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Sulfated Polysaccharides from Red Microalgae Have Antiinflammatory Properties In Vitro and In Vivo

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Abstract

The primary goal of the present research was to determine whether sulfated polysaccharides derived from red microalgae possess antiinflammatory properties when directed against specific parameters of human skin inflammation. These unique biopolymers were studied in both in vitro and in vivo models of skin inflammation. Human subjects were recruited to participate in a study in which the polysaccharide material was applied topically and shown to inhibit cutaneous erythema induced by a known irritant. Leukocyte migration from capillary blood into sites of inflammation is an essential component of the inflammatory process and occurs in a series of steps, two of which are adhesion and chemotaxis. In vitro, the polysaccharide material primarily inhibited the migration of polymorphonuclear leukocytes (PMNs) toward a standard chemoattractant molecule and also partially blocked adhesion of PMNs to endothelial cells. The data obtained strongly suggest that sulfated polysaccharides derived from red microalgae have significant beneficial potential for use in topical products. In addition, the data suggested that the antiinflammatory mechanism for the polysaccharide was, at least in part, due to inhibition of circulating immune cell recruitment toward inflammatory stimuli.

Index Entries: Polysaccharide; algae; chemotaxis; cutaneous inflammation; topical antiinflammatory; dermatitis.

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Introduction

One of the most important issues addressed by the cosmetic industry is that of premature skin aging. As indicated by prior research conducted by our laboratory and others, one of the key factors contributing to premature skin aging is chronic low-level inflammation resulting from environmental stressors *(1–3)*. These stressors include ultraviolet (UV) and infrared radiation, ozone, and bacterial byproducts, which are all capable of initiating inflammatory cascade processes in the skin tissue. One end point of stimulating this inflammatory cascade is the release of metalloproteinases such as collagenase and elastase both from circulating immune cells and from cutaneous keratinocytes and fibroblasts. Chronic activation of these enzymes is believed to contribute to an increased rate of skin aging, as measured by lines and wrinkles, irregular pigmentation, skin laxity, and loss of resilience *(4,5)*.

To reduce the rate of skin aging, most skin care products contain a variety of compounds designed to be nonirritating and "soothing," or modest antiirritants. In the present series of experiments, a new type of ingredient was analyzed for its potential to reduce skin inflammation by measuring the effect of specific sulfated polysaccharides on three parameters important for this response.

Chemotaxis and adhesion of neutrophils to endothelial cells is an essential component of all inflammatory responses. Chemotaxis is the directed movement of a cell toward a chemoattractant molecule and occurs (in the skin) when these chemoattractant signaling molecules are released either from bacteria or from cells stimulated by physical damage, neuropeptides, or UV radiation. Inflammatory cytokines stimulate the expression of adhesion molecules on the surface of dermal microvascular endothelial cells and allow polymorphonuclear leukocytes (PMNs) and T-lymphocytes from the peripheral blood stream to "stick." After contact between the cell types, adhesion occurs, mediated by the specialized surface adhesion molecules. Extravasation then takes place, the movement of the immune cells into dermal and epidermal tissue. An agent that interferes with either directed migration or the binding of complementary adhesion molecules would lead to decreased inflammation. Examples of compounds that are very potent at reducing adhesion by interfering with this binding process are some simple sugars and the sialyl lactose molecule. For example, sucrose, lactose, and dextrose appear to exert their antiinflammatory effects through inhibition of PMN cell adhesion to endothelial cells *(6)*.

In the present set of experiments, balsam of Peru was used to induce an inflammatory erythema. Balsam of Peru is a naturally occurring mixture of resins that exudes as a dark brown, viscous liquid from incisions into a Central American tree, *Myroxylon pereirae (7)*. Its primary component is cinnamein, an essential oil containing cinnamic acid and vanillin. The North American Contact Dermatitis Group found 7.5% of their patients to be allergic to this *(7)*. Balsam of Peru is also thought to be an immediate urticariant.

