Characterization of Aloeride, a New High-Molecular-Weight Polysaccharide from *Aloe vera* with Potent Immunostimulatory Activity

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We have characterized a new immunostimulatory polysaccharide called Aloeride from commercial aloe vera (*Aloe barbadensis*) juice. Aloeride is between 4 and 7 million Da, and its glycosyl components include glucose (37.2%), galactose (23.9%), mannose (19.5%), and arabinose (10.3%). At 0.5 μg/mL Aloeride increased NF-kappa B directed luciferase expression in THP-1 human monocytic cells to levels 50% of those achieved by maximal concentrations (10 μg/mL) of LPS. Aloeride induced the expression of the mRNAs encoding IL-1β and TNF-α to levels equal to those observed in cells maximally activated by LPS. Acemannan, the major carbohydrate component from aloe, used at 200 μg/mL in the macrophage assay resulted in negligible NF-kappa B activation. Analysis of acemannan and Aloeride using size-exclusion chromatography suggests that the low activity of acemannan is due to trace amounts of Aloeride. Although Aloeride comprises only 0.015% of the aloe juice dry weight, its potency for macrophage activation accounts fully for the activity of the crude juice.

Keywords: *Polysaccharide; aloe vera; immunostimulatory; nuclear factor kappa B; THP-1 human monocytic cells; acemannan; macrophage*

INTRODUCTION

The genus *Aloe* (Liliaceae) is a shrubby tropical/subtropical plant which has succulent and elongate leaves. Of the more than 360 *Aloe* species known, *Aloe barbadensis* Miller (*Aloe vera* Linne) is the most widely used both commercially and for its therapeutic properties. Commonly referred to as aloe vera, these plants contain two major juice materials: first, a yellow exudate containing a high concentration of anthraquinone compounds that has been used throughout the centuries as a cathartic and for medicinal purges; and, second, a clear mucilaginous gel that has been used since ancient times to treat burns and other wounds where it is thought to increase the rate of healing and reduce the risk of infection (*1* and *2*).

Several chemical components of the aloe gel are thought to be responsible for its wound healing and immunostimulatory properties. For example, the glycoprotein Aloctin A is reported to have antitumor and antiulcer effects (*3*), and a 29KD glycoprotein has been found to increase proliferation of normal human dermal cells (*4*). Clearly, however, the major composition of aloe gel consists of a mucilage of polysaccharide substances. Most of these polysaccharides are glucomannans, mannans, or pectins having a range of molecular weights. The major carbohydrate fraction isolated from aloe gel, "acemannan", consists of a polydispersed β-1,4-linked acetylated mannan interspersed with O-acetyl groups.

One mechanism by which aloe components may enhance wound healing is by activation of macrophages. Macrophages function as phagocytes and debridement agents, and they produce growth factors that influence the angiogenesis stage of wound repair (*5*). The production of cytokines by macrophages requires activation of these cells, and prolonged wound healing times in aged mammals may be symptomatic of impaired macrophage function (*6*). Pharmacological activities of acemannan include antiviral effects (*7*), activation of macrophages (*8*), stimulation of T cells (*9*), and induction of nitric oxide production (*10*). Thus, acemannan may exert some of its therapeutic properties through macrophages. However, very high concentrations of acemannan (e.g., 200 to 2000 μg/mL) are typically required to achieve modest activation of macrophages (*11*). This indicates that either acemannan is not very potent or that trace amounts of a potent compound are present as a contaminant within acemannan preparations.

Preliminary findings from our lab suggested that detectable levels of immunostimulation in crude aloe juice and gel could not be accounted for by known aloe components such as acemannan. Therefore, the objective of this research was to isolate and characterize the compound(s) responsible for this observed immunostimulatory activity of crude aloe vera juice.

MATERIALS AND METHODS

Materials and Reagents. Aloe vera extracts (*Aloe barbadensis*) were purchased from the following sources: Nature's Sunshine Products Inc., Spanish Fork, UT (whole leaf aloe juice, Lot Nos. 9120378A and 9080548A); Lily of the Desert Inc., Irving, TX (whole leaf aloe juice, Lot No. B22908, and aloe gel, Lot No. B25809). Carrington Laboratories Inc. (Irving, TX) provided two different preparations of acemannan: *Aloe vera mucilaginous polysaccharide* (AVMP, Lot No. 11586) and Manapol (Lot No. 116018). Aloe anthraquinones (aloin, aloe-emodin, and emodin) were purchased from Sigma Chemical Co. (St. Louis, MO).

