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Melanin: dietary mucosal immune modulator from *Echinacea* and other botanical supplements

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Abstract

The agents responsible for the therapeutic effects of many botanical supplements have not been established in spite of their popularity. Here we show that melanin is a previously unrecognized immunostimulatory compound that is a major component of botanicals traditionally used to enhance immune function. While melanin is present in commonly consumed vegetables, its specific activity is several orders of magnitude less than melanin extracted from these botanicals. The major reason that this agent has eluded detection is its solvent-specific requirement for extraction/solubility. Melanin activates NF-kappa B in monocytes in vitro through a toll-like receptor 2-dependent process. Ingestion of melanin by mice for four days increases production ex vivo of interferon-γ by spleen cells and IgA and interleukin-6 by Peyer's patch cells. The identification of this new class of mucosal immune stimulants will allow further characterization of botanical products and advances our understanding of the basis for their traditional use.

Keywords: Plant melanin; Toll-like receptor; Monocyte; Mucosal immune; Pyrolysis–GC–MS

1. Introduction

A complex network of cells and cellular structures known as the mucosa-associated lymphoreticular tissues (MALT) has evolved within higher organisms to protect mucosal surfaces from invasion by pathogens. This interconnected system of inductive and effector sites includes systems within the gastro-

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intestinal, respiratory and nasal systems [1]. The recognition of pathogens by cells within inductive sites is mediated at least partly by a family of pattern-recognition receptors called toll-like receptors (TLR). These receptors recognize specific pathogen-associated molecular patterns and allow cells within the innate immune system to distinguish self-molecules from pathogen-associated non-self structures [2]. Ligands for these receptors include bacterial components such as lipopolysaccharides (LPS), flagellin, lipoproteins and DNA, as well as fungal components and double stranded viral RNA.

One of the major factors influencing mucosal immunity is alteration of gut micro-flora. This is most dramatically seen in the germ-free mouse. Compared to conventional animals, germ-free mice exhibit suppressed immunological characteristics such as decreased lymph node, spleen and Peyer's patch size, reduced mucosal IgA production, decreased blood clearance of microorganisms and delayed immune response after antigenic challenge [3].

Experimental evidence suggests that dietary components can also influence mucosal immunity. Increasing the amount of fermentable fiber in the diets of laboratory animals has been shown to alter the type and function of various immune cells in different regions of the intestinal MALT. Animals fed this diet exhibited more CD8+ cells in their Peyer's patches and CD4+ cells in their mesenteric lymph nodes [4], decreased CD4+:CD8+ ratios in spleen [5], increased IgA positive cells and IgA secretion in cecum [6], and increased IgA in spleen and mesenteric lymph nodes [7]. It has been suggested that fermentable fiber acts indirectly to enhance these immune parameters through mechanisms such as the alteration of gut micro-flora, by enhancing short chain fatty acid production from fiber fermentation, or by modulation of mucin production [8]. It also appears that some dietary components may act directly on various immune cells populating the gut. For example oat β-glucan stimulates macrophage interleukin-1 (IL-1 β) production and spleen cell production of interleukin-2, interferon-γ (IFN-γ), and interleukin-4 in vitro [9].

Components of several botanical herbal supplements purported to enhance immune function also appear to act directly on immune cells. For example polysaccharides extracted from *Platycodon grandiflorum* [10] and safflower petals [11] activate macrophages in vitro via a TLR4-dependent pathway. The interaction of these dietary constituents with TLR on cells within intestinal inductive sites may "mimic" the interaction of bacteria and fungal components with these receptors and could influence mucosal immunity in a similar fashion.

In this communication, we report that the polymeric material melanin, isolated from various botanical supplements, activates monocytes through a TLR2-dependent process and enhances several immune parameters when ingested by mice. Evidence that led to the discovery of this previously unrecognized immune enhancing compound was initially obtained in studies with the popular botanical *Echinacea*. In these experiments, substantial activation of monocytes was observed when finely ground *Echinacea* plant material (but not plant material from seven other botanicals) was added in suspension to these cells. The only solvent that was found to quantitatively extract this activity from this material was aqueous phenol. The inability to extract melanin using solvents normally used in natural product chemistry laboratories is one of the main reasons this highly active material has eluded detection.

