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Structural and immunological studies of a pectin and a pectic arabinogalactan from *Vernonia kotschyana* Sch. Bip. ex Walp. (Asteraceae)

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Abstract—Two polysaccharides, a pectin (Vk100A2b) and a pectic arabinogalactan (Vk100A2a) with mean M_w 2×10^4 and 1.15×10^6 Da, respectively, were isolated from the dried powdered roots of *Vernonia kotschyana* Sch. Bip. ex Walp. by hot water extraction followed by fractionation on DEAE-Sepharose fast flow and Sephacryl S-400 HR. The pectin showed low-complement fixation activity and no influence on proliferation of B or T cells, while the pectic arabinogalactan showed a potent, dose-dependent complement fixation activity and a T cell independent induction of B-cell proliferation. Both polysaccharides induced chemotaxis of human macrophages, T cells and NK cells. *exo*-α-L-arabinofuranosidase and *exo*-β-D-galactosidase digestion followed by component sugar and methylation analysis indicated that Vk100A2a consisted of a highly branched rhamnogalacturonan core with approximately 50% of the rhamnose 1,2,4-substituted, side chains rich in terminal-, 1,5-linked and 1,3,5-branched arabinose and terminal-, 1,4-, 1,6-linked and 1,3,6-branched galactose. The enzyme resistant part of Vk100A2a still showed strong complement fixating activity, suggesting that this activity may at least in part be expressed by carbohydrate structures present in the enzyme resistant, inner portion of the polymer.

Keywords: *Vernonia kotschyana*; *Baccharoides adoensis var. kotschyana*; Polysaccharide; Pectin; Pectic arabinogalactan; Chemotaxis; Complement fixation; Mitogenic activity; Immunomodulating activity

1. Introduction

Baccharoides adoensis var. kotschyana (Sch. Bip. ex Walp.) M. A. Isawumi, G. El-Ghazaly and B. Nordenstam, mainly described in the literature as *Vernonia kotschyana* Sch. Bip. ex Walp. or *Vernonia adoensis* Sch. Bip., is a shrub up to 2 m high, of the savanna, from Senegal to Nigeria extending across Africa to Ethiopia.[1] In traditional medicine a decoction of the roots of *V. kotschyana* (Asteraceae) is usually taken internally (orally) and widely used in the treatment of gastric-ulcers or externally as a wound healing remedy in Mali,[2] as a digestive in Nigeria and against stomach-pain in Tanzania.[1] A butanol fraction of an aqueous extract of the roots has shown gastro-protective activities in different types of gastric ulcers induced in rats, and steroidal glycosides are suggested to contribute to this

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activity.[3] The biology of wound healing is much more complex than initially assumed and involves soluble mediators, extracellular matrix components, resident cells and circulating immune cells.[4] Immune cells are critical for the outcome of healing,[5] and agents that modulate immune function may have a significant effect on the reparative process.[6]

Low-molecular weight compounds have for a long time been considered as the active principles of medicinal plants, but they often cannot account for the effects observed alone. Some types of plant polysaccharides have shown some interesting pharmacological activities, especially regarding immune stimulation. Polysaccharides from *Plantago major* L., a plant used traditionally to promote wound healing, have shown effects on the human immune system and are suggested to at least in part account for the wound healing capacity of this plant. The reported polysaccharides are pectins and type II arabinogalactans.[7,8] Complement fixation by pectic arabinogalatans has been reported from the plants *Trichilia emetica* and *Entada africana*, which are used in the treatment of wounds in Mali.[9,10] An arabino-3,6-galactan from the rhizomes of medicinal herb, *Atractylodes lancea* DC., have shown intestinal immune system modulating activities,[11] and potent anti-ulcer pectic polysaccharides with immunomodulating activities have been isolated from *Bupleurum falcatum*.[12–14]

Several acidic polysaccharide fractions isolated from the roots of *V. kotschyana* showed immunomodulating activities in a previous study,[2] and we now report on the further purification and characterisation of polysaccharides from one of these fractions, Vk100A2. In addition, we have tested the purified polysaccharides for immunomodulation such as induction of chemotaxis of leucocytes, stimulation of lymphocyte proliferation and for complement fixation activities. Optimal host defence against infection is understood as a high capacity of leucocytes to respond by chemotaxis and random migration. Very often, chronic and recurrent infections are associated with diminished chemotaxis in vitro. Therefore, agents that improve or stimulate leukocyte locomotion are of interest. The stimulation of lymphocyte proliferation has clinical relevance in patients with depressed cellular immunity and a variety of bacterial and viral infections as well as cancer.[15] The complement system is an important effector pathway of the nonspecific humoral immune response, and the complement fixation test has been widely used for location of possible immunomodulating compounds of polysaccharide nature. The complement system is associated with various important biological systems, such as the thymus-dependent anti-body response, regulation of specific cyclical anti-body production, regulation of IgM-IgG switch, modulation of T and B cell proliferation, induction of suppressor or helper T cells and modulation of monokine or lymphokine release.[16]

2. Results

2.1. Characterisation of the isolated and purified polysaccharide fractions

Two polysaccharides, Vk100A2a and Vk100A2b, were isolated from the crude polysaccharide fraction Vk100A2,[2] according to their molecular weight on a gel-filtration column, Sephacryl S-400 HR (Fig. 1). The molecular weight of Vk100A2b was estimated to about 20 kDa on a Superose 6 column using a FPLC-system with different dextrans as standards (data not shown). As Vk100A2a was eluted very closely to the void, the molecular weight of Vk100A2a was determined by SEC/MALLS to 1150 ± 20 kDa with a polydispersity $M_w/M_n = 1.44$. The monosaccharide-composition determined by methanolysis, trimethylsylation and gas chromatography is given in Table 1. The presence of arabinogalactan type II was identified in the high-molecular weight polymer Vk100A2a only, by the reactivity with Yariv antigen and linkage analysis (Table 2). The protein content in Vk100A2a and Vk100A2b determined by the Lowry method was very low, <0.5% and <0.3%, respectively. The amino acid composition of Vk100A2a is presented in Table 3. The degree of sugars (mol %) acetylated in Vk100A2a and Vk100A2b was, 11% and 7%, respectively. The amount

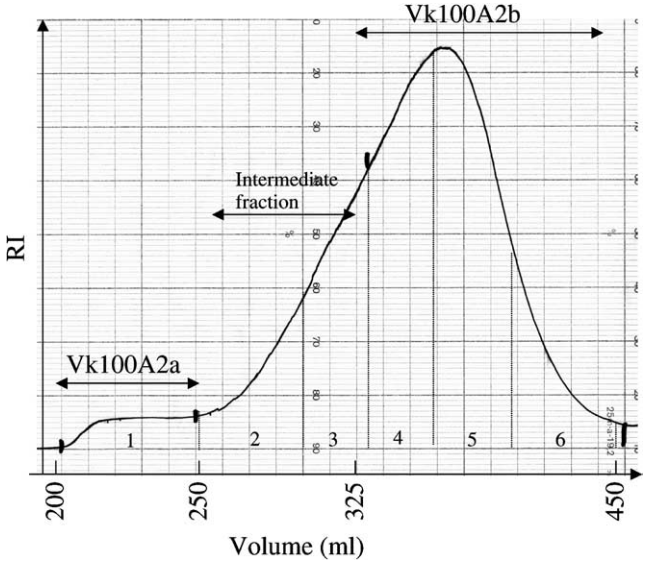

Figure 1. Separation of Vk100A2 on a Sephacryl S-400 HR column (15 mM NaCl solution). The elution profile was monitored by a refractive index detector (RID). The phenol–sulfuric acid test was also performed and showed approximately the same profile. Originally six fractions were collected according to the dashed lines, dialysed and lyophilised. The six fractions (1–6) were finally pooled into 3 fractions, Vk100A2a, the intermediate fraction and Vk100A2b, according to their monosaccharide compositions and complement fixation activities. The intermediate fraction(s) were not investigated further.

Table 1. Yield (%, w/w), total carbohydrate content (%, w/w) and monosaccharide composition (mol %) of the fractions Vk100A2a and Vk100A2b obtained after size exclusion chromatography on Sephacryl S-400 of Vk100A2

Polysaccharide fraction	Vk100A2	
	a	b
Yield (%, w/w)[a]	0.05	0.34
Total carbohydrate (%, w/w)	86	68
Monosaccharide composition (mol %)		
Arabinose	31.3	3.9
Rhamnose	11.0	4.9
Galactose	24.4	4.9
Xylose	0.7	Trace
Mannose	0.3	Trace
Glucose	5.7	1.8
Fucose	—	—
Glucuronic acid	Trace	Trace
Galacturonic acid	26.4	84.6
Reaction with Yariv-reagent	+	–
Protein content (%, w/w)[b]	<0.5	<0.3

[a] Based on dried and pulverised root.
[b] Based on the Lowry method.