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10.1021/jf001036d CCC: $20.00
Published on Web 12/23/2000

Bacterial lipopolysaccharide (*E. coli*, serotype 026:B6) was obtained from Sigma Chemical Co. THP-1 human monocytes were obtained from American Type Culture Collection (Rockville, MD). LucLite luciferase reporter gene assay kit was purchased from Packard (Downers Grove, IL). NF-kappa B plasmid construct (pBIIXLUC), a gift from Dr. Riccardo Dalla-Favera, contained two copies of NF-kappa B motif from HIV/IgK (*12*). Reverse transcriptase (RT)-PCR kits were obtained from Promega (Madison, WI), and for RNA isolation the TRI Reagent system was used (Molecular Research Center, Inc., Cincinnati, OH). RT-PCR primers for IL-1β, TNF-α, and GAPDH were purchased from Integrated DNA Technologies, Inc. (Coralville, IA).

Isolation Procedure. One L of aloe vera juice (Nature's Sunshine, Lot No. 9120378A) was filtered through a 0.2 -μm membrane prior to use. Fractionation was accomplished by using two consecutive ultrafiltration devices from Millipore (Bedford, MA). Low-molecular-weight material (<100 000 Da) was removed by passing crude aloe juice through a Centriplus-100 concentrator (15-mL volume) with a regenerated cellulose membrane. The retentate was then passed through a Centricon-500 concentator (2 mL volume) with a regenerated cellulose membrane to remove all material with molecular weight less than 500 000 Da.

Final purification of active material (referred to as Aloeride) from the high-molecular-weight aloe fraction (> 500 000 Da) employed size-exclusion chromatography (SEC). The setup consisted of a model 600E system controller, UK6 injector, model 600 solvent delivery system, model 401 differential refractometer, and a model 3396A Hewlett-Packard integrator. Analysis was performed at a flow rate of 1 mL/minute using HPLC-grade water and a Shodex Ohpak KB-800 series SEC column held at 30 °C. The molecular weight of Aloeride was estimated by comparison with retention times of high-molecular-weight dextran standards (Sigma).

Structural Characterization. Carbohydrate composition of Aloeride was estimated using a colorimetric assay based on reaction with phenol (5% w/v in water) and concentrated sulfuric acid. Absorbance was determined at 450 nm and 490 nm (*13*). Glycosyl composition and glycosyl linkage analyses were performed by The University of Georgia Complex Carbohydrate Research Center using traditional methods (*14*–*16*).

Macrophage Assay. Macrophage activation was measured using a luciferase reporter gene assay in THP-1 human monocytic cells. This assay measures immunostimulatory activity as indicated by increased expression of a NF-kappa B-driven luciferase reporter. THP-1 cells were cultured in RPMI 1640 medium supplemented with fetal bovine serum (10% v/v) and amikacin (60 mg/L) at 37 °C, under 5% CO_2 and 95% air. Actively growing cells were transiently transfected using DEAE-dextran (*17*) (10 μg/1 $\times$ 10^6 cells) and the pBIIXLUC reporter plasmid (1 μg/1 $\times$ 10^6 cells) containing two binding sites for NF-kappa B. The transfection solution containing THP-1 cells was incubated for 7 min in a 37 °C water bath. The transfected cells were then resuspended in RPMI 1640 medium containing 10% FBS and plated out in 96-well plates at a cell density of 2 $\times$ 10^5 cells per well. After 24 hours, aloe vera extracts and fractions, and pure Aloeride polysaccharide were added to transfected cells. Four hours after addition of the samples, cells were harvested, and luciferase activity was measured. Cells were harvested using Packard filter plates and lysed using 30 μL of luciferase mix (1:1, LucLite luciferase:1xPBS, 1mM Ca and Mg). Luciferase light emission was measured using a Packard microplate scintillation counter in single photon mode. Activation is reported as a percentage relative to maximal activation of NF-kappa B by 10 μg/mL LPS.