Materials and Methods

Sulfated Polysaccharide

The test material was supplied by The Institute for Applied Biosciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel. The material referred to as "polysaccharides" was derived from cultures of the red microalgae *Porphyridium*. It was previously observed that during normal growth, cells of the red microalgae are encapsulated within sulfated polysaccharides that ultimately dissolve into the growth medium *(8–10)*. The polysaccharides are composed of 10 sugars, primarily xylose, glucose, and galactose, in addition to glycoproteins and sulfate. The polysaccharide has an apparent molecular mass of from 5 to 7×10^6 Daltons, is negatively charged, and is highly viscous in aqueous solution (approx 970 cP).

Porphyridium cells were cultivated in vertical polyethylene sleeves as previously described *(11)*. The cultures were centrifuged and the polysaccharide was obtained from the medium after dialysis *(12)*. For some experiments, the initial polysaccharide solution was further processed by ultrasonic degradation. The solution (having an original viscosity of 970 cP) was subjected to pulses of energy of 20 kHz, 20,000 cycles/s with a regime of 5 s on, 10 s off. This procedure was carried out at 4°C to obtain the final viscosity levels indicated in Fig. 4.

In Vitro Models of Inflammation

Leukocyte migration has been shown to be one of the first steps in the initiation of an inflammatory/immune response and is essential for accumulation of active immune cells at sites of inflammation *(13)*. Because leukocytes are known to contribute to the erythema and edema characterizing inflammation by the further secretion of proinflammatory cytokines and growth factors, inhibition of leukocyte accumulation is believed to be important in reducing the extent of skin response to irritants and allergens. The chemotaxis assay used to analyze the test material is designed to assess the ability of a test material to inhibit the migration of PMNs toward a known chemotactic agent.

The chemotaxis protocol followed that described previously *(14)*. Heparinized peripheral venous blood (20–30 mL) was collected from healthy human donors (who had been requested to refrain from caffeine intake for the previous 12 h), layered over a density gradient (mono-poly resolving media; ICN, Costa Mesa, CA), and spun at 400*g* for 30 min. The PMN-rich fraction was removed and red blood cells were lysed with hypotonic saline. The PMNs were washed twice with Hank's balanced salt solution (HBSS) and then resuspended in 5.0 mL of HBSS with ions supplemented with 0.4% bovine serum albumin (BSA) (Sigma, St. Louis, MO). The concentration of cells was adjusted to 10×10^6 PMN/mL. Collected PMNs were >95% pure and 98% viable as assessed by the trypan blue exclusion assay.

The assay was performed using the Boyden chamber apparatus with blind well chambers fitted with 5-µm pore size filters (Millipore, Bedford, MA). The apparatus consists of two vertical chambers separated by a filter that contains pores of a size chosen such that the holes are large enough for the cells to actively crawl through them but not so large that the cells can physically fall through into the lower chamber. PMNs were then preincubated ± polysaccharide at the indicated concentrations. A 200-µL PMN cell suspension was layered on the top of the filter, and 100 µL of chemotactic factors was added to the lower compartment. The chemoattractant used was 10^{-7} f-Met-Leu-Phe (fMLP) (Sigma). Following incubation at 37°C for 90 min, under a humidified atmosphere with 5% CO_2, the filters were fixed with propanol and stained with hematoxylin and eosin. The PMN chemotactic response was determined by the distance to the leading front and the number of cells that migrated to the front. The distance to the leading front was determined at ×400 magnification by the distance the majority of the cells migrated through the filter. The results were expressed as the average number of cells per high-powered field (HPF) at the leading (migratory) front (PMN/HPF).

Adhesion of PMNs to human dermal microvascular cells is a required step in the recruitment of leukocytes into the site of infection or irritation. Human dermal microvascular endothelial cells (HDMECs) were obtained from Clonetics and maintained according to specifications until confluent. PMNs were collected as just described for chemotaxis and resuspended in HBSS with ions supplemented with 0.4% BSA (Sigma). The concentration was adjusted to 10×10^6 PMN/mL in the incubation medium. Collected PMNs were >96% pure and 98% viable as assayed by the trypan blue dye exclusion assay. PMNs were incubated for 30 min with the test material before being placed on the endothelial cells. Polysaccharide at the concentrations used did not affect viability of PMNs or endothelial cells. In preliminary experiments, the optimum concentrations of stimulatory agents (10 U/mL of interleukin-1β [IL-1β] and 5 ng/mL of tetradecanoyl phorbol acetate [TPA]) were determined.