Table 2. Methylation analysis of the original polysaccharides, Vk100A2b (b) and Vk100A2a (a), and the enzyme resistant products from Vk100A2a obtained after enzymatic digestion with *exo*-α-L-arabinofuranosidase (a-AF) or *exo*-β-galactosidase (a-GN) or both *exo*-α-L-arabino-furanosidase and *exo*-β-galactosidase (a-AF/GN)

Glycosyl residue	Deduced linkage	Mol %				
		b	a	a-AF	a-GN	a-AF/GN
Ara	Terminal *f*	1.4	8.0	0.6	1.1	3.5
	Terminal *p*	0.6	Trace	Trace	Trace	Trace
	1,3	Trace	0.2	Trace	Trace	Trace
	1,5	1.4	11.2	2.0	17.9	0.1
	1,3,5	0.6	11.8	—	—	—
Rha	Terminal	2.0	0.3	0.3	0.6	0.2
	1,2	1.8	5.2	5.3	5.4	4.9
	1,3	0.7	—	—	—	—
	1,2,4	0.4	5.5	4.9	5.6	6.5
Gal	Terminal	0.3	3.2	6.2	3.9	10.9
	1,3	0.1	0.6	0.6	0.5	0.5
	1,4	4.2	15.7	14.7	6.3	3.2
	1,6	0.1	1.7	3.8	0.4	0.6
	1,3,6	0.1	2.9	1.2	0.2	0.3
	1,3,4	—	0.4	—	—	—
Xyl	Terminal *p*	Trace	0.7	0.7	0.6	0.5
Man[a]		Trace	0.3	0.5	0.3	0.4
Glc	Terminal	0.1	1.2	1.2	0.2	0.4
	1,4	1.7	4.6	2.1	1.7	2.4
Fucose	Terminal	Trace	trace	Trace	Trace	Trace
GlcA[b]	Terminal	Trace	Trace	Trace	Trace	Trace
GalA[b]	Terminal	1.6	—	—	1.5	1.8
	1,4	79.7	25.2	24.5	26.8	25.7
	1,2,4	1.7	0.6	1.6	0.7	0.9
	1,3,4	1.5	0.8	1.4	0.8	0.5

The molar percentages of the glycosyl residues in the enzyme resistant products are correlated to the total amounts of arabinose and galactose found in the original polysaccharide, Vk100A2a.
[a] The linkage was not deduced.
[b] The uronic acids were reduced with sodium borodeuteride prior to methylation and GC–MS.

Table 3. Amino acid composition (mol %) of Vk100A2a

Amino acid	Mol %
Serine	17.1
Alanine	14.9
Glutamic acid	13.4
Glycine	12.1
Aspartic acid	8.6
Hydroxy proline	7.4
Valine	4.7
Threonine	4.7
Leucine	3.7
Proline	3.1
Isoleucine	2.2
Lysine	2.1
Arginine	2.0
Phenylalanine	1.5
Methionine	0.9
Tyrosine	0.9
Histidine	0.8

(mol %) of galacturonic acids methylesterified was relatively high in Vk100A2b (20%) compared to Vk100A2a (8%).

Since the crude acidic polysaccharide fraction, Vk100A2, originally also contained inulin, expressed by the content of about 20% fructose, a quantitative ketose determination of the purified fractions was performed as described previously.[2] Vk100A2a and Vk100A2b contained 11% and 4% ketose, respectively. The ketose was identified as fructose on the Dionex system[2] after weak acid hydrolysis (sample treated with 0.2% oxalic acid at 100 °C for 2.5 h).

2.2. Complement fixation

Vk100A2a showed dose-dependent and high-complement fixation activities expressed as high % inhibition of hemolysis at low concentrations of the sample (Fig. 2) compared with the positive control, a pectin fraction from *Plantago major*,[7] PMII. PMII typically showed a 50% inhibition of hemolysis at 27 μg/mL, while Vk100A2b did not reach 50% inhibition of hemolysis at the highest concentration tested (500 μg/mL, data not shown).

The polysaccharide samples were tested for lipopolysaccharide (LPS) contamination by the Limulus Amebocyte Lysate test (LAL-test). The content of LAL-positive substances was estimated to between 0.25% and 0.125% in Vk100A2a and between 0.001% and 0.0005% in Vk100A2b. Various concentrations of LPS from *E. coli* (Sigma) were tested for complement fixation activities, but no activity could be observed for the concentrations tested (0.05, 0.50 and 5.00 μg/mL) (data not shown). The concentrations of LAL-positive substances in Vk100A2a tested for complement fixation activity was <0.08 μg/mL. We also evaluated a possible synergistic effect of LPS and Vk100A2a. Various amounts of LPS were added to Vk100A2a, and the activity was

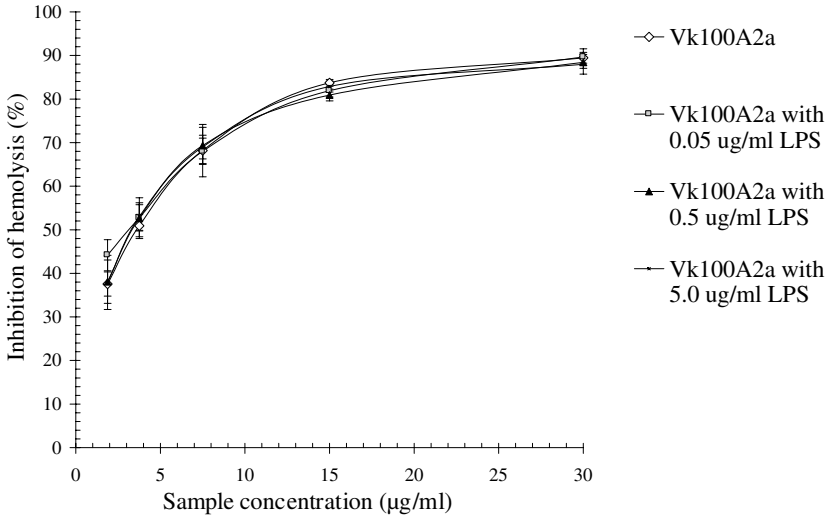

Figure 2. Investigation of a possible synergistic or additive effect on complement fixation by LPS from *E. coli* and Vk100A2a polysaccharides. The % inhibition of hemolysis of three different concentrations of LPS (□: 0.05; ▲: 0.5 and ■: 5.0 μg/mL) together with Vk100A2a were compared with the activity of pure Vk100A2a (◇). No synergistic or additive effects of LPS on the complement fixation activity could be observed at the concentrations tested.

recorded. No synergistic effects could be observed at any of the concentrations tested (Fig. 2).

2.3. Structure and complement fixation activities of the enzyme digested Vk100A2a

In order to elucidate the structure and structure–activity relationships of the bioactive Vk100A2a enzymatic degradation with *exo*-α-L-arabinofuranosidase or *exo*-β-D-galactanase or a combined digestion with both enzymes was performed. The monosaccharide composition of the enzyme resistant products and their sugar linkages are given in Table 2. The *exo*-α-L-arabinofuranosidase selectively removed about 90% of the arabinose in Vk100A2a. As the amount of arabinose decreased, a slight decrease in 1,3,6-branched galactose and a concomitant increase in 1,6-linked galactose could be observed in addition to a slight decrease in 1,4-linked galactose and an increase in terminally-linked galactose (Table 2). The degradation of Vk100A2a by *exo*-β-D-galactanase reduced the galactose content from 24.4% to 11.3%, with 1,4- and terminally-linked galactose as the most resistant units for degradation. The 3-linkage in the 1,3,5-branched arabinose subunits were also

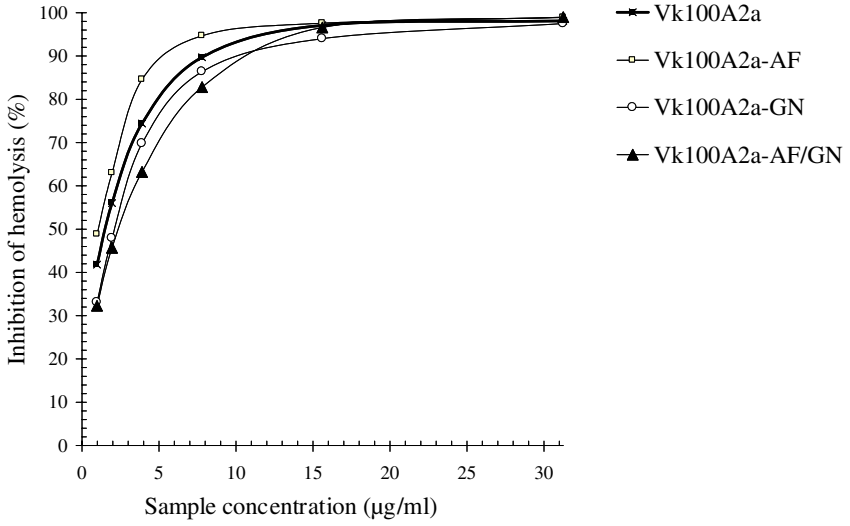

Figure 3. Complement fixation activity expressed as % inhibition of hemolysis of Vk100A2a (■) and the products obtained after enzymatic digestion with α-L-arabinofuranosidase (□: a-AF) or β-galactosidase (○: a-GN) or both α-L-arabinofuranosidase and β-galactosidase (▲: a-AF/GN). Vk100A2b did not obtain 50% complement fixation by the highest concentration tested (500 μg/mL). A complement fixating polysaccharide (PMII) from *Plantago major* was used as positive control, and showed 50% complement fixation at 27 μg/mL.

susceptible to degradation, leaving the major part of the remaining arabinose on the 1,5-linked form (Table 2). In a combination the *exo*-α-L-arabinofuranosidase and the *exo*-β-D-galactanase removed about 88% of the arabinose and 36% of the galactose in Vk100A2a. In the enzyme resistant part of Vk100A2a, the remaining arabinose was almost completely terminally-linked (97%), and so was the remaining galactose residues (70%) in addition to 1,4-linked (21%) (Table 2).