Sequence of PCR Primers. Sequences for the primers were described in Su et al (*18*). IL-1β forward (5′-ATG-GCA-GAA-GTA-CCT-AAG-CTC-GC-3′); IL-1β reverse (5′-ACA-CAA-ATT-GCA-TGG-TGA-AGT-CAG-TT-3′); TNF-α forward (5′-GAG-TGA-CAA-GCC-TGT-AGC-CCA-TGT-TGT-AGC-3′);TNF-α reverse (5′-GCA-ATG-ATC-CCA-AAG-TAG-ACC-TGC-CCA-GAC-T-3′); GAPDH forward (5′-TGA-AGG-TCG-GAG-TCA-ACG-GAT-TTG-GT-3′); GAPDH reverse (5′-CAT-GTG-GGC-CAT-GAG-GTC-CAC-CAC-3′).

RT-PCR for IL-1β, TNF-α, and GAPDH. Actively growing THP-1 cells (3 mL, 1 $\times$ 10^6 cells/mL) were incubated for 2 h: control, LPS at 10 μg/mL, and Aloeride polysaccharide at 10 μg/mL. Total RNA was isolated using the TRI Reagent kit in which cells are lysed using a combination of phenol and guanidine thiocyanate. After the addition of bromochloropropane, RNA is separated into the aqueous phase and subsequently precipitated with 2-propanol. Total RNA recovered using this method is about 30 μg.

RT-PCR reactions were run using kit reagents from Promega. Each reaction used the same components (total volume of 30 μL): 6 μL of AMV/Tfl 5x reaction buffer, 0.6 μL of dNTP mix (10 mM), 1.2 μL of $MgSO_4$ (25 mM), 0.6 μL of AMV reverse transcriptase (5 units/μL), 0.6 μL of Tfl DNA polymerase (5 units/μL), 1.2 μL of each primer (15 pmol/μL), and 2 ng total RNA (IL-1β, TNF-α) or 5 ng total RNA (GAPDH). The RT-PCR protocol used a Techne Unit Progene automatic thermal cycler. The first cycle consisted of 45 min at 48 °C, followed by 2 min at 94 °C. Amplification was achieved using 35 cycles: denature at 94 °C for 45 s, anneal at 60 °C for 1 min, and extend at 68 °C for 2 min. The final cycle held samples at 68 °C for 7 min. Electrophoresis of RT-PCR products (mRNA IL-1β, TNF-α, and GAPDH) was accomplished using 12 μL of reaction mix on 5% polyacrylamide gels and ethidium bromide as the staining agent.

RESULTS AND DISCUSSION

The immunostimulatory properties of aloe vera were evaluated and characterized using a luciferase reporter-gene-based bioassay where luciferase expression is driven by the binding of NF-kappa B. The activation of transcription factor NF-kappa B controls the expression of multiple genes in activated monocytes/macrophages (*19*). Target genes regulated by NF-kappa B include proinflammatory cytokines, chemokines, inflammatory enzymes, adhesion molecules, receptors, and inhibitors of apoptosis (*20*).

This reporter-gene assay was used to evaluate and compare the immunostimulatory activity of crude aloe vera juice and previously known compounds isolated from aloe vera. Three major aloe anthraquinones (aloin, aloe-emodin, and emodin) were inactive at 0.1 μg/mL, 1.0 μg/mL, and 10 μg/mL. At higher concentrations they inhibited NF-kappa B directed luciferase expression (results not shown). Two different preparations of acemannan, the major carbohydrate fraction from aloe, were also evaluated: *Aloe vera mucilaginous polysaccharide* (AVMP) and Manapol. AVMP and Manapol are two different preparations where AVMP is a higher purity grade acemannan (personal communication, Carrington Laboratories). At 200 μg/mL AVMP exhibited 2.2% and Manapol exhibited 3.5% activation of NF-kappa B directed luciferase expression, respectively, as compared with those achieved by maximal concentration of LPS at 10 μg/mL (Figure 1). This agrees with previous reports that very high concentrations of acemannan (200 to 2000 μg/mL) are required to achieve modest activation of macrophages (*11*). Higher levels of macrophage activation can be achieved if acemannan is combined with interferon-γ (*10*).