2. Materials and methods

2.1. Melanin extraction and purification

Plant material was extracted twice with 90% aqueous phenol for 30 min at 70 °C and active material was precipitated with two volumes of ethyl acetate. The precipitate was washed three times with ethyl acetate and three times with isopropanol. The pellet was redissolved in 90% phenol at 70 °C, and undissolved material removed by centrifugation. The phenol layer was then partitioned against equal volumes of water. The water partition was conducted at 70 °C and repeated until the top water layer was clear. A protein-free melanin fraction precipitates out of the phenol during the water partitions. Melanin that remains dissolved was precipitated from the phenol layer by addition of two volumes of ethyl acetate and four volumes of ether:acetone (1:5), washed extensively with ethyl acetate and isopropanol and dried under vacuum. For testing in cell culture dried melanin was resuspended in isopropanol. For size

fractionation, melanin was dissolved by heating (98 °C) in a buffer composed of 50 mM Tris, 6 M guanidine, and 50 mM cysteine at pH 8.5. For crude fractionation, the melanin solution is centrifuged in Amicon Ultra-4 (regenerated cellulose) centrifugal filter devices (Millipore). For chromatography, the melanin extract was loaded on a Superose 6 10/300 GL size exclusion column connected to an AKTA purifier (Amersham Pharmacia Biotech). The solvent system consisted of an isocratic flow of buffer at 2 ml min^{-1} and the melanin was detected by monitoring the UV absorbance at 280 nm. Carbohydrate analysis (glycosyl composition) was performed by the University of Georgia, CCRC, and protein analysis was performed by AAA Laboratories, Mercer Island, WA. Synthetic (oxidation of tyrosine with H_2O_2) and *Sepia* melanin was purchased from Sigma.

2.2. Monocyte activation assay

The THP-1 human monocyte cell line was transfected with a luciferase reporter gene construct containing two copies of NF-kappa B motif from HIV/IgK as described previously [12]. Response to a sample is reported as an EC_{50} value and represents the concentration at which light output equals 50% of those achieved with maximal concentrations of LPS (10 μg/ml, *E. coli*, serotype 026:B6, Sigma). For determination of IL-1 β production, THP-1 cells were treated with the indicated concentration of reagent for 24 h and then the medium was harvested for ELISA (Assay Designs).

Monoclonal antibodies to TLR2 (clone TL2.1) and TLR4 (clone HTA125) and control antibody IgG_{2a} (clone eBM_{2a}) were obtained from *e*Bioscience and the monoclonal antibody to human CD14 (MY4) and control antibody ($MsIgG_{2b}$) was purchased from Coulter.

2.3. Mouse treatment

C3H/He male mice (6–10 weeks of age, Harlan Sprague/Dawley) were housed one per cage and food consumption (Research Diets AIN-76A) per day was monitored by weight. Mice (4 per treatment) were fed for 4 days with either AIN-76A alone or AIN-76A mixed with amounts of the melanin preparation to result in intakes of ~10 mg/day (alfalfa sprout) or ~25 mg/day (American ginseng) per mouse. Average consumption per day over the 4 day period for all mice in each group for alfalfa sprout melanin was 11.7 mg/day (range 11.1–12.2) and for American ginseng melanin 27.7 mg/day (range 22.7–32.7). The EC_{50}s for NF-kappa B activation in the monocyte assay for the melanin preparations used in the experiments were 0.25 and 0.5 μg/ml, for alfalfa sprout and American ginseng melanin, respectively. Animal studies were approved by the animal welfare committee at the University of Mississippi.

2.4. Spleen cell IFN-γ production

Mice were sacrificed by CO_2 asphyxiation, spleens removed and crushed between the frosted ends of two microscope slides in PBS. Following the lysis of red blood cells at 4 °C in buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 10 μM EDTA), remaining cells were filtered through a 200 μm nylon mesh. Viability was determined with trypan blue and cells were cultured (0.6×10^6 cells/200 μl medium/well) in RPMI 1640 containing 10% FBS, 2 mM glutamine, and 1% penicillin/streptomycin. After 48 h the cell culture medium was collected and stored at −70 °C for later determination of IFN-γ concentration by ELISA (R&D systems).

2.5. Peyer's patch IgA and IL-6 production

Mice were sacrificed by CO_2 asphyxiation and Peyer's patches were excised from the serosal side of the intestine and teased apart using forceps in ice cold RPMI 1640 medium containing 10% FBS and 1% penicillin/streptomycin (growth medium). Peyer's patch fragments and released cells were layered onto 1 ml of FBS to stand for 10 min, during which time large fragments settled to the bottom of the FBS. The upper cell suspension was washed with growth medium by centrifugation. The large fragments were washed with serum-free medium and then treated with 50 U/ml of type I collagenase (Sigma) in serum-free RPMI media for 30 min at 37 °C with constant rocking and the supernatant was collected. This digestion process was repeated two more times with fresh collagenase in serum-free medium. After each collagenase digestion, the cell suspensions were passed through nylon filters and washed with growth

medium. Cell suspensions from all the steps were pooled, washed three times with growth medium and cell number was counted using flow cytometry. Cell viability was determined by propidium iodide staining. Cells were cultured in 96 well plates (0.4×10^6/well) for 48 h and then the medium was collected and stored at −70 °C for later determination of total IgA (Bethyl Laboratories) and IL-6 (BD Bioscience) by ELISA.