The complement fixation activities of the enzyme resistant parts of the polymer were investigated. All the fractions were very active with high inhibition of

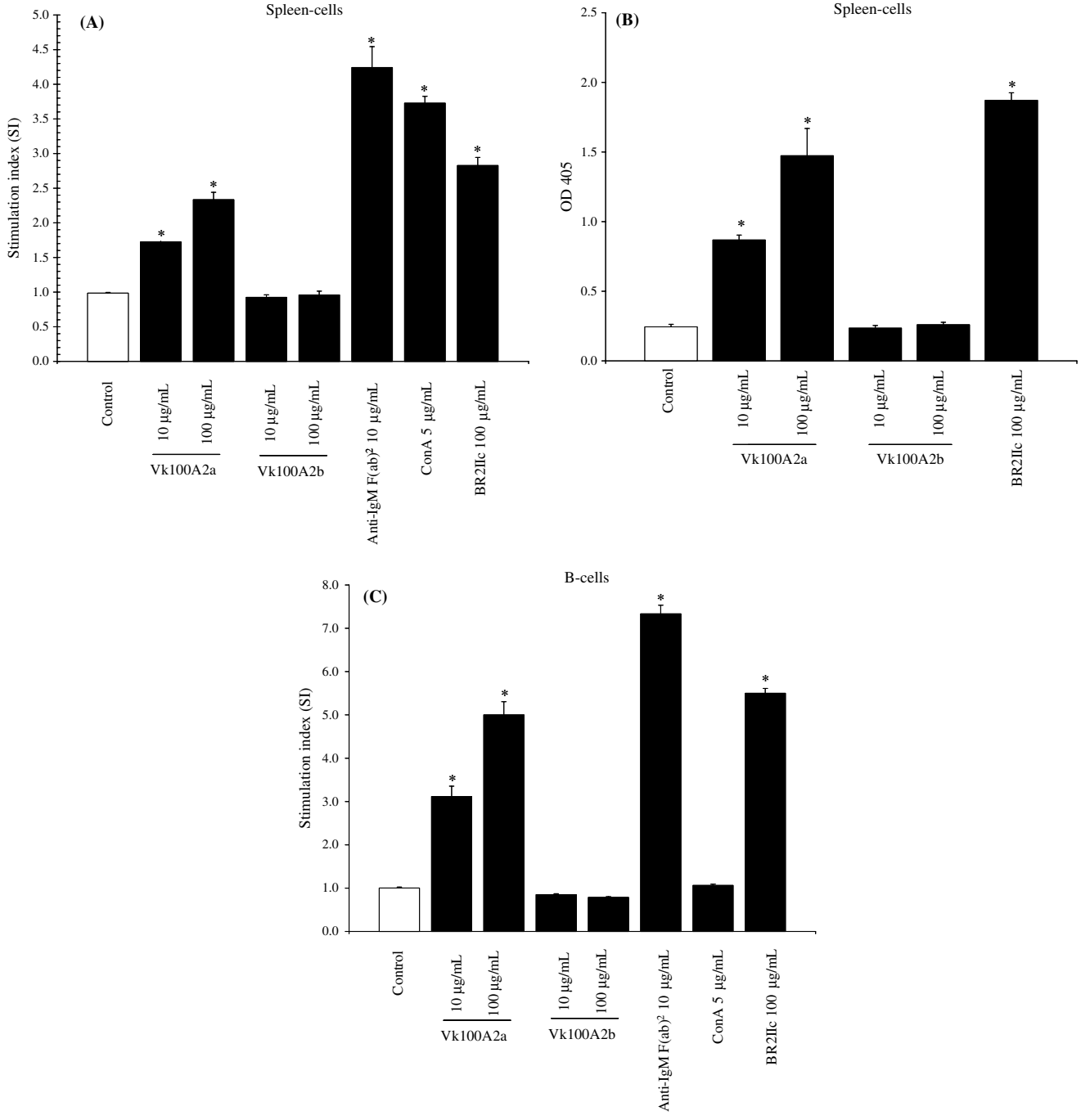

Figure 4. Mitogenic activities of Vk100A2a and Vk100A2b on spleen cells (A and B) and on purified B-cells (C). The cells (2 millions of cells/well, in a 96 well plate) isolated from spleens from C3H/HeJ mice were cultured with various concentrations of Vk100A2a, Vk100A2b, water (unstimulated control), Anti-IgM $F(ab')_2$, ConA or BR2IIc for 3 days, and the proliferative responses were assessed either by the AlamarBlue™ reduction assay (A, C) measuring the relative fluorescence intensity with an excitation wavelength at 544 nm and emission wavelength at 590 nm, with the stimulation index (SI) calculated as described in Experimental, or the Alkaline Phosphatase assay (OD 405) (B) as described in Experimental. Each value is presented as mean ± SD. Asterisks indicate significant ($^*P < 0.001$) compared to unstimulated control.

hemolysis at low concentrations (Fig. 3). At 50% inhibition of hemolysis no clear differences in activities could be observed between the enzyme resistant parts. The assay was repeated twice with similar results.

2.4. Mitogenic activities

Vk100A2b did not show any mitogenic activities on spleen cells from C3H/HeJ mice at the concentrations tested (Fig. 4A–C). But the high-molecular weight fraction, Vk100A2a, showed high-mitogenic activity on the spleen cells in a dose-dependent manner (Fig. 4A). A dose-dependent induction of alkaline phosphatase, which is a selective marker for B cell proliferation, could be observed in the spleen cell suspension by Vk100A2a (Fig. 4B). Mitogenic activity was thereafter shown on purified B cells (Fig. 4C), indicating that Vk100A2a polysaccharides may stimulate B cells independently of other immune cells such as macrophages and T cells. Vk100A2a showed mitogenic activity on B cells in the same range as the well-known pectin, bupleuran 2IIc, from *B. falcatum*. The proliferative response was confirmed by flow cytometry, which showed an increase in the CD45R/B220 (B cell)-positive cell number after stimulation of the spleen cell suspension with Vk100A2a (Fig. 5). The microscopic observation did not show an increase in apoptotic cells, which suggested that the increase in B cell population was not due to a decrease in the population of T cells.

2.5. Chemotaxis of macrophages, NK-cells and T-cells

In order to study whether the polysaccharides isolated from *V. kotschyana* would affect macrophages, we generated macrophages from human monocytes (Fig. 6A). Both Vk100A2a and Vk100A2b attracted macrophages in a chemotaxis assay (Fig. 6B). We experienced some donor variations in the ability of Vk100A2a and Vk100A2b to chemoattract macrophages. Macrophages from two out of six donors were only reactive towards 10 μg/mL of the polysaccharides, while macrophages from three other donors reacted towards both Vk100A2a and Vk100A2b with optimal concentrations at both 10 and 100 μg/mL (Fig. 6B). Macrophages from the last donor were only reactive towards 1 μg/mL of both polysaccharides (data not shown).

The ability of Vk100A2a and Vk100A2b to chemoattract human T cells and NK cells was also tested. The purity of T cells and NK cells was examined by flow cytometry (Fig. 7A, left and right panel, respectively). As shown in Figure 7B, the two Vk100A2 fractions induced chemotaxis of T cells. While the optimal concentration of Vk100A2b for T cell migration was 10 μg/mL for four out of five donors tested, we observed donor variations for Vk100A2a. Two donors responded optimally at 10 μg/mL (Fig. 7B, represented in the left panel), while T cells from the three other donors migrated towards 100 μg/mL of Vk100A2a (Fig. 7B, represented in the right panel). In Figure 7C, we demonstrate chemotaxis of IL-2 cultured NK cells, towards the Vk100A2 fractions. Vk100A2a seem to most potently induce chemotaxis of NK cells, as compared with Vk100A2b. Both fractions induced chemotaxis within 10–100 μg/mL.