In contrast, two different lots of crude whole-leaf aloe vera juice (Nature's Sunshine Products) at 200 μg/mL enhanced NF-kappa B activation by about 7% (Figure 1). Comparable levels of NF-kappa B activation were also observed with crude aloe vera juice (4.2%) and aloe vera gel (4.9%) from Lily of the Desert, also run at 200 μg/mL. Because it has been reported (*21*) that acemannan comprises approximately 5% of the dry weight of

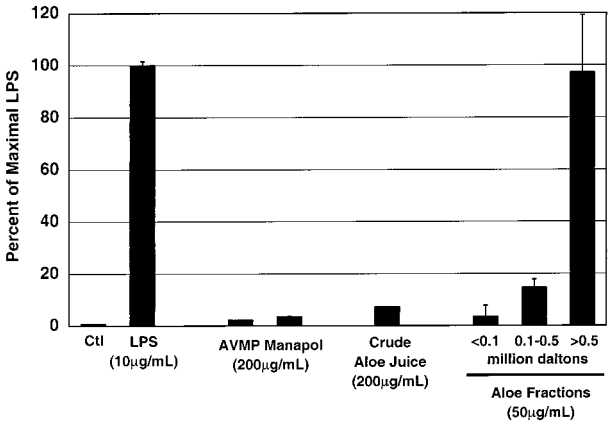

Figure 1. Evaluation and comparison of acemannan preparations with crude aloe vera fractions. AVMP and Manapol cause weak activation of NF-kappa B directed luciferase expression in THP-1 cells at 200 μg/mL. In contrast, the high-molecular-weight fraction (>500 000 Da) from crude aloe vera juice exhibits potent immunostimulatory activity at 50 μg/mL that is equivalent to that achieved by maximal concentrations of LPS (10 μg/mL). Samples run in duplicate in one experiment. Means ± range.

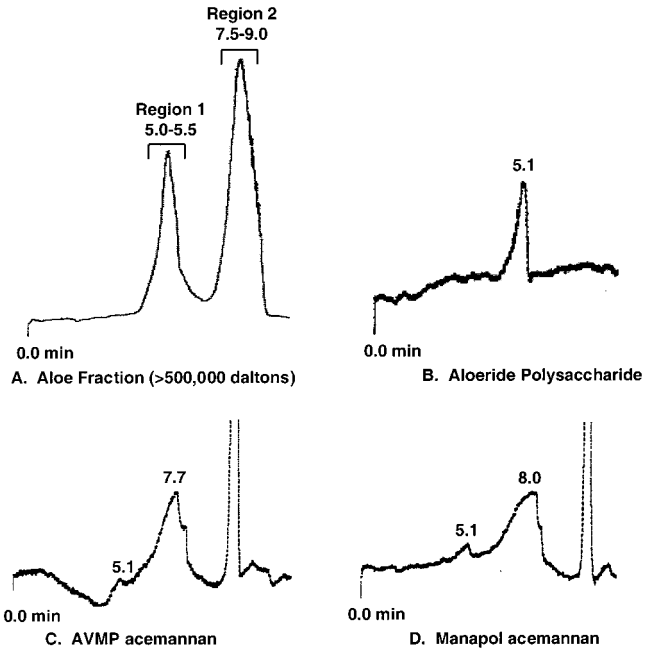

Figure 2. Size-exclusion HPLC chromatograms: (a) aloe vera juice high-molecular-weight fraction (>500 000 molecular weight cutoff), 75 μL injection at 10 mg/mL; (b) purified Aloeride polysaccharide, 20 μL injection at 1.8 mg/mL; (c) AVMP acemannan preparation, 100 μL injection at 1 mg/mL; d) Manapol acemannan preparation, 100 μL injection at 1 mg/mL.

aloe gel, these results suggest that there is another component responsible for the observed level of NF-kappa B activation in crude aloe vera juice.