2.6. Plant culture

Micropropagated *Echinacea* plants were derived from accessions donated by The North Central Regional Plant Introduction Station (*Echinacea angustifolia* PI 312814, *E. pallida* PI 597603 and *Echinacea purpurea* PI 061 AMES 23967). The hypocotyl of each seedling was used as an explant to produce a single clone that was cultured on Murashige and Skoog [13] half-strength salt medium supplemented with benzyladenine at concentrations varying according to each species' requirement [14,15].

Alfalfa seeds were purchased from Johnny's Selected Seeds, Winslow, Maine (lot number 25089, Germ/Date 88%, 6/03).

3. Results

3.1. Melanin isolated from Echinacea activates monocytes

To detect and guide the isolation of immunomodulatory compounds within botanicals, we utilized a sensitive in vitro monocyte activation assay, where activation of the proinflammatory transcription factor NF-kappa B drives the expression of the luciferase reporter gene in the widely studied human monocyte cell line THP-1. This assay was used to optimize the extraction and purification of a potent monocyte activating material from the roots of *E. purpurea*. Aqueous phenol quantitatively extracted this activity since the previously active plant material remaining following extraction (marc material) was essentially inactive when tested in suspension in the monocyte assay. Attempts to extract this activity with all other solvents ranging from polar (methanol) to more nonpolar (hexane) were ineffective. In commercially relevant *Echinacea* species, the isolated material constitutes 5–10% of the plant dry weight. Based on its physical properties and structural analysis, the active compound was identified to be a melanin. It is an amorphous, dark-colored pigment (reddish brown and similar to pheomelanin), insoluble in most solvents, bleached by oxidizing agents (H_2O_2), and soluble in alkali and phenol [16]. Elemental analysis indicated 50% carbon, 13% nitrogen, 7% hydrogen, 0.8% sulfur and 0.08% phosphorus. The material contained less than 1% carbohydrate and no detectable fatty acids (including the LPS component 3β-hydroxymyristate). Therefore, this substance is not a lipopolysaccharide or polysaccharide. In addition, monocyte activation by this material was not altered by the lipid A-binding agent polymyxin B, indicating that small amounts of LPS possibly contaminating this extract did not contribute to this activity. Extensive treatment of the material with DNase I, RNase A, proteinase K, trypsin, α-chymotrypsin, pronase E, or nagarse or by heating at 98 °C for 2 h did not reduce its activity in the monocyte assay, indicating that the biological activity was not due to contaminating proteins or nucleic acids.

Pyrolysis–GC–MS has been successfully used in melanin identification and structural analysis [17] and together with other approaches has determined that melanin polymers are based on the indole structural unit [18]. Fig. 1 shows the thermal degradation products resulting from filament pyrolysis–GC–MS of melanin extracted from in vitro propagated *E. angustifolia* plants. High amounts of indole as well as indole derivatives (e.g. 7-methylindole, 3-methylpyrrole) were identified. These results are consistent with melanin pyrolysis products described by others [19,20].

3.2. Melanin activity varies substantially within botanicals and vegetables

Our first experiment to analyze melanin within the different plant parts of several *Echinacea* species revealed differences in melanin content of two- to three-fold, but these differences were minor in comparison to the dramatic variations in the activity of the extracted melanin. For example, we found differences in activity of up to 100-fold between