As both Vk100A2a and Vk100A2b are contaminated by small amounts of LPS and inulin, we tested one

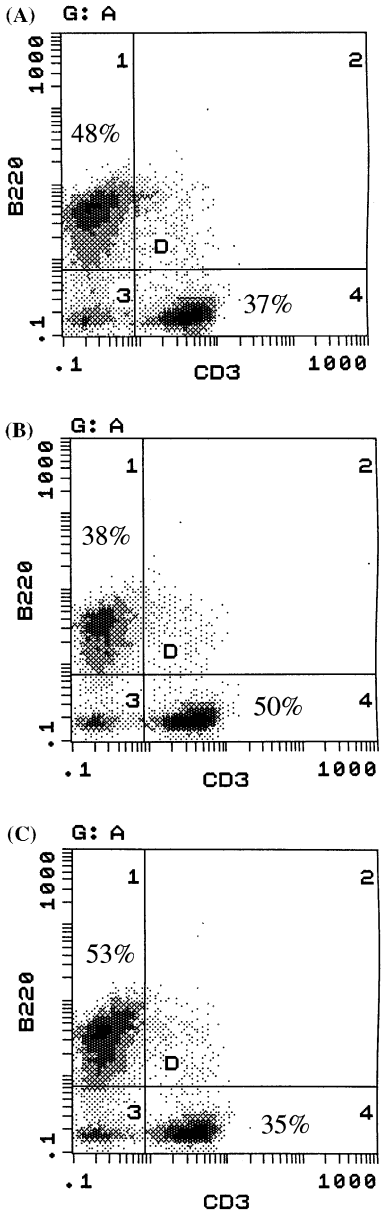

Figure 5. Flow cytometric analysis of Vk100A2a induced proliferation of B-cells. Spleen cells (2 millions of cells/well, in a 96 well plate) from C3H/HeJ mice were cultured with Vk100A2a (100 μg/mL) (A), unstimulated control (B), or positive control (bupleurum 2IIc, 100 μg/mL) (C) for 3 days. After blocking with anti-CD16/32 mAb, lymphocytes were stained with FITC-conjugated anti CD3ε mAb, and PE-conjugated anti-CD45R/B220.

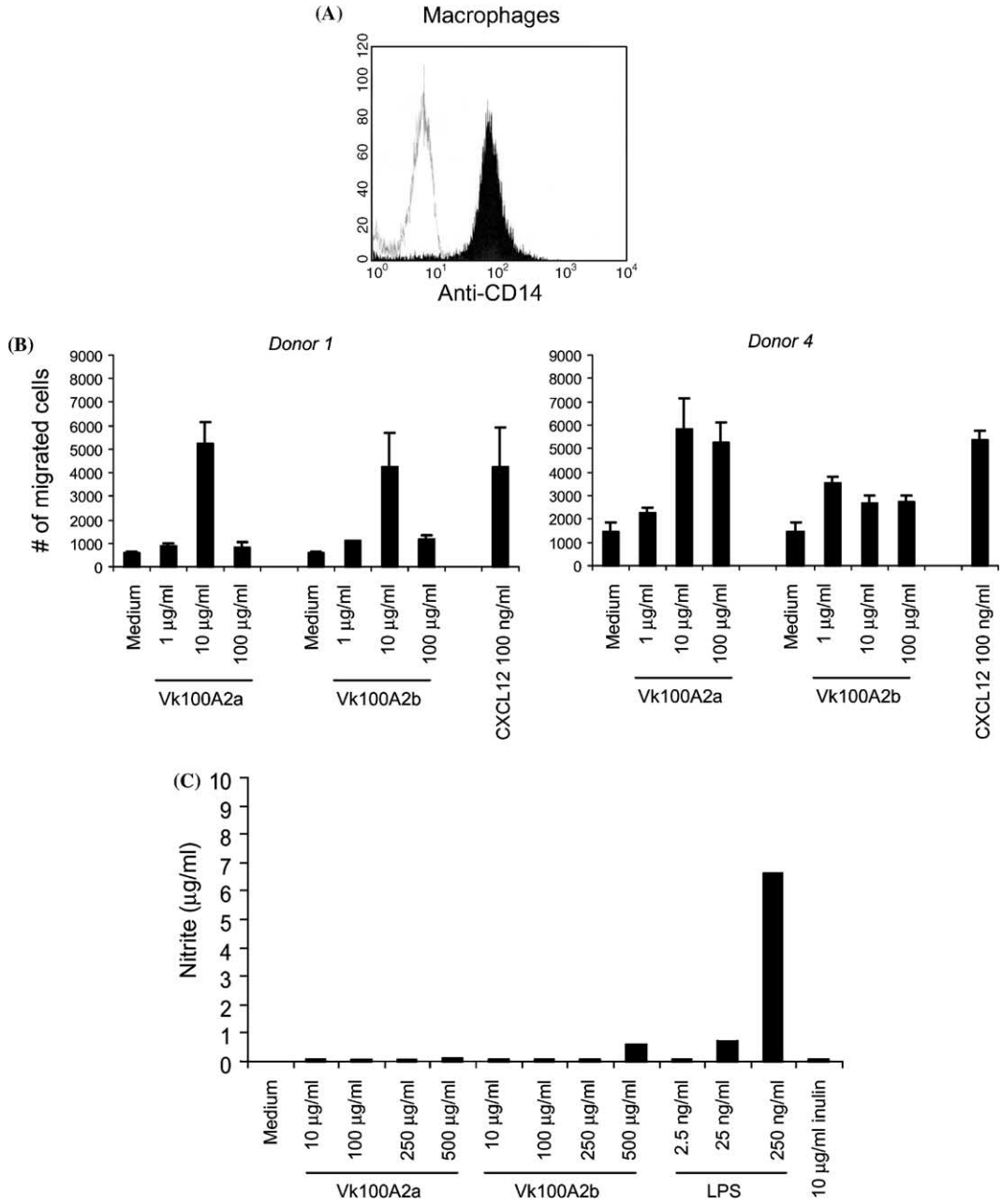

Figure 6. Stimulation of macrophages with Vk100A2 fractions. The purity of macrophages was examined by flow cytometry using CD14-FITC antibody (A). Chemotaxis of macrophages in response to increasing concentrations of Vk100A2a and VK100A2b was measured in Boyden chambers as described in Experimental. Two representative donors of six tested are shown (B). Measurement of nitric oxide release from macrophages after overnight stimulation with Vk100A2 fractions, LPS or inulin (C).

donor for chemotactic effects of various concentrations of LPS and inulin. Some activity was observed both for LPS (1 ng/mL) and inulin (0.4 µg/mL) on macrophage chemotaxis, and LPS (1 ng/mL) induced some effect on T cell chemotaxis (data not shown). Corresponding the amounts of LPS and inulin tested to the amounts present in the Vk100A2a and Vk100A2b samples, it seems unlikely that LPS and inulin account for the chemotaxis observed. It is however interesting to note that chemotaxis of the Vk polysaccharides is dependent on the individual donors, and for this reason it would be interesting for future research to investigate if there are donor variations also for the chemotaxis induced by LPS and inulin.

2.6. Nitric oxide measurement

We tested the ability of the samples to induce the release of nitric oxide (NO) from human macrophages, measured through nitrite, which is a stable breakdown