After incubation (30 min) with the polysaccharide plus TPA (5 ng/mL), polysaccharide alone, TPA alone, or vehicle, PMNs (350,000/well) were added to the wells of a 96-well microtiter plate in which endothelial cells had been allowed to reach confluence. Endothelial cells had been preincubated with IL-1β (10 U/mL) for 60 min at 37°C in 5% CO_2. After the two cell types had been in contact for 2 h, the supernatant was removed, the remaining cells were gently rinsed, and 100 µL of 0.25% rose bengal (ICN) stain in phosphate-buffered saline (PBS) was added for 5 min at room temperature. Nonadherent cells were removed by two subsequent washes (Medium 199 with 25 m*M* HEPES and 10% fetal bovine serum). Stain incorporated into cells was released by the addition of 200 µL of ethanol:PBS (1:1). After 30–45 min, the wells were read in an enzyme-linked immunosorbent assay reader (Bio-Tek; Winooslei, VT) at 570 nm. The level of adherence was given as the mean optical density (OD) reading at an OD_{570} for

wells containing endothelial cells plus PMN minus the mean OD_{570} of wells containing endothelial cells alone.

In Vivo Model of Inflammation

Skin responsiveness to topical balsam of Peru was used as a marker for skin irritation *(15,16)*. Female volunteers who had a history of skin sensitivity to balsam of Peru, ages 21–55, were recruited and selected using the following criteria: no evidence of acute or chronic disease, including dermatologic or ophthalmologic complaints. The test sites were examined and required to be devoid of warts, nevi, moles, sunburn, suntan, scars, and active cutaneous lesions. Pregnant or lactating volunteers were also excluded. Written informed consent was obtained from each volunteer before entrance into the study. On the day of the test, volunteers were instructed to refrain from using any lotions, creams, or other topical products on their volar forearms.

To initiate the assay, polysaccharide or vehicle was applied to selected test sites on the volar forearm. The material was allowed to remain for 20 min, and then balsam of Peru (8% [w/w] in petrolatum) was applied on the test sites (4 mg/cm^2). On sensitive individuals, redness appeared within 20–40 min, at which point the material was wiped off with a wet towel. Skin irritation was determined from an increase in skin redness as measured by a Minolta chromameter (Minolta, Ramsey, NJ, USA). Polysaccharide-treated sites were compared with vehicle-treated sites (negative control) and with sites treated with balsam of Peru alone (positive control). For comparison to a material previously found to reduce erythema induced by balsam of Peru, one site was treated with *Cola nitida* (10% in water:ethanol [1:1]), a powdered extract of the cola nut species *C. nitida* (Indena); the data obtained from those sites is reported in Results.

Results

In the first set of experiments, it was critical to determine whether the polysaccharide material had any effect on either the general viability of PMNs or on their intrinsic mobility. Trypan blue staining revealed that there was no change in viability (data not shown). PMNs treated for 30–60 min with either the vehicle or polysaccharide in the absence of fMLP had a migratory front measurable at 65 µm, which is typical under non-stimulated conditions in which cells are allowed to undergo random movement. This also demonstrated that polysaccharide did not enhance PMN movement or act as a chemoattractant molecule itself. Cells that do not migrate to the leading edge were not enumerated and reported but were always noted in order to confirm the viability of the PMNs. Because every experiment must be performed with "fresh" PMNs from a different human donor, differences in intrinsic mobility do occur. For that reason, a representative experiment from over six experiments is shown in Fig. 1. Concentrations of polysaccharide from 0.005% (w/v) to 1% were incubated