Initial fractionation of crude whole-leaf aloe vera juice (Nature's Sunshine Products) was accomplished using two different ultrafiltration devices with molecular-weight-cutoff membranes of 100 000 and 500 000 Da. The following three fractions were obtained for evaluation of immunostimulatory activity: first, extract material less than 100 000 Da; second, extract material between 100 000 and 500 000 Da; third, extract material greater than 500 000 Da. The low-molecular-weight material (<100 000 Da) was inactive at 50 μg/mL, and the intermediate-molecular-weight material (100 000−500 000 Da) displayed marginal NF-kappa B activation (15.4%) at 50 μg/mL (Figure 1). However, the high-molecular-weight material (>500 000 Da) contained very potent activity (Figure 1). At 50 μg/mL this fraction increased NF-kappa B directed luciferase expression to levels equivalent to those achieved by the maximal concentration of LPS (10 μg/mL).

The high-molecular-weight material (>500 000 Da) was further fractionated using HPLC size-exclusion chromatography. A typical chromatogram of this fraction (Figure 2a) gave two major peak regions: 5.0−5.5 min (estimated molecular weight between 4 and 7 million), and 7.5−9.0 min (estimated molecular weight between 0.1 and 1 million). Both regions were evaluated for NF-kappa B activation and the first region (between 5.1 and 5.7 min) was at least 10 times more active than the second region (between 7.5 and 9.0 min). The single peak in the first region (referred to as Aloeride) was isolated, through repeated injections (Figure 2b), in sufficient quantity for structural characterization and immunostimulatory evaluation. Purified compound Aloeride is a white powder and is fairly soluble in water at 10 mg/mL. By comparison, acemannan preparations (AVMP and Manapol) are very difficult to dissolve in water even at 1 mg/mL.

Chromatographic analysis of acemannan (AVMP and Manapol preparations) using the HPLC size-exclusion separation system gave a major peak between 6.5 and 8.5 min (Figure 2c and 2d) that agrees with previous studies (*22*). The isolated compound Aloeride (retention time between 5.0 and 5.5) is thus chromatographically distinct from this acemannan preparation. On the basis of these chromatograms it is obvious that the molecular weight of Aloeride (between 4 and 7 million) is much larger than that of acemannan and any previously reported substance isolated from aloe vera. Interestingly, there is also a minor peak with a retention time of 5.1 on the AVMP and Manapol chromatograms (Figure 2c and 2d) that elutes in the same area as Aloeride. If this "contaminant" peak is the same as Aloeride, then it is probable that the weak immunostimulatory activity of acemannan is due to the presence of Aloeride. Pharmaceutical-grade acemannan is typically prepared by a series of alcohol precipitation and centrifugation steps that would obviously not remove minor components that are chemically similar (such as other polysaccharides).

Aloeride represents about 0.015% of the crude aloe juice material (dry weight). This percentage is slightly higher due to the fact that a minor amount of Aloeride "leaked" through the 500 000 molecular-weight-cutoff ultrafiltration device (HPLC chromatogram not shown). Small amounts of Aloeride within the intermediate molecular weight fraction (100 000 to 500 000 Da, Figure 1) would explain its low level of activity. The amount of "inactive" high-molecular-weight material between 0.1 and 1 million Da (second region, Figure 2a) varies between 10% and 70% of the total area of all chromatographic peaks of this fraction, depending on the aloe vera juice lot preparation. This observation is in agreement with previous findings that report between 0 and 1.30 mg/mL of acemannan present in 18 different commercial aloe vera products (*22*). This wide variability may be attributed to depolymerization (hydrolysis) of aloe polysaccharides that can occur in the presence of water and by the action of naturally occurring enzymes