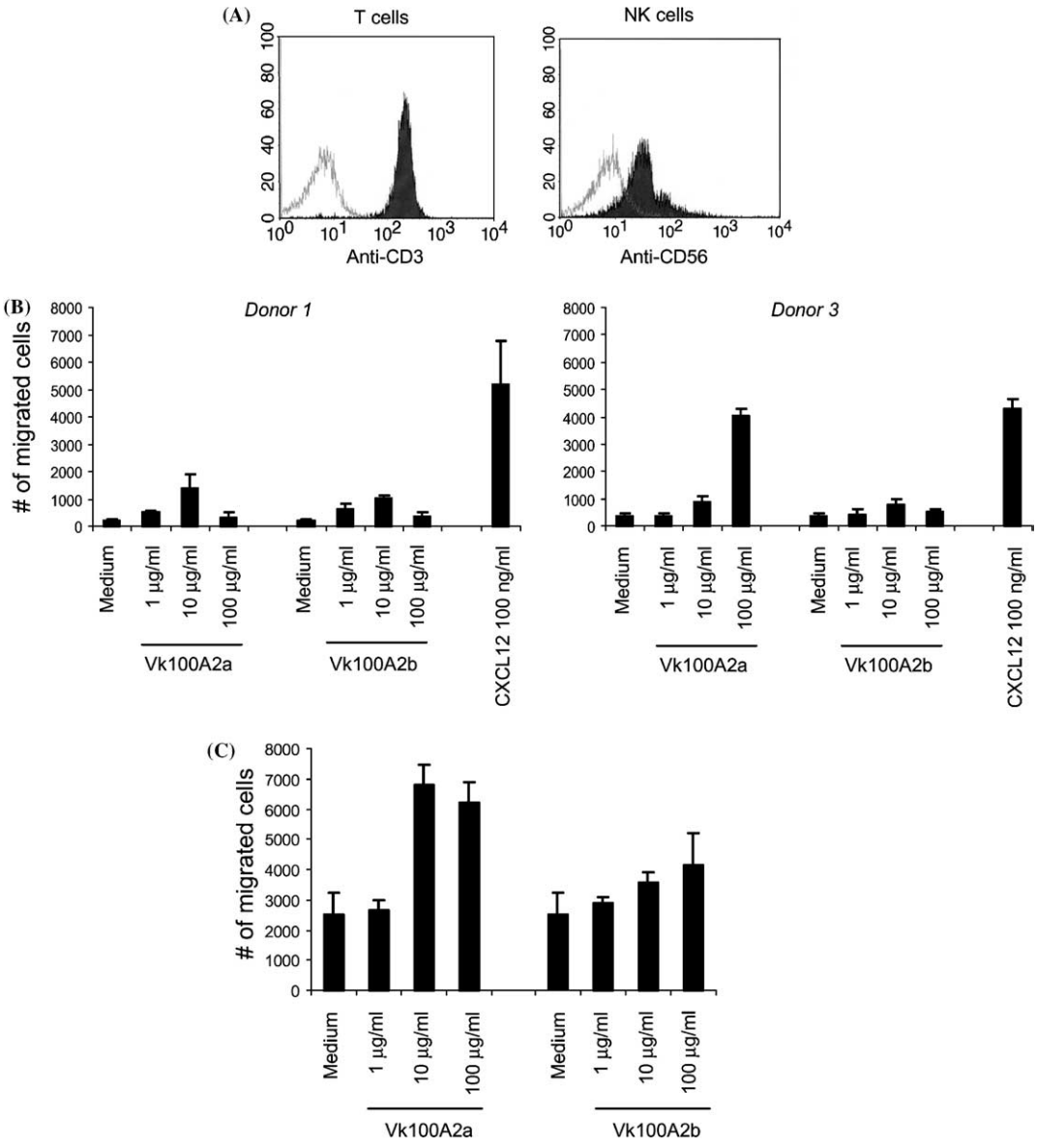

Figure 7. Lymphocytes chemotaxe in response to Vk100A2. The purity of T cells (A, left panel) and NK cells (A, right panel) was examined by flow cytometry. Chemotaxis of T cells in response to increasing concentrations of Vk100A2a and VK100A2b was measured in Boyden chambers as described in Experimental. Two representative donors of five tested are shown (B). Chemotaxis of NK cells in response to increasing concentrations of Vk100A2a and VK100A2b. One representative donor of three tested is shown (C).

product of NO. We were unable to detect nitric oxide release under a wide range of concentrations of both Vk100A2a and Vk100A2b, while the positive control LPS led to some nitrite formation (Fig. 6C).

3. Discussion

In this study we have isolated two very distinctly different polysaccharides from an acidic polysaccharide fraction, Vk100A2, which originated from ion-exchange chromatography of the hot water extract of the powdered roots from the medicinal plant *V. kotschyana*. The polysaccharides denominated Vk100A2a and Vk100A2b are similar in the way that they contain the same types of monosaccharides (Table 1), but there are clear differences regarding the relative amounts (mol %) of the different monosaccharides present, their ability to react with the Yariv-reagent, their molecular weights and their different properties regarding immunological activities. Vk100A2b is a typical pectin, which typically comprise large portions of polygalacturonan regions as the backbone chain, with small portions of rhamnogalacturonan core.[17] Neutral side chains may be attached to C-4 of rhamnose residues but have also been found at C-2 or C-3 of galacturonic acid.[18] The carboxylic groups of about 20% of the galacturonic acids in Vk100A2b are esterified by methanol. The

degree of O-acetylation of the Vk100A2b sugar residues was determined to about 7% (about 11% if calculated as acetylgroups per galacturonic acid residues only). There has been found considerable diversity in the pattern of pectin O-acetyl esterification and the location of the O-acetyl ester residues is not evident. The neutral sugars may also be acetylated,[19,20] although it is generally assumed that the acetyl groups are located on the hydroxyl groups of galacturonic acid present in the pectin backbone.[21] Most commonly reported in pectins are 2-O-acetylated or 3-O-acetylated galacturonic acid residues, mainly in the homogalacturonan regions. Some studies indicate that methyl esterified domains of homogalacturonans are 2-O-acetylated whereas the domains that lack methyl esters are 3-O-acetylated.[22]

The other polysaccharide isolated, Vk100A2a, may be defined as a pectic arabinogalactan or as an acidic arabinogalactan-protein. Pectic arabinogalactans contain a lower amount of galacturonic acids relative to rhamnose residues, and are reported to mainly consist of a rhamnogalacturonan core substituted with neutral sugar chains (ramified region).[23] Vk100A2a fulfil the three criteria that are generally set to define arabinogalactan-proteins (AGP), which are the presence of arabinogalactan chains, together with the ability to precipitate the β-glycosyl Yariv reagent, and finally a typical amino acid composition,[24] with serine (17.1%, mol %), alanine (14.9%) and hydroxyproline (7.4%) (Table 3). The protein part of AGP is generally linked to the arabinosyl and galactosyl residues in the carbohydrate moiety via hydroxyproline.[25] Typically, AGPs contain less than 10% (w/w) protein. Since the protein part of Vk100A2a is very small (<0.5%) we choose to define the polysaccharide as a pectic arabinogalactan.

As the pectin, the pectic arabinogalactan is also esterified, with about 8% of the galacturonic acids esterified by methanol and 11% of the sugars O-acetylated (about 50% if calculated as acetylgroups per galacturonic acid residues only). For both samples the degree of methylesterification is quite low. Recently, bioadhesive properties of pectins have been demonstrated against colonic epithelial membranes with homogalacturonide region of the pectin as the main adhesive structural part. Low degree of esterification was a structural requirement and highly branched polymers had limited effects.[26] Vk100A2b fulfil these structural requirements, and may possess bioadhesive properties. It is suggested that mucoadhesion may protect the epithelia against physical and microbial irritations leading to better healing properties.[26]

The finding of fructose (inulin) in the very high-molecular weight pectic arabinogalactan, Vk100A2a, was quite surprising since the highest degree of polymerisation (DP) of inulin in the crude polysaccharide fraction Vk100A2 was determined to about 50. We therefore expected the inulin present in Vk100A2 to be co-eluted with the low-molecular weight fraction, Vk100A2b. We therefore speculate if oligomers of inulin may interact noncovalently with the pectic arabinogalactan and partly be trapped or intertwined in the long and branched side chains of the polymer. The apparent homogeneity of the molecular weight of Vk100A2a also supports this theory.

Inulinase from *Aspergillus niger* (EC 3.2.1.7, Fluka) was used in order to remove inulin from the samples as described by Azhari et al.[27] with a thermal pre-treatment (60 °C for 2 h) for the removal of contaminating pectolytic activities. Unfortunately, in addition to the breakdown of inulin (a 73% reduction in ketose content), the enzymatic treatment also resulted in the removal of 60% of the arabinose subunits from the Vk100A2a polysaccharides. The complement fixation activity of the inulinase-treated Vk100A2a did not change compared to the original fraction (data not shown). We therefore concluded that the inulin present in the Vk100A2a fraction was not important for the complement fixation activities observed in this fraction.

The crude pectic polysaccharide fraction, Vk100A2, having complement fixation activities and selective B cell proliferation activities was previously isolated from the important Malian medicinal plant, *V. kotschyana*.[2] The present results indicate that the active ingredient both regarding complement fixation (Fig. 2) and mitogenic activities (Figs. 4 and 5) is the pectic arabinogalactan polysaccharide, Vk100A2a.

The most evident differences between the typical pectin, Vk100A2b, which was inactive, and Vk100A2a are the huge difference in molecular weight (20 kDa vs 1150 kDa) and the presence of arabinogalactan type II (arabino-3,6-galactan) in Vk100A2a. According to the linkage analysis the pectin, Vk100A2b, contains only trace amounts of 1,3,6-branched galactose (Table 2), and the negative reaction between the Yariv-reagent and Vk100A2b may be due to the low amount of 3,6-linked galactose in the molecule.

While Vk100A2b seems to consist mainly of smooth region (almost 80% 1,4-linked galacturonic acid) and only small, ramified regions with some side chains, Vk100A2a seems to be heavily branched by neutral sugars, and the relative amounts of rhamnose versus galacturonic acid (1:2.4) may indicate a backbone-chain consisting mainly of rhamnogalacturonan I (RGI) regions.[28,29] Structural analyses of the enzyme resistant parts support the assumption that Vk100A2a consists of a backbone of rhamnose and galacturonic acid, which is heavily branched by arabinosyl and galactosyl units (Table 2).