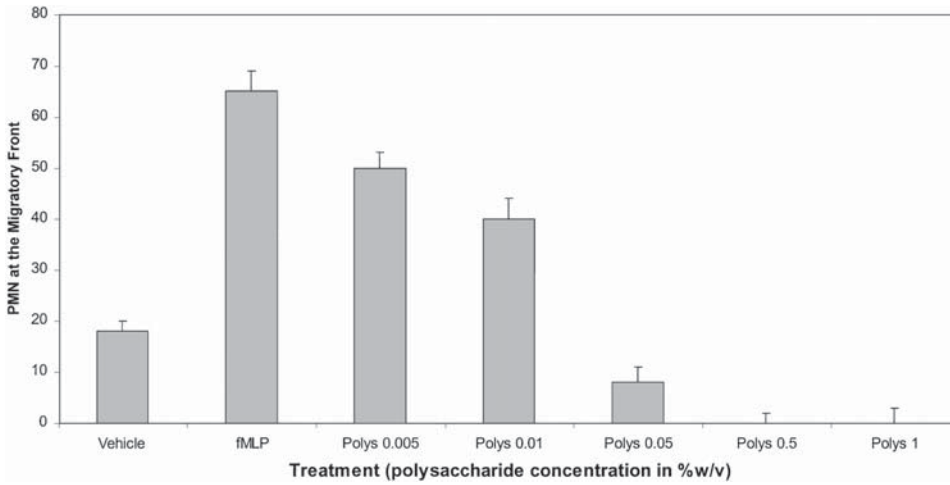

Fig. 1. Effect of polysaccharide on fMLP-induced chemotaxis of PMNs. PMNs were treated with the chemoattractant fMLP plus vehicle, or the indicated concentrations of polysaccharide (% [w/v]). Inhibition of chemotaxis is shown by decreasing numbers of PMNs at the migratory front, determined by the leading edge of cells in untreated wells. Each bar represents the average of triplicate determinations ± SD.

with PMNs prior to placing the cells in a Boyden chamber (upper) with the lower chamber either containing or devoid of fMLP (the latter data were discussed earlier but are not shown). It is evident in Fig. 1 that the presence of polysaccharide inhibited the movement of PMNs toward fMLP in a dose-related fashion. At a concentration of 0.5% or greater, no cells were detectable at the migratory front. Therefore, inhibition of chemotaxis by polysaccharide concentrations of 0.005, 0.01, 0.05, 0.5, and 1% were 23, 38, 87, 100, and 100%, respectively.

The ability of polysaccharides to inhibit activated PMN adherence to activated endothelial cells was then determined. Because every experiment is performed with PMNs from a different donor, the results of adhesion are summarized from three representative experiments. Each data point represents the average of three separate experiments. Data for each test condition were first compared to the maximally stimulated vehicle group for that experiment and expressed as percentage of maximum adhesion before combining all three percentages for Fig. 2. As shown in Fig. 2, on average, polysaccharide concentrations of 1% were required for significant inhibition of adhesion. Figure 3 shows the variation in response to the polysaccharide between individual donors, represented by data from two separate experiments performed with different donor PMNs. Under conditions in which both cell types were maximally stimulated (top two data lines in Fig. 3), increasing concentrations of polysaccharide resulted in increasing inhibition of adhesion as measured by absorbance at 570 nm. Figure 3 includes data showing that the adhesion that occurs in the absence of activation (by TPA or IL-1β) was not affected by any concentration of polysac-