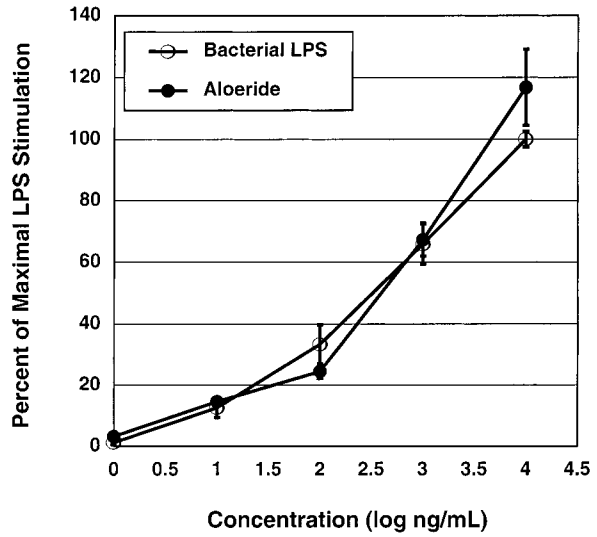

Figure 3. Dose response for Aloeride polysaccharide and bacterial LPS for activation of NF-kappa B in THP-1 monocytes/macrophages at 4 hours. Samples run in quadruplicate. Means ± standard deviation.

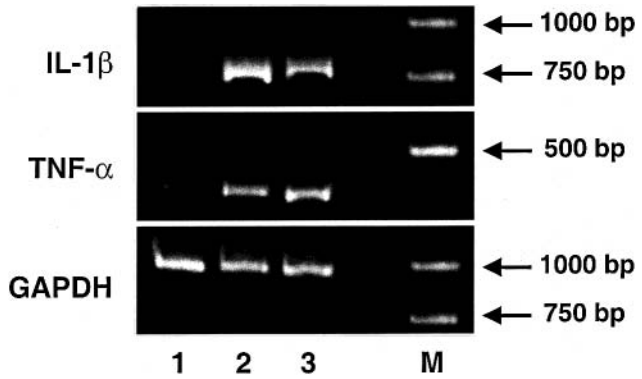

Figure 4. Aloeride polysaccharide enhances proinflammatory cytokine production. RT-PCR results for IL-1β mRNA, TNF-α mRNA, and GAPDH mRNA in THP-1 cells at 2 h: (1) control, (2) bacterial LPS at 10 μg/mL, (3) Aloeride polysaccharide at 10 μg/mL, and (M) PCR Marker.

(*23*). Further research on Aloeride will need to address the issue of its stability and procedures to optimize its concentration. Obviously, aloe formulations containing excessive amounts of interfering high-molecular-weight material will make it difficult to isolate Aloeride.

Figure 3 presents a dose response for both LPS and purified Aloeride. The EC_{50} (50% of maximal LPS induction) value for NF-kappa B directed luciferase expression for purified Aloeride is 0.5 μg/mL. To confirm THP-1 macrophage activation by Aloeride, mRNA levels of proinflammatory cytokines IL-1β and TNF-α were measured using RT-PCR (Figure 4). Treatment of THP-1 cells with either LPS (10 μg/mL) or Aloeride (10 μg/mL) resulted in a dramatic increase of both IL-1β mRNA (810 bp) and TNF-α mRNA (444 bp), as compared with that of the control. The mRNA levels of the housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH, 1000 bp) was the same for all samples (Figure 4).

It is possible that the observed NF-kappa B activation by Aloeride is due to an endotoxin-mediated response. To address this possibility the presence of β-hydroxymyristate was measured in the glycosyl composition analysis. In two separate sample preparations of Aloeride, there were no detectable levels of β-hydroxymyristate. Thus, it is unlikely that the observed macrophage activation by Aloeride is due to endotoxins.

Table 1. Glycosyl Composition and Glycosyl Linkage Data for Aloeride (Data Obtained from One Experiment)

glycosyl residue	mole %	glycosyl linkage	% total area
glucose	37.2	1,6-linked glucose	24.2
galactose	23.9	1,4-linked mannose	19.2
mannose	19.5	3,6-linked galactose	11.2
arabinose	10.3	terminal 1-glucose	7.6
rhamnose	5.8	3-linked galactose	6.4
glucuronic acid	3.3	terminal 1-arabinofuranose	5.1
		terminal 1-galactose	4.5
		1,6-linked galactose	4.3
		3,6-linked glucose	3.8
		1,4-Ara*f* or 1,5-Ara*p*	2.1
		terminal 1-rhamnose	1.8
		1,4-linked glucose	1.8
		4,6-linked glucose	1.4
		3,4-linked galactose	1.3
		3-linked glucose	1.1
		3,4,6-linked galactose	0.9
		terminal mannose	0.8
		4,6-linked mannose	0.8
		3-linked arabinofuranose	0.7
		terminal arabinopyranose	0.6
		4,6-linked galactose	0.4