An almost complete loss of arabinose and no reduction in the amounts of the other monosaccharide residues present in the polymer after the α-L-arabinofuranosidase treatment indicate that the arabinose

subunits are located in the outer part of the molecule. An increase in 1,6-linked galactose and a simultaneous decrease in 1,3,6-branched galactose (Table 2) after enzymatic treatment indicate that at least some of the arabinose side chains in Vk100A2a are linked to position 3 of 1,6-linked galactose. A relative increase in terminally linked galactose may also indicate that arabinose may be linked to the nonreducing end of the 1,6-linked galactan chain or to the 1,4-linked galactan chain since a slight decrease in 4-linked galactose could be observed in the enzyme resistant part of the polymer. Since the amount of 1,3-linked galactose residues remained unchanged, arabinose does not seem to be attached to position 3 of nonreducing terminally-linked galactose in the native Vk100A2a. No change in complement fixation activity could be observed in the arabinose stripped Vk100A2a (Fig. 3), therefore the arabinose side chains present in the outer part of the molecule do not seem to be important for this bioactivity. Previously published reports on α-L-arabinofuranosidase digested pectic polysaccharides from *B. falcatum* and *Angelica acutiloba* and arabinogalactans from *Angelica acutiloba* have shown an enhancement or no change in activity on the complement system.[14,30–32]

Only trace amounts of 1,6-linked and 1,3,6-branched galactose remained after *exo*-β-D-galactanase digestion of Vk100A2a (Table 2). Although the enzymatic procedure partly removed the arabinose subunits, the 1,5-linked arabinose seemed to be quite resistant. The treatment with *exo*-β-D-galactanase only, left about 40% of the 1,4-linked galactose undigested, but with a treatment consisting of a combination of *exo*-α-L-arabinofuranosidase and *exo*-β-D-galactanase only trace amounts of 1,5-linked arabinose could be detected and the amount of 1,4-linked galactose decreased whereas the terminally linked galactose increased. This indicates that the 1,5-linked arabinose chains may be linked to the nonreducing end of 4-linked galactose. Some of the 1,5-linked arabinose present in Vk100A2a-GN (*exo*-β-D-galactanase resistant part of Vk100A2a) also seems to originate from the breakdown of the 3-linkage of the 1,3,5-branched arabinose residues present in the native polymer (Table 2).

The removal of arabinose and the partial removal of galactose residues did not change the complement fixation activities of Vk100A2a (Fig. 3). For the effect on the complement system several reports indicate that the β-3,6-galactan moiety, which is present in arabinogalactans type II, is one of the typical units and may even be essential for expression of the activity.[16] The galactanase resistant part of Vk100A2a still contained 1,3,6-branched galactose, but the amounts were very small (0.2% or 0.3%, mol %) (Table 2) and we are not sure whether the remaining 1,3,6-branched galactose can be responsible for the expression of the potent complement fixation activity. For the type II arabinogalactan chains isolated from a pectic arabinogalactan from *Angelica acutiloba* roots, 6-linked galactosyl chains are suggested to contribute to the expression of the complement activation.[33] According to this study a certain chain length of the galacto-oligosaccharide seemed to be necessary in order to obtain a potent activity. The amounts of 6-linked galactose in the complement-activating arabinogalactan chains from *A. acutiloba* were high, varying from 29.6% to 37.5% (mol %), compared with the amounts (0.4%, mol %) present in the *exo*-β-galactosidase resistant part of Vk100A2a. There may therefore also be other structural characteristics in Vk100A2a that are important for the complement fixation activity.

There is however a wide range of medicinal plant polysaccharides with effect on the complement system, which comprise both pectins, arabinogalactans including pectic arabinogalactans, arabinans and other heteroglycans. Although the majority of the complement-activating polysaccharides reported contains 1,3,6-branched or 6-linked galactosyl residues, there are exceptions like the arabinans and several heteroglycans.[16] A common feature for all active polymers is a backbone with a high degree of branch points, which may make the molecule quite stiff giving a three dimensional structure and a surface that may be important for the activities observed.[34]

Complete removal of arabinose and galactose from Vk100A2a was not obtained by enzymatic digestion with the *exo*-α-L-arabinofuranosidase and *exo*-β-D-galactanase enzymes. Enzymatic digestion of the pectin, Vk100A2b, with *exo*-α-L-arabinofuranosidase or *exo*-β-D-galactanase or a combination of both enzymes was also performed, but no change in the amounts of arabinose or galactose subunits could be detected (data not shown). The fact that enzymatic digestion (*exo*-α-L-arabinofuranosidase and *exo*-β-D-galactanase) does not remove all arabinose or galactose residues seems to be a quite commonly reported problem.[35,36] It seems that there must at least be a minimum amount of the monomers present for the enzymes to succeed in at least a partial degradation. When very low amounts of arabinose and galactose are present it might be necessary with additional enzymes, which are highly specific for the Ara-Rha and the Gal-Rha linkage, respectively, for example, RG-arabinofuranosidases and RG-galactosidases,[37] which to the authors' knowledge are not commercially available.

Since LPS is known as an immunostimulant and probably a contaminant in all plant material, the polysaccharide samples were tested for LPS contamination by the Limulus Amebocyte Lysate test (LAL-test). LPS is known as the major activator of LAL, but other molecules such as β-1,3-glucans may also activate LAL,[38] giving false positive results. Since the results from the LAL-test indicated the highest content of

LPS or LPS-like molecules in the most bioactive fraction, Vk100A2a, we wanted to investigate whether LPS (*E. coli*) could be responsible for or at least partly responsible for the potent effects observed on the complement system. Since relevant concentrations of LPS showed no dose-dependent activity, nor could a synergistic effect of LPS and the polysaccharide, Vk100A2a, be demonstrated (Fig. 2), we concluded that LPS present in Vk100A2a could not be responsible for the dose-dependent complement fixation activities observed. Vk100A2a and Vk100A2b was isolated from the crude polysaccharide fraction Vk100A2 by gel-filtration, and the fact that lipopolysaccharides have a tendency to form aggregates in aqueous solutions with a minimum molecular weight of approximately 1000 kDa,[39] which corresponds approximately to the molecular weight of Vk100A2a, may explain the higher content of LAL-positive substances in Vk100A2a compared to Vk100A2b.

Modulation of T and B cell proliferation is one effect associated with activation of the complement system.[23] In this study Vk100A2a showed a potent dose-dependent induction of B cell proliferation, which was independent of the complement system. The mitogenic activity against B cells was T cell independent, as shown by the activation of B cells in the T cell depleted cell suspension (Fig. 4C). Results from the flow cytometry suggest that Vk100A2a increased the proliferation of B cells, but not of T cells (Fig. 5). Since LPS-low-responsive mice (C3H/HeJ) were used in these assays we did not have to consider the possible effect of LPS present in the samples tested.

The mitogenic activity test clearly demonstrated a difference in the activity of Vk100A2a and Vk100A2b. For the typical pectin of low-molecular weight, Vk100A2b, no mitogenic activities could be observed. It has been suggested by some studies that a combination of the polygalacturonic acid region and the 'ramified' region are essential for the induction of a potent mitogenic activity of the pectic polysaccharides from *Glycyrrhiza uralensis* and *B. falcatum*.[40,41] An antigenic epitope of the *B. falcatum* polysaccharide, bupleuran 2IIc (BR2IIc), is suggested to be 6-linked galactosyl chains containing terminal GlcA or 4-OMe-GlcA attached to β-D-1,3-linked galactosyl chains of the 'ramified' region.[13,42] Even though both polysaccharides isolated from *V. kotschyana* investigated in this study contained the component sugars and sugar linkages that are suggested to be responsible for the mitogenic activity expressed by bupleurum 2IIc, they may differ in detailed carbohydrate sequences. The reason for the pectin, Vk100A2b, not expressing mitogenic activity is not known, but the amount of the relevant sugar residues is low compared to Vk100A2a and they may also differ in the fine chemical structure.

Due to the large molecular weight of Vk100A2a a penetration of the cell membrane is unlikely, and B cell activation may therefore be caused by the surface binding of Vk100A2a to receptors expressed on the B cell surface. Pectic polysaccharides from the roots of *B. falcatum* (BR2IIc)[13,14] and from *G. uralensis* roots[40] have also shown complement activation in addition to selective induction of B cell proliferation. Due to the expression of complement activation, B cell activation may at least partly be mediated through complement receptors (CD19/21 and/or CD21/35) on the B cell surface. Recently, Han et al.[43] reported the complement receptor CD19 to be involved in the induction of selective B cell proliferation by a plant polysaccharide, AK, isolated from the roots of *Acanthopanax koreanum*. Interestingly toll-like receptor 4 (TLR4), which plays a pivotal role in LPS activation of B cells, was also partially responsible for the induction of B cell proliferation by AK. The use of TLR4-deficient B cells resulted in a decreased response to the AK polysaccharide. In addition to CD19 and TLR4, TLR2 and CD79b receptors also seemed to be involved in the activation of B cells by AK, and therefore AK seems to possess broader receptor specificity than LPS.[43] Whether the results from this study are valid for other plant polysaccharides selectively inducing B cell proliferation, remains to be investigated. In our study TLR4-deficient B cells from C3H/HeJ mice were used. We can therefore conclude that for the induction of B cell proliferation by Vk100A2a other B cell receptors than TLR4 have to be involved, possibly CD19, TLR2 and CD79b.