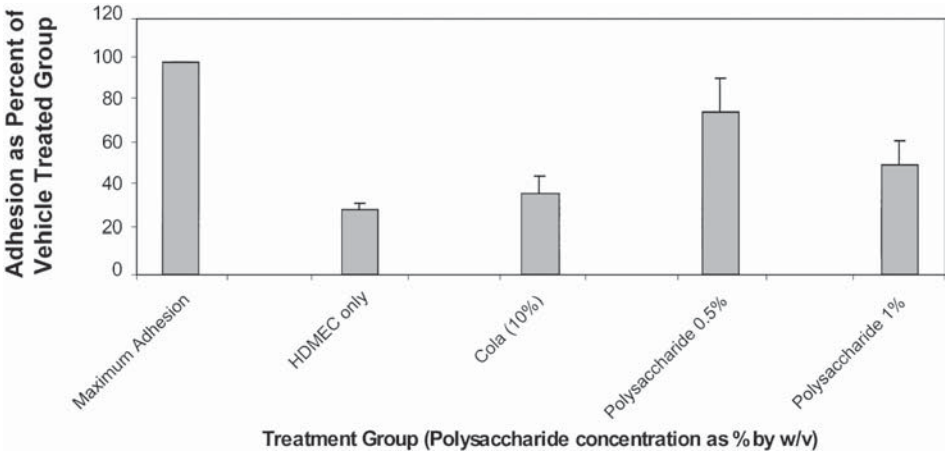

Fig. 2. Effect of polysaccharide on adhesion of PMNs to HDMECs. PMNs and HDMECs were stimulated by IL-1β and TPA, respectively, except for cells represented by the "HDMEC only" group. HDMEC only represents the unstimulated condition in which no PMNs adhere to HDMECs and would be the level at which the test groups would obtain if there were 100% inhibition of adherence. The control group represents the fully activated cells that were pretreated with vehicle alone. PMNs in the other two groups were pretreated with polysaccharide at the concentrations indicated. Each data point is the average of three experiments ± SD.

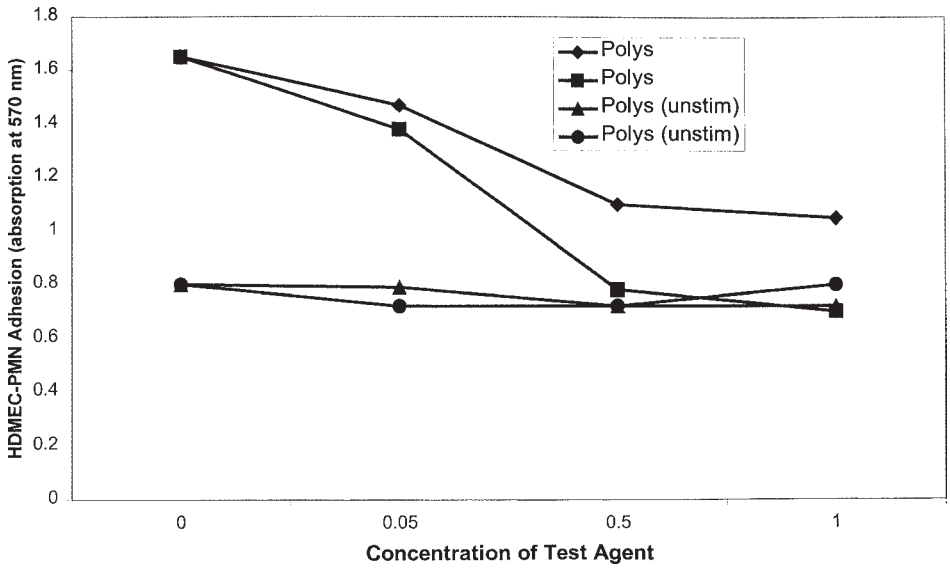

Fig. 3. Effect of polysaccharide on adhesion of PMNs to HDMECs. Two separate experiments are shown, each conducted with PMNs and HDMECs under both stimulated and nonstimulated conditions, treated with 0, 0.05, 0.5, or 1% (w/v) polysaccharide. (—◆—) and (—▲—), Data obtained from donor no. 1; (—■—) and (—●—), data obtained from donor no. 2.