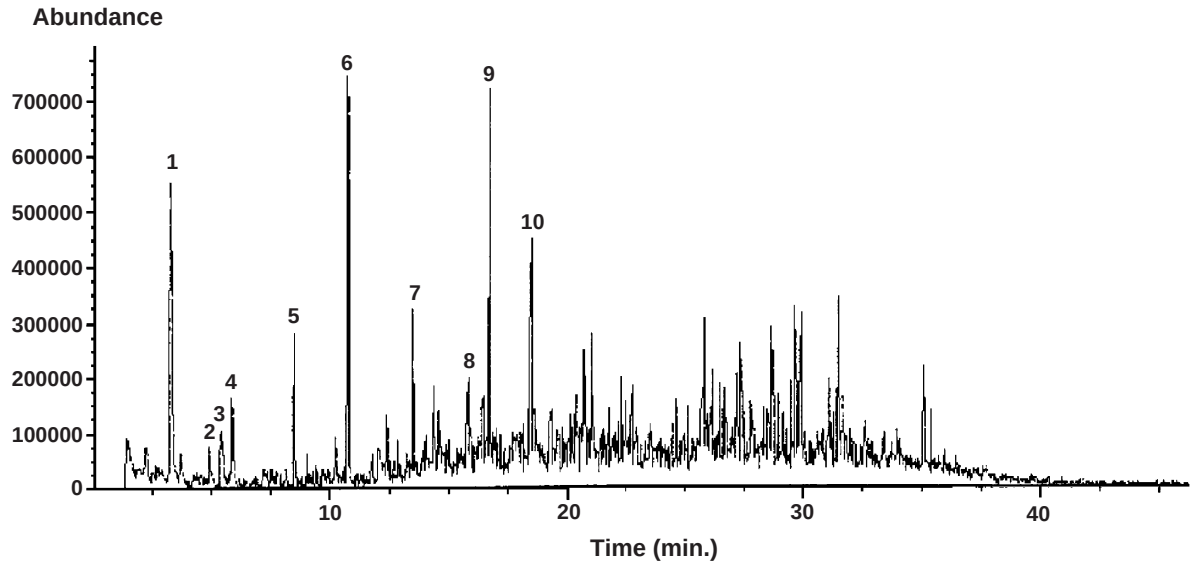

Fig. 1. Total ion chromatogram of melanin pyrolysis products. Melanin was extracted from cloned in vitro propagated *Echinacea angustifolia* plants. The sample was analyzed by pyrolysis–GC–MS using a CDS Analytical, Pyroprobe® 2000 at 700 °C for 10 s with a temperature rise of 10 °C/ms. Compound identification was accomplished by comparison with mass spectra from the Wiley library. Peaks correspond to the following thermal decomposition products: toluene (1), ethylbenzene (2), 3-methylpyrrole (3), styrene (4), phenol (5), 4-methylphenol (6), benzene acetonitrile (7), benzene propanenitrile (8), indole (9), 7-methylindole (10).

melanin extracted from the roots and leaves of individual plants. Similar differences were observed between the same plant part within the same species and also between the three major *Echinacea* species. Since numerous environmental influences could have accounted for these substantial variations, we examined plants propagated in vitro. Among twenty *E. angustifolia*, *E. purpurea* and *E. pallida* clones, some contained half as much melanin than the average but, again, greater differences existed in the activity of this material (EC_{50}=1.6 to >1000 μg/ml). We also examined melanin content/activity in other popular botanicals, as well as in commonly used vegetables to determine if melanin within these plants also exhibited this activity. Since only one to two samples of each botanical were analyzed in this survey, the data indicate only general trends with respect to the types of plants that contain melanin with high activity. The data in Table 1 show that melanin with a high specific activity for monocyte NF-kappa B activation is not found within all plants, and that botanicals traditionally used to enhance immune function contain melanin with the highest activity. None of the melanin extracted from vegetables exhibited significant activity. Percent recovery of melanin preparations from common herbs ranged from 0.5% to 20% and for vegetables 0% to 5% of plant dry weight. As a side note, synthetic melanin was inactive in the monocyte assay and *Sepia* melanin exhibited moderate activity.

3.3. TLR2 is involved in NF-kappa B activation by botanical melanin

Fig. 2a compares the response to several of the more active melanin extracted from botanicals with that of *E. coli* LPS, a known potent activator of monocytes mediated by NF-kappa B. The EC_{50} values for melanin from alfalfa sprouts, American ginseng and *E. angustifolia* clone were 0.1, 0.4, and 1.0 μg/ml, respectively, while that of LPS was 0.25 μg/ml. Fig. 2b confirms monocyte activation by these melanins in that similar to LPS they substantially increased the secretion IL-1 β in a dose-dependent fashion.

Table 1
Activity of melanin preparations extracted from selected herbs and common vegetables