A general activation of the complement cascade results in generation of the chemotactic cleavage product C5a. Through such activation, polysaccharides have shown to be able to increase leukocyte migration in vitro when incubated in the presence of serum.[44] In contrast to chemotaxis mediated by activation of complement, our study showed that both Vk100A2a and Vk100A2b were able to stimulate human leukocyte migration, independent of complement (Figs. 6 and 7). The content of inulin and LPS or LPS-like molecules in the samples did not seem to be responsible for the effects observed, and the results obtained suggest that both Vk100A2a and Vk100A2b may function as chemoattractants to recruit leukocytes. An effect of the polysaccharides may therefore be to increase the rate of leukocyte infiltrations into the wound site, which may play a role in the healing process as recruitment and activity of inflammatory cells is central in wound healing.[45] Wagner et al. have reported an α-glucan from the rhizome of *Urtica dioica* (USP I) to increase leukocyte migration in an in vitro chemotaxis assay.[46] A β-glucan from *Ganoderma lucidum* submitted to a chemotaxis assay increased neutrophil migration.[47] The reasons for the donor variations experienced in our study and the polysaccharide structure versus chemotaxis activity should be investigated further. Both the secondary and tertiary structure of the polysaccharide, whether it has a helix formed

structure, a linear backbone only or a branched structure may be important for the expression of activity.

We also examined another effect of Vk100A2a and Vk100A2b on macrophages, since macrophages are known to be one of the target cells for polysaccharides. During a previous assay a significant but low induction of acid phosphatase in macrophages from C3H/HeJ mice was observed by the crude polysaccharide fraction Vk100A2.[2] In this study the purified fractions Vk100A2a and Vk100A2b were investigated for their ability to induce nitric oxide release from human macrophages. For both polysaccharides we were unable to detect nitric oxide release from macrophages under a wide range of concentrations, while the positive control LPS led to some nitrite formation (Fig. 6C). This result may indicate that Vk100A2a and Vk100A2b are not acting as general activators of human macrophages.

In this study we have isolated a pectin Vk100A2b and a pectic arabinogalactan Vk100A2a with immunomodulating activities. Vk100A2a showed potent activity on the complement system and on B cell proliferation in vitro. Both polysaccharides induced chemotaxis in macrophages, T cells and NK cells. The plant, *V. kotschyana*, is widely used in traditional medicine as a wound healing remedy both for dermal wounds and ulcers,[2] and the potential immunoregulatory activity may have some bearing on this. Leukocytes are not only immunological effector cells against invading environmental pathogens but also a source of growth promoting cytokines, which contribute to the regulation of epithelisation, tissue remodelling and angiogenesis.[4] Wound repair in the gastrointestinal tract also involves a close interplay between cell migration and proliferation,[48] and agents that modulate immune function may therefore have a significant effect both on the reparative process of dermal wounds[6] and gastrointestinal ulcers.[48]

V. kotschyana seems to have a great potential as a medicinal plant, but further studies are needed in order to evaluate its effectiveness as a wound healing remedy.

This study demonstrates the presence of immunomodulating polysaccharides in the roots of *V. kotschyana*. The polysaccharides, especially the pectic arabinogalactan, Vk100A2a, are highly complex compounds and further characterisation is important for elucidation of structure–activity relationships. For the effect on the complement system, this study suggests that carbohydrate structures present in the inner portions of Vk100A2a, which are resistant to both *exo*-α-L-arabinofuranosidase and *exo*-β-D-galactanase digestion, are important for expression of the activity.

Investigation of potentially important carbohydrate sequences for the expression of the potent mitogenic activity are ongoing, for example, by employing specific enzymes like endo-α-D-(1,4)-polygalacturonase and *exo*-β-D-(1,3)-galactanase for partial degradation of Vk100A2a.

4. Experimental

4.1. Material

Roots from *V. kotschyana* were collected near the Baoulé river in the Kolokani area, Mali, in February 1998 and identified by the Department of Traditional Medicine (DMT), Bamako, Mali. A voucher specimen is deposited at the herbarium of DMT. The polysaccharide fraction, Vk100A2, was isolated from *V. kotschyana* Sch. Bip. ex Walp. by hot water extraction and ion-exchange chromatography as described previously.[2]

4.2. Fractionation of the crude polysaccharide fraction, Vk100A2

Vk100A2 (30 mg) was dissolved in a 15 mM NaCl-solution, filtered (0.22 μm, Millex®-GV, Millipore) and applied to a column (2.4 × 85 cm) of Sephacryl S-400, which had been equilibrated with the same solution. The elution pattern was monitored by refractive index and the carbohydrate elution pattern was determined by the phenol–sulfuric acid method.[49] The eluate was fractionated into six fractions, dialysed (MWCO 3.500) and lyophilised. The fractions were investigated for complement fixation activity and the carbohydrate composition was determined. According to the complement fixation activity and carbohydrate composition, the relevant fractions were pooled to give two fractions, Vk100A2a and Vk100A2b (yield from Vk100A2, 5% and 50%, respectively). The intermediate fraction, which consisted of a mixture of Vk100A2a and Vk100A2b, was not investigated further.

4.3. Analysis of carbohydrate content and composition

Samples were methanolysed by 4 M HCl in anhydrous MeOH at 80 °C for 24 h followed by trimethylsilylation as described by Chambers and Clamp,[50] with the modifications described by Samuelsen et al.[7] Mannitol as internal standard was included throughout the total procedure.

4.4. Molecular weight

The approximate sizes of the polysaccharides were determined by size exclusion chromatography on a Superose 6 column coupled to a FPLC-system (Pharmacia, Sweden) as described by Nergard et al.[2] Dextran polymers (Pharmacia) of 2000, 223, 98, 16, 12 and 6 kDa were used as calibration standards.

The polysaccharides were also subjected to size exclusion chromatography/multi-angle laser light scattering (SEC/MALLS) for the determination of weight-average molecular weight as described by Hokputsa et al.[51]

4.5. Detection of type II arabinogalactans

The presence of type II arabinogalactan structures were identified by the interaction of samples (16, 32 or 48 μg) with a β-glucosyl-Yariv reagent (30 μg/mL) in an agarose gel by single radial diffusion as described by van Holst and Clarke.[52]

4.6. Protein content and composition

The protein content of the samples was determined by the protein assay of Lowry et al.[53] using bovine serum albumin (BSA) as standard.

For determination of amino acid composition the sample, Vk100A2a, was subjected to hydrolysis under vacuum in 6 M HCl at 110 °C for 24 h. After removal of HCl under reduced pressure, the amino acid composition was determined using a Biocal JC 5000 automatic amino acid analyser. The analysis was performed by Ola Rumohr Blingsmo at the Biotechnology Centre of Oslo.

4.7. Degree of esterification (methyl ester and O-acetyl groups)

The degree of esterification (methyl ester and O-acetyl groups) was determined by the release of MeOH and acetic acid after treatment of the samples with acid by gas chromatography on a Carlo Herba 6000 Vega series with control module ICU 600, a split-splitless injector and a flame ionisation detector coupled to a PC with software Chrom-Card. Nitrogen was used as carrier gas (25 mL/min). A glass column (2 m × 0.3 mm i.d.) was packed with 10% SP1200/1% H_3PO_4 on 80/100 Chromosorb WAW (Supelco) and the column temperature was 125 °C while the injector and detector temperature was 150 and 190 °C, respectively. The polysaccharide sample (2 mg) was hydrolysed with 1 M HCl (0.1 mL) at 100 °C for 2 h before applied directly on the GC. Propionic acid was used as internal standard.

4.8. Enzymatic digestion of Vk100A2a

4.8.1. *exo*-α-L-Arabinofuranosidase digestion. Vk100A2a (5 mg) in 30 mM acetate-buffer (pH 4.2) (1 mL) was digested with *exo*-α-L-arabinofuranosidase (10 μL) (EC 3.2.1.55 from *Aspergillus niger*, Megazyme, Ireland) and incubated for 72 h at 40 °C; 3 μL of enzyme was supplemented at 36 h as described by Pellerin et al.[54] The enzyme was inactivated by boiling, and the enzymatic digests were separated into a high-molecular weight enzyme resistant fraction (VK100A2a-AF) and a low-molecular weight (LMW) fraction on a PD-10 column with a Sephadex G-25 medium (Amersham Pharmacia Biotech, Sweden), and lyophilised. Only arabinose was detected in the LMW fraction.

4.8.2. *exo*-β-D-Galactanase digestion. Vk100A2a (5 mg) in 30 mM acetate-buffer (pH 4.4) (1 mL) was digested with *exo*-β-D-galactanase (50 μL) (EC 3.2.1.23 from *Aspergillus niger*, Megazyme, Ireland) and incubated for 48 h at 40 °C. The enzyme was inactivated by boiling, and the large enzyme resistant part (VK100A2a-GN) was isolated as described above. Only arabinose and galactose were detected in the LMW fraction.

4.8.3. *exo*-α-L-Arabinofuranosidase and *exo*-β-D-galactanase digestion. Vk100A2a (6 mg) was simultaneously digested with both *exo*-α-L-arabinofuranosidase (10 μL) and *exo*-β-D-galactanase (50 μL) at 40 °C for 48 h in 30 mM acetate-buffer (pH 4.4) (1 mL) and incubated for 48 h at 40 °C. The enzymes were inactivated by boiling, and the large enzyme resistant part (VK100A2a-AFGN) was isolated as described above. Only arabinose and galactose were detected in the LMW fraction.

