kept at this condition for 20 min before allowing the samples to cool. Subsequently, the samples were dialyzed against MQ-water to remove salts and further reduce the pH. These samples were referred to as SCX_Y, where Y specifies the highest molar NaOH concentration in the dialysis solvents. The previous subscript NaOH in the sample designations [35] was omitted here because NaOH was the only type of alkaline used for denaturation. Some of the samples, denoted SCX_YA_Z, were annealed by heating in pressurized bottles at 100 or 133 °C (Z in the sample designation) for 2 h and cooled by being left to stand in air at rt.

Scleroglucan gels were cast (curing time 24 h, rt) in sterile 24 well tissue plates using aq autoclaved sterilized scleroglucan solutions, $c_p = 10$ and 18 mg/mL. The scleroglucan sample in this series of experiments was purified from a different broth from the same source as described above, and the M_w was determined to be 500×10^4 g/mol. The gels were prepared by cross-linking scleroglucan with sodium-*meta*-periodate [41]. The gels were washed with sterile PBS medium three times before the immunological testing.

Structural analysis.—Analyses of the distribution of the hydrodynamic volumes were carried out using aq HPLC, as previously described [35], employing serially connected TSK Gel G6000 PWXL and TSK Gel G5000 PWXL columns (TosoHaas). The relative polysaccharide concentrations were determined by the refractive index increment. Sample loss in the filtration was determined by integration of the HPLC elution profiles. Transmission electron micrographs of the scleroglucan samples were obtained as previously detailed [49,50]. The relative abundance of the linear triplex and circular topology of the samples was estimated by counting these topologies in the micrographs (of the order of 1000 observations for each sample).

^{13}C NMR.—The degree of branching of selected scleroglucan samples was determined by ^{13}C NMR. The scleroglucan was freeze-dried and dissolved (30 mg/mL) in Me_2SO-d_6. Proton-decoupled ^{13}C NMR was performed at 90 °C on a Bruker Advance DPX spectrometer at 78.48 MHz. Chemical shifts were assigned as previously reported for scleroglucan [51,52]. The degree of branching (DB) was determined as the mole of glucose in the side-chains per mole of glucose in the main chain from the integrated intensities of the C-2, C-3 and C-5 carbons in the a, b, c and d residues of the repeating unit (Fig. 1). The DBs were determined as the mean $\pm$ S.D. from the data obtained on the individual carbons.

Monocyte cultivation.—Monocytes were isolated from human A^+ blood buffy coat (The Bloodbank, The University Hospital of Trondheim) [53]. Monolayers of monocytes were cultured in 24 well culture plates with RPMI 1640 medium (Gibco) containing 1% glutamine, 40 μg/mL gentamicin sulfate and 20% A^+ serum (The Bloodbank, The University Hospital of Trondheim). In some experiments monocytes were isolated and cultivated in RPMI 1640 medium without serum. Sterile filtered (0.22 μm, Millex-GV) scleroglucan samples were added to the monocyte cultures at three different concentrations. The loss of scleroglucan in the filtration step was corrected when determining the c_p in the monocyte cultures by integrating the HPLC elution profiles of filtered compared with non-sterile filtered samples. The supernatants were harvested and assayed for TNF-α after 6–9 h incubation (37 °C, 5% CO_2). Determination of monocyte stimulation by the scleroglucan gels was carried out by incubating the monocytes on top of the scleroglucan gels as described above.

TNF-assay.—TNF-α concentration in the supernatants was determined by the cytotoxic effect of TNF-α on the fibrosarcoma cell line WEHI 164 clone 13, according to Espevik and Nissen-Meyer [54]. Recombinant TNF-α (r-TNF-α, Genentech) was used as standard and medium from non-stimulated monocytes was included as a blank control. The fibrosarcoma cells were incubated (37 °C, 5% CO_2) for 12–18 h and their viability was determined by adding 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT] [55] and determining the resulting optical density ($\lambda =$ 570 nm). The concentration of TNF-α in the scleroglucan stimulated monocyte cultures was determined from the r-TNF-α standard curve. A LPS sample (1.0 and/or 0.1 μg/mL) from *E. Coli* was included as a positive control. In some experiments, mAbs against CD11b or CD14, Mn41 and 3C10, respectively, were added to the monocyte cultures just before scleroglucan or LPS (2.5 μL mAbs/2×10^6 cells). The Mn41 mAb was generously provided by Dr G.D. Ross, University of Louisville, USA. Results are presented as mean and $\pm$ S.D. of triplicate determinations.

To exclude endotoxin stimulating effect of scleroglucan samples, a *Limulus amoebocyte* lysate (LAL) assay was carried out. The LAL assay indicated a low endotoxin content in the scleroglucan samples (less than 0.02% by weight).

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