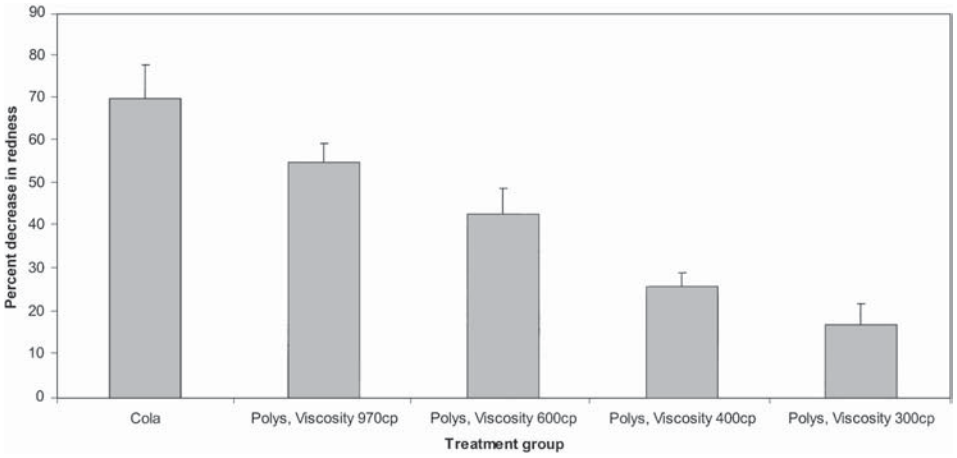

Fig. 4. Inhibition of balsam of Peru–induced inflammation by topical polysaccharide. Human volunteers were treated with either a known topically active antiinflammatory agent (cola) or a 1% solution of polysaccharide in order to determine the degree to which erythema induced by balsam of Peru could be reduced. Experimental groups are indicated by the viscosity of polysaccharide fractions used to pretreat the test area. The data are portrayed as percentage decrease in redness (compared with untreated control) as measured by a Minolta chromameter.

charide tested. This is demonstrated by the bottom two data lines that remain constant at approx 0.8 absorbance at 570 nm. These results indicate that polysaccharide does have modest inhibitory effects on the ability of PMNs to adhere to activated endothelial cells, although at concentrations higher than were necessary to inhibit PMN chemotaxis. Figure 3 also illustrates that although there is individual variation among human subjects regarding the adhesion response and inhibition, the polysaccharide was always effectively inhibitory at concentrations of 0.5% and higher.

Figure 4 represents the data obtained from a representative clinical irritancy study in which polysaccharide was applied to a site on the ventral forearm prior to treatment with a standard inflammatory agent. The polysaccharide was always applied as a solution of 8% (w/w) in petrolatum at a concentration of 4 mg/cm^2. A solution of cola was used as a positive control to compare the efficacy of polysaccharide to a known topical antiinflammatory agent. Pretreatment with native polysaccharide resulted in a 55% decrease in redness, as measured by a chromameter. In additional experimental groups, aliquots of polysaccharide broken by ultrasonic exposure and having lower viscosity were also used to pretreat test sites. These experiments showed that solutions containing nonfragmented polysaccharide were more effective at reducing erythema than solutions with lower viscosity, and presumably shorter sugar chain lengths. Viscosity correlated directly with the ability to inhibit balsam of Peru–induced irritancy.

Discussion

Skin inflammation is accompanied by leukocyte infiltration, which occurs via a cascade of events, including adhesion of leukocytes to the capillary endothelial cell wall and migration into the interstitial space. Leukocyte infiltration is necessary for antiinfectious processes and wound healing but can also produce tissue injury. For example, tissue damage caused by a chronic inflammatory condition is seen in psoriasis, allergic contact dermatitis, and atopic dermatitis. It is therefore desirable to develop topical agents that antagonize overexuberant acute or chronic inflammation. In previous work, e.g., extracts of green tea have been shown to reduce parameters of photodamage relevant to those studied in the present work *(17,18)*.

Selectins, a family of cell adhesion molecules, mediate leukocyte–endothelial cell adhesion by virtue of their recognition of and binding to sialylated derivatives of the Lewis X oligosaccharide. It was previously found by our laboratory that certain carbohydrates can induce a dose-dependent inhibition of leukocyte migration toward a chemoattractant as well as inhibition of leukocyte binding to endothelial cells *(6)*. It was for these reasons that the sulfated polysaccharides from red microalgae were considered as a potential antiinflammatory material.

Together, the results of our investigation indicate that the test material, a sulfated polysaccharide obtained from red microalgae, should be considered an effective antiinflammatory for topical use. In human subjects, the polysaccharide inhibited the development of erythema induced by a powerful irritant. In vitro, polysaccharide inhibited two aspects of cutaneous inflammation: recruitment and adhesion of PMNs. The in vitro models used are considered to mimic cytokine-induced inflammation, as occurs, e.g., from exposure to UV radiation. Because leukocyte aggregation at the site of inflammation is known to contribute to additional cytokine release and nitric oxide induction, both factors involved in increasing blood vessel permeability and extravasation, this suggests that inhibition of leukocyte involvement may be, at least in part, the mechanism of action for polysaccharide's antiinflammatory effect.

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