Herbs	EC_{50} (μg/ml)
Alfalfa sprouts (*Medicago sativa*)	0.1
Black walnut hulls (*Juglans nigra*)	0.1
Green tea leaves (*Camellia sinensis*)	0.2
Parthenium integrifolium root	0.3
Korean ginseng root (*Panax ginseng*)	0.4
American ginseng (*Panax quinquefolius*)	0.4
Ginger root (*Zingiber officinalis*)	0.5
Echinacea angustifolia leaf	1.0
Echinacea purpurea root	1.0
Goldenseal root (*Hydrastis canadensis*)	2.7
Red clover blossoms (*Trifolium pretense*)	3.0
Parthenium integrifolium leaf	3.2
Dandelion shoot (*Taraxacum officinale*)	3.2
Black cohosh root (*Actea recemosa*)	3.2
Licorice root (*Glycyrrhiza glabra*)	3.5
Chamomile flower (*Matricaria recuita*)	4.0
Milk thistle seeds (*Silybum marianum*)	4.4
Echinacea pallida root	5.0
Alfalfa herb (*Medicago sativa*)	5.0
Horsetail stems (*Equisetum arvense*)	8.5
Astragalus membranaceus root	9.0
Gotu kola herb (*Centella asiatica*)	15.0
Feverfew herb (*Tanacetum parthenium*)	25.0
Valerian root (*Valeriana officinalis*)	82.0
Hawthorn fruits (*Crataegus monogyna*)	100
Black tea leaves (*Camellia sinensis*)	500
Rosemary leaves (*Rosmarinus officinalis*)	1000
Saw palmetto berries (*Serenoa repens*)	1000
St. John's wort leaves (*Hypericum perforatum*)	>1000
Garlic cloves (*Allium sativum*)	>1000
Ginkgo biloba leaves	>1000
Mahuang herb (*Ephedra sinica*)	>1000
Pau D'arco inner bark (*Tabebuia* spp.)	>1000
Vegetables	
Swiss chard stem	>1000
Red leaf lettuce	>1000
Carrot	>1000
Iceberg lettuce	>1000
Green bean	>1000
Spinach leaf	>1000
Celery stem	>1000
Swiss chard leaf	>1000
Broccoli floret	NA
Cabbage leaf	NA
Tomato	NA
Green bell pepper	NA
Green pea	NA
White jasmine rice	NM
Red potato	NM
Asparagus	NA
Butternut squash	NA
Yellow corn kernel	NA

Notes to Table 1:
Melanin was tested in the monocyte test system at concentrations ranging from 0.1 to 100 μg/ml. Melanin preparations that exhibited less than 20% activation when run at 100 μg/ml are assigned an EC_{50} value of >1000 μg/ml since a doubling of percent activation requires an order of magnitude increase in melanin concentration in this assay system. NA indicates not active at 100 μg/ml. NM indicates no melanin could be extracted. All botanical specimens were identified by a trained plant taxonomist (V. Joshi) and authenticated vouchers deposited in the NCNPR, University of Mississippi.

At present, it is thought that many bacterial components such as LPS and lipoproteins are recognized by the innate immune system due to the binding of these agents to TLR2 and TLR4. The experiment presented in Fig. 2c suggests that TLR2 is involved in monocyte activation by melanin extracted from *Echinacea*, alfalfa sprouts and American ginseng, since antibodies to this receptor suppress activation. Antibodies to CD14 also suppressed activation by these melanins. This is consistent with its role in mediating the action of this TLR [21]. TLR4 antibody was ineffective at suppressing melanin-dependent activation, indicating the specificity of these antibodies. The control IgG fractions for these antibodies ($MsIgG_{2b}$ and IgG_{2a}) were not effective at suppressing activation. Activation by ultra pure *Salmonella minnesota* LPS, a known TLR4 ligand [22], was suppressed by TLR4 antibody, but not by TLR2 antibody.

3.4. Oral intake of melanin enhances immune parameters in mice

IgA secreted by the small intestine prevents the adherence of viruses, bacteria and toxic molecules to the mucosal surfaces and is thought to play a major role in eliminating infectious agents. Fig. 3a shows that IgA production is enhanced from Peyer's patch cells isolated from mice that had consumed either American ginseng or alfalfa melanin for 4 days. The production of interleukin-6 (IL-6) is also enhanced in cells from melanin treated mice (Fig. 3b). Another immune parameter commonly used to assess the immunological impact of orally consumed botanical immune stimulants is the production of IFN-γ from spleen cells. Fig. 3c shows that spleen cells from these mice produced more IFN-γ in culture than cells from untreated mice.

3.5. Alfalfa melanin activity is enhanced by plant defense elicitors

Plant defense strategies against pathogens involves protective mechanisms that are both structural and chemical. Compounds produced by certain soil microbes, microbial cell wall fragments and host-induced endogenous signaling compounds can serve as signals for eliciting plant defense mechanisms. Melanin extracted from alfalfa sprouts that had been treated with chitin, a commonly used elicitor that mimics pathogen or insect exposure [23], exhibited activities in the monocyte assay approximately 10–25 times greater than melanin from untreated sprouts. Plant defense signaling mediators (salicylic acid or methyl jasmonate) increased the activity of the extracted melanin by 3–5 times (data not shown). Fig. 4 shows the time course of the induction of this heightened melanin activity after treatment with chitin. The EC_{50} values for NF-kappa B activation for the purified melanin at the 48 and 72 h time points in these experiments were 50 ng/ml for elicited and 500–1250 ng/ml for non-elicited sprouts (data not shown). Treatment of THP-1 cells with chitin did not influence NF-kappa B activation.