4.9. Sugar linkage analysis

Prior to methylation, samples containing uronic acids were reduced to primary alcohols on the polymer level. To distinguish between reduced uronic acids and the corresponding neutral sugars in GC–MS, sodium borodeuteride was used. In the methylation procedure free hydroxyl groups in the carbohydrates were de-protonated and methylated, then the glycosidic linkages were hydrolysed and the partially methylated monosaccharides were reduced to alditols and acetylated as described by Kim and Carpita.[55] The partially methylated alditol acetates were analysed by GC–MS Fisons GC 8065 on a SPB-1 fused silica capillary column (30 m × 0.20 mm i.d.) with film thickness 0.20 μm. The injector temperature was 250 °C, the detector temperature was 300 °C and the column temperature was 80 °C when injected, then increased with 20 °C/min to 170 °C, followed by 0.5 °C/min to 200 °C and then to 30 °C/min to 300 °C. Helium was the carrier gas with a flow rate of 0.9 mL/min. E.I. mass spectra were obtained using Hewlett-Packard Mass Selective Detector 5970 with a Hewlett-Packard GC. The compound at each peak was characterised by an interpretation of the characteristic mass spectra and retention times in relation to the standard sugar derivatives.

4.10. Complement fixation assay[56,57]

The effect on human complement was measured by complement consumption and degree of red blood cell lysis by the residual complement using pectic polysaccharides, PMII, from *Plantago major* L. as positive control.[7] The % inhibition of lysis is given by the formula $100 \times ((A_{\text{control}} - A_{\text{sample}})/A_{\text{control}})$. The results were plotted as initial sample concentration when added to the wells versus % inhibition of lysis.

4.11. Mitogenic activity

Mitogenic activity was measured as described previously.[2] Specific-pathogen-free C3H/HeJ female mice (LPS low-sensitive) were purchased from SLC (Shizouka, Japan) and used at 6–8 weeks of age for the spleen cell cultures. B cells were isolated and purified from the spleen cell suspension by a magnetic cell sorting (MACS) system as described by Guo et al.[58] The efficiency of the separation was determined by flow cytometry (FCM), and the cell fraction was shown to be 95% CD45R/B220 and CD19 double positive B cells (data not shown).

Three methods were employed for measurement of proliferation: (a) Cell growth was measured by means of a fluorometric assay, the Alamar Blue™ reduction assay.[13,59] Spleen cells or purified B cells (2 millions of cells/mL) were cultured with samples (10 μg/mL and 100 μg/mL), concanavalin A (ConA, 5 μg/mL, Sigma, USA), bupleuran 2IIc[12,14] (BR2IIc, 100 μg/mL) or anti-IgM F(ab′)$_2$ (10 μg/mL, Jackson ImmunoResearch Laboratories, Inc., USA). Four hours before culture termination 20 μL Alamar Blue™ (Alamar Bio-Science, Inc., USA) was added to each well. The fluorescence intensity was measured by Fluoroscan II (Labosystems Oy, Finland) at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. The delta soft II (Version 4.13 FL, BioMetallics, Inc., USA) was used for data management. The Stimulation Index (SI) was calculated as follows: SI = (relative fluorescence with stimuli)/(relative fluorescence without stimuli). (b) Selective measurement of B cell proliferation in the spleen cell suspension was measured by the Alkaline phosphatase assay (APase assay).[60,61] Spleen cells (2.0 millions of cells/mL) were cultured with samples (10 μg/mL and 100 μg/mL), or bupleuran 2IIc[12,14] (BR2IIc, 100 μg/mL). Cell cultures were terminated by spinning the plates at 400*g* for 5 min. The culture medium was removed by aspiration. Thereafter, 50 μL 1% Triton X-100 and 150 μL 1 mg/mL *p*-nitrophenyl phosphate disodium salt in 0.1 M diethanolamin buffer (pH 9.5) was added to each well. The reaction was terminated by adding 50 μL 3 M NaOH to each well and the optical density at 405 nm was measured using a microplate reader (Bio-Rad, Model 250, Nippon Bio-Rad, Japan). (c) Flow cytometry.[2] Briefly, a spleen single-cell suspension (2.0 millions of cell/mL) was cultivated with samples (100 μg/mL) or bupleuran 2IIc[12,14] (BR2IIc, 100 μg/mL). The surface phenotype of the cells was identified by using monoclonal antibodies (mAbs). Anti-mouse CD16/32 (clone 2.4G2) was added as Fc receptor blocking, and then the cells were stained with fluoroscein isothiocyanate (FITC)-labelled anti-mouse CD3ε (145-2C11), and phycoerythrin (PE)-labelled anti-mouse CD45R/B220 (RA3-6B2) (PharMingen, USA). All cells were analysed by flow cytometry on EPICS ELITE with logarithmic amplifier (Coulter Corp., USA). Lymphoid cells of spleen were gated by the forward- and side-scatter gating method for the analysis of the lymphocyte population. Typically 10,000 cells were analysed.

4.12. Isolation of leukocyte subsets

Buffy coats from healthy human volunteers (The Red Cross Blood Bank, Ullevaal Hospital, Oslo, Norway) were underlaid with Lymhoprep (Nycomed Pharma, Oslo, Norway) in order to generate peripheral blood mononuclear cells (PBMC). After density centrifugation at 1700 rpm for 30 min, interface cells were collected and washed three times with RPMI 1640 supplemented with 2% fetal calf serum (FCS). Monocytes were isolated by adherence for 1–2 h at 37 °C to plastic flasks suspended in AIM-V medium at 7 millions of cells/mL. Nonadherent cells were removed by rigorous washes with RPMI 1640, after which macrophages were generated by incubating monocytes in AIM-V medium containing 40 ng/mL M-CSF for 6–8 days. The cells were fed medium supplemented with M-CSF after 3–4 days. The nonadherent cells were further incubated on nylonwool to remove B cells and residual monocytes. T cells were separated from the nylonwool nonadherent cells after incubation with CD3-coated Dynabeads (Dynal, Oslo, Norway) for 30 min at 4 °C. The T cells were separated in a magnetic field, and incubated overnight in RPMI 1640 containing 10% FCS in order to induce release of the Dynabeads prior to functional analysis. NK cells were generated by incubating the nylonwool nonadherent cells overnight in AIM-V supplemented with 500 IU/mL IL-2. Nonadherent cells were removed, and the adherent NK cells incubated for a further 14 days in AIM-V containing 500 IU/mL IL-2. The phenotypes of macrophages, T cells and NK cells were all confirmed to be more than 90% pure populations by analysis in flow cytometry against their respective markers CD14, CD3 and CD56. Human M-CSF was obtained from R&D Systems (UK). CD14-FITC and CD56-PE antibodies were from Immunotools (Friesoythe, Germany), while the anti-CD3 antibody OKT3 were generated from its hybridoma.

4.12.1. Chemotaxis assays. Chemotaxis of cells was assayed using a 48-well transwell chemotaxis chamber, with either 5 μm (macrophages, NK cells) or 3 μm (T cells) pore-sized polyvinylpyrrolidone-free filters (NeuroProbe, Gaithersburg, MD). The bottom chambers were filled with 28 μL Iscove's Modified Dulbecco's Medium (IMDM) containing 0.5% BSA with or without increasing concentrations of the polysaccharide samples or as positive control the chemokine CXCL12 (100 ng/mL), and medium as negative control. Relevant concentrations of lipopolysaccharide (LPS) and inulin were

also assayed, corresponding to the amounts present in the samples. Cells (1×10^5) in 50 μL were loaded in the upper chambers. After 2 h incubation at 37 °C and 5% CO_2, transmigrated cells were harvested from the bottom chambers and counted in a Bürker chamber. All experiments were performed in triplicate. CXCL12 was purchased from PeproTech (Rocky Hill, NJ), and LPS from *Salmonella typhosa* was from Sigma (St. Louis, MO).

4.12.2. Nitric oxide release from macrophages. 1×10^5 cells were seeded into 96 well plates, and stimulated overnight in duplicates with increasing concentrations of samples and LPS. The plate was afterwards spun and 50 μL of supernatants were transferred to a fresh plate and added 50 μL of Griess reagent (1% sulfanilamide, 0.1% *N*-(1-naphtyl)ethylenediamine and 2.5% phosphoric acid). The samples were left for 10 min at room temperature protected from light, and the absorbance was measured at 540 nm. A dilution series of nitrite ($NaNO_2$) was performed as a standard reference curve.

4.13. Limulus Amebocyte Lysate test (LAL-test)

The polysaccharide samples were tested for LPS contamination by the Limulus Amebocyte Lysate test (LAL-test, Gel-clot method, European Pharmacopeia). The assay was performed by Margrethe Steenberg at the National Institute of Public Health (Norway), and the content of LAL-positive substances was expressed as % (w/w) of LPS-equivalents from *E. coli* (Sigma) in each sample.

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