Using a colorimetric assay (*13*) with phenol−sulfuric acid at 450 nm and 490 nm, the carbohydrate content of Aloeride was estimated at between 80% and 100%. It is concluded from this result that Aloeride is a very-high-molecular-weight polysaccharide. Glycosyl composition and glycosyl linkage analysis for Aloeride polysaccharide are summarized in Table 1. The major glycosyl components are glucose (37.2%), galactose (23.9%), mannose (19.5%), and arabinose (10.3%). For linkage analysis, the major derivatives included 1,6-linked glucose (24.2%), 1,4-linked mannose (19.2%), and 3,6-linked galactose (11.2%). Although the polysaccharide was methylated by a protocol designed to detect uronic acid linkages, no such glycosyl linkages were observed. However, if the glucuronic acid was present as a methyl ester, it would have been destroyed during the methylation procedure. Because of the complex nature of this polysaccharide, having a variety of glycosyl linkages (refer to Table 1), the anomeric configurations for each linkage have not yet been determined.

The structure of acemannan consists of a polydispersed β-1,4-linked acetylated mannan interspersed with O-acetyl groups. The degree of acetylation is about 0.91 acetyl groups per monomer. The ratio between mannose and galactose is about 20:1 (*24*). Although Aloeride polysaccharide contains 1,4-linked mannose (19.2%), it also has a variety of other major sugar units that exist only as minor constituents in acemannan.

A number of other polysaccharides isolated from *Aloe vera* have been published in the patent literature. For example, a polyuronide with a molecular weight between 275 000 and 374 000 is reported to be useful in treatment of surface wounds (*25*). The 70KD polysaccharide, Aloeferon, has also been reported to have therapeutic potential (*26*). Other active components isolated from aloe include a polysaccharide between 420 000 and 520 000 Da comprising equal amounts of glucose and mannose (*27*). In addition, several groups have enzymatically prepared altered polysaccharide compositions from the naturally occurring carbohydrates in aloe (*28*). Clearly, Aloeride is a new polysaccharide that is distinctly different both in molecular weight and glycosyl composition from any of these other aloe polysaccharides that have been isolated or prepared.

In summary, this paper reports the isolation of a new high-molecular-weight polysaccharide (Aloeride) from commercial aloe vera juice that is distinct in both molecular weight and glycosyl composition/linkage from any aloe polysaccharide previously isolated. In the NF-kappa B transcription factor-based bioassay, Aloeride polysaccharide robustly activates THP-1 macrophages. Although this polysaccharide comprises only 0.015% of the original dry weight, its potency in this assay accounts fully for the activity observed in the crude aloe juice. Acemannan exhibited very weak NF-kappa B directed luciferase expression which supports previous observations that very high concentrations of acemannan are required to achieve modest macrophage activation (*11*). On the basis of our results, we propose that the low immunostimulatory activity of acemannan is due to a very potent substance (most likely Aloeride polysaccharide) that is present in trace amounts as a "contaminant". Pharmaceutical development of Aloeride as an immunostimulant, either alone or in combination with other aloe components, may have significant potential for wound healing and immunotherapy. Aloeride polysaccharide could also be valuable for standardization of commercial aloe products, instead of the presently used, and most likely inactive, acemannan.

ACKNOWLEDGMENT

We are grateful to Carrington Laboratories, Irving, TX, for providing reference samples of AVMP and Manapol.

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Received for review August 24, 2000. Revised manuscript received November 14, 2000. Accepted November 14, 2000. This work was supported in part by the U.S. Department of Agriculture, Agricultural Research Service Specific Cooperative Agreement No. 58-6408-7-012. Glycosyl composition and linkage analyses performed by the University of Georgia, Complex Carbohydrate Research Center, was supported in part by the Department of Energy-funded (DE-FG09-93ER-20097) Center for Plant and Microbial Complex Carbohydrates.

JF001036D