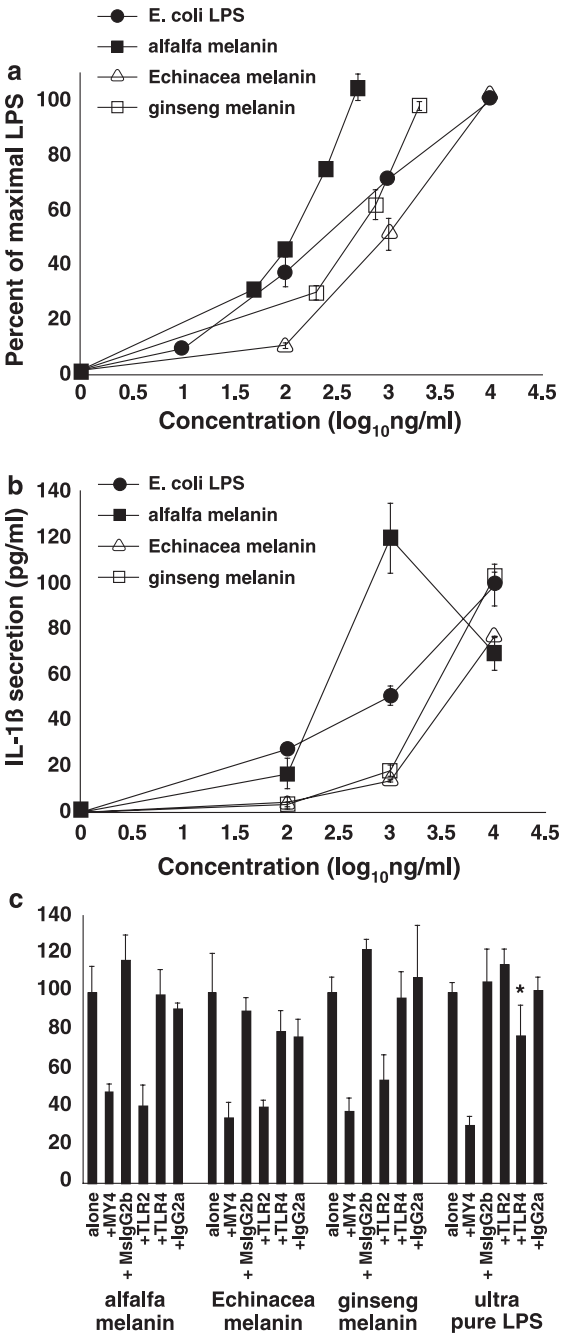

Fig. 2. Response of THP-1 monocytes to botanical melanin. Melanin was extracted from American ginseng root, alfalfa sprouts and cloned *E. angustifolia*. (a) Twenty-four hours following transfection with the NF-kappa B luciferase reporter plasmid, cells were treated with the indicated agents for 4 h. Luciferase activity was determined and is reported as percent of maximal light output from LPS-treated cells. (b) THP-1 cells were incubated with the indicated concentration of melanin or LPS for 24 h. Culture medium was analyzed for IL-1 β by ELISA. Values represent the average of duplicate determinations and error bars represent range. (c) THP-1 cells were treated with antibodies to CD14 (MY4), TLR2, TLR4, or control IgG fractions for these antibodies ($MsIgG_{2b}$, IgG_{2a}) for 30 min. (all at 10 μg/ml) prior to addition of the indicated melanin preparation (0.4 μg/ml and 0.2 μg/ml for alfalfa), or ultra pure LPS (0.01 μg/ml). Four hours later cells were harvested for luciferase assay. The results are the average±s.d. of two experiments with each sample performed in duplicate or triplicate. Statistical differences between treatments with antibody and control antibody were determined by unpaired two-tailed Student's *t*-test (*=$P<0.05$, **=$P<0.01$ and ***=$P<0.001$).

3.6. Melanin activity is irreversibly inactivated by strong base

A procedure commonly used for solubilization and extraction of pheomelanins from various sources is with aqueous solutions containing sodium or potassium hydroxide at concentrations ranging from 0.5 to 3.0 M. *Echinacea* melanin dissolved in 0.5 M NaOH

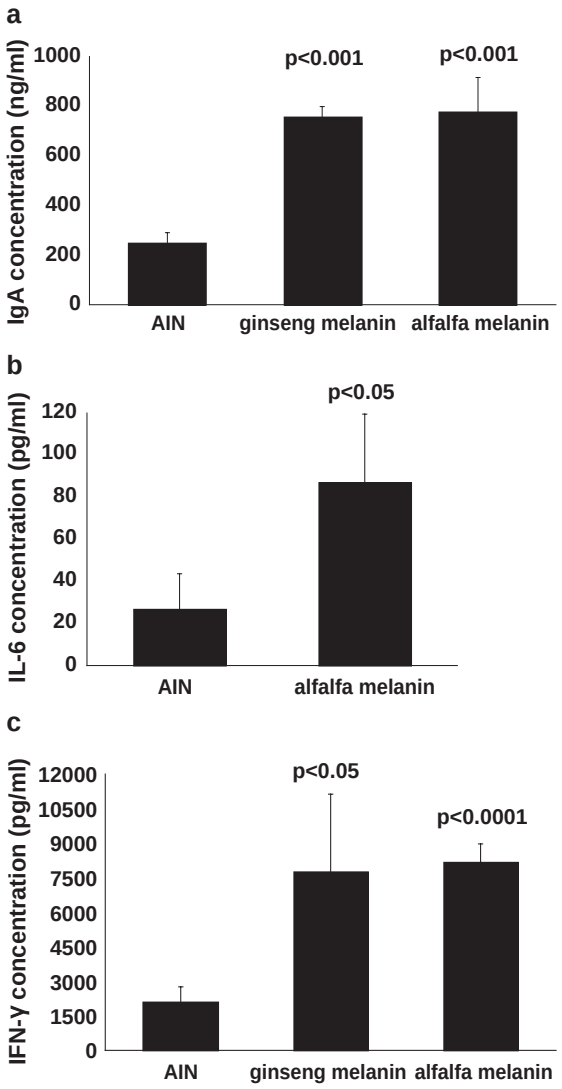

Fig. 3. Effect of dietary melanin on mouse immune parameters. C3H/He mice (four per treatment group) were treated for 4 days with either American ginseng (25 mg/day) or alfalfa melanin (10 mg/day) mixed with chow (AIN-76A) or chow alone. Peyer's patch cells isolated from these mice were cultured for 2 days and the culture medium analyzed for IgA (a) or IL-6 (b) by ELISA. Spleen cells isolated from these mice were cultured for 2 days and the culture medium analyzed for IFN-γ by ELISA (c). Values are average±s.d. and statistical analysis was by a Student's *t*-test.

is inactive in the monocyte assay system. In previous studies on the isolation and characterization of *Echinacea* polysaccharides, aqueous extracts were prepared by an overnight incubation in 0.5 M NaOH at room temperature, followed by ethanol precipitation [24]. These conditions probably extracted the melanin, but in an inactive form. This may explain why this extremely potent immunostimulatory component was missed by these investigators. All melanin preparations that we have tested from other botanicals also exhibit this sensitivity to NaOH. However, botanical melanin dissolved in up to 10% ammonium hydroxide retains activity.

3.7. Melanin activity and molecular weight

Fractionation of solubilized alfalfa sprout melanin using molecular weight cutoff spin columns indicated that approximately 36% of the melanin was over 100,000 Da while 25% fell between 10,000 and 100,000 Da. The larger molecular weight alfalfa melanin fraction had an EC_{50} value of 20 ng/ml while melanin within the smaller molecular weight fraction had an EC_{50} value of 4000 ng/ml. Melanin extracted from American ginseng exhibited similar properties in that material between 30,000 and 100,000 Da (40% of the material) was 10 times less active than melanin over 100,000 Da (2.2% of the material). Similar results are obtained with size exclusion chromatography. Analysis of melanin from

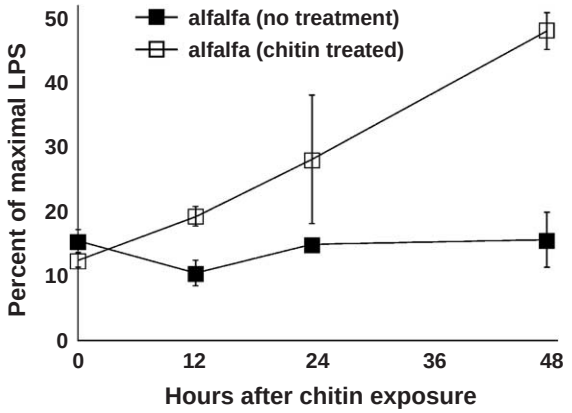

Fig. 4. Time course of enhancement of melanin activity by treatment with chitin. Alfalfa seeds were germinated until approximately 1 in. in length and then treated with chitin (50 mg/ml) for 12 h. The sprouts were grown for the specified time and then freeze dried and melanin extracted. The data represent the average±s.d. of triplicate determinations from one experiment that was repeated three times. Harvest time of 0 h indicates the termination of the 12 h chitin treatment period.

several different botanicals showed that within each plant, melanin ranged in size from less than 10,000 to 1,000,000 Da. Melanin of all sizes was active in the monocyte assay, but material over approximately 100,000 Da was ~10–100 times more active than the lower molecular weight material. This indicates that differences in activity between some melanin preparations is due, at least in part, to differences in the average size of melanin molecules within these preparations.

4. Discussion

Melanin is commonly thought of as the agent that protects numerous life forms from solar UV radiation, but recent studies suggest that this polymer can have diverse functions in various organisms. For example in invertebrates, a major aspect of the innate immune defense system against invading pathogens involves melanin. Within minutes after infection, the microbe is encapsulated within melanin (melanization), and the generation of free radical byproducts during the formation of this capsule is thought to aid in their killing [25]. The production of melanin by pathogenic fungi such as *Cryptococcus neoformans* enhances virulence, protects against antifungal drugs and promotes survival in toxic environments [26]. A recent study indicates that fungal melanin is immunologically active, with evidence for eliciting both humoral and cellular responses [27]. Plant melanin may have structural similarities to fungal melanin and, when ingested, may activate pathways similar to that elicited by pathogenic fungi within the gut. Our research also suggests that plant melanin represents a new class of polymers recognized by the toll-like family of receptors that is structurally distinct from other known ligands (i.e. bacterial DNA, double stranded RNA, lipopolysaccharides, polysaccharides, lipoproteins, etc.).

We have found only one report of an immune-enhancing material extracted from black tea that was said to be "melanin-like." However, no structural evidence was given to support melanin identity, and since black tea is a fermented product, whether the immune enhancing material was derived from the plant material or was of microbial origin is unclear [28]. In addition, we show that melanin from green tea leaves was at least 100 times more active than black tea melanin (Table 1).

Although melanin has been identified previously within plants [29] the functional role of this polymer is not known. The dramatic increase in the immunostimulatory activity of alfalfa sprout melanin following exposure to an elicitor of plant defensive mechanisms suggests that some structural feature of this molecule has been altered. Whether this structural change is important for conferring resistance to infectious agents remains to be determined.

The structural characterization of melanin polymers has been notoriously difficult, due to their general insolubility in most solvents and because the subunits are linked by non-hydrolyzable bonds. Although pyrolysis–GC–MS has been used to some success for melanin identification and structural analysis [17], most melanin preparations are contaminated with proteins [29]. We have found that purified proteins, due to their content of aromatic amino acids, give thermal degradation product signatures similar to that of plant melanin but degradation product yield is less than 10% of that seen with melanin (data not shown). The melanin purification procedure described in Materials and Methods will separate a protein-free melanin fraction (as determined by amino acid analysis of acid hydrolyzed material) from the bulk of the melanin. Our protein-free melanin preparations give pyrolysis thermal degradation profiles identical to protein containing preparations (data not shown) and further support the identity of this material.

The ability of plant-derived melanin to activate human monocytes through a TLR-dependent pathway suggests that it may be detected by cells of the innate immune system within the gut. Both macrophages and dendritic cells express TLR and are crucial cellular components for the detection of pathogens within the main inductive sites of the intestinal MALT known as Peyer's patches. Both particulate and soluble immunogenic material is transported into these structures from the intestinal lumen by M cells, a single layer of epithelium separating the intestinal lumen from the cells within the Peyer's patches. Antigen presenting cells (macrophages and dendritic cells) within the Peyer's patches are thus exposed to this intestinal material for potential recognition of pathogens, pathogen components, or herbal components by the

TLR. Activation of the dendritic cells or macrophages by ligands of the TLRs results in the production of cytokines that can influence the course of the immune response. In mice, dendritic cells from Peyer's patches are able to induce high levels of IgA secretion from naive B cells. A specific subset of CD11b(+) dendritic cells was responsible for this effect, in that they secreted higher levels of the IgA-inducing cytokine IL-6 than other subsets of dendritic cells [30]. The enhanced secretion of IgA and IL-6 ex vivo by cells isolated from the Peyer's patches of mice that had ingested botanical melanin may have resulted from either increased numbers of CD11b+ dendritic cells or higher IL-6 production by these cells as a result of melanin ingestion. The greater production of IFN-γ by spleen cells from mice treated with these melanins suggests that dietary consumption of this polymer would skew Th1/Th2 balance in favor of Th1. This is consistent with a similar study in which mice ingesting the TLR4 agonist lambda carrageenan exhibited reduced antibody production to allergens (suppressed Th2), while spleen cell production of the Th1 cytokine IFN-γ was increased [31].

Due to the growing popularity of herbal supplements, it has become increasingly recognized worldwide that their characterization is an integral part of advancing the quality of botanicals. In spite of this popularity, the lack of definitive understanding of active constituents, the inherent variability in botanical sources, species variability, plant part used, and the processing/formulation make the design and interpretation of botanical efficacy studies problematic. Our discovery of high activity melanin within botanical supplements and its influence on mucosal immune function could help explain the traditional use of these plants and may aid in the design of more relevant clinical studies.

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