Vaccine 26 (2008) 4860–4865

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

# Evaluation of widely consumed botanicals as immunological adjuvants

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ARTICLE INFO

*Article history:*
Received 10 January 2008
Received in revised form 21 April 2008
Accepted 13 June 2008
Available online 18 July 2008

*Keywords:*
Astragalus
Botanicals
Conjugate vaccine
Cancer vaccine
β-Glucan
Immunological adjuvant
PSK
Saponin

ABSTRACT

*Background:* Many widely used botanical medicines are claimed to be immune enhancers. Clear evidence of augmentation of immune responses *in vivo* is lacking in most cases. To select botanicals for further study based on immune enhancing activity, we study them here mixed with antigen and injected subcutaneously (s.c.). Globo H and GD3 are cell surface carbohydrates expressed on glycolipids or glycoproteins on the cell surface of many cancers. When conjugated to keyhole limpet hemocyanin (KLH), mixed with an immunological adjuvant and administered s.c. the magnitude of the antibody responses against globo H, GD3 and KLH depend largely on the potency of the adjuvant. We describe here the results obtained using this s.c. immunization model with seven botanicals purported to have immune stimulant effects.
*Methods:* Groups of 5–10 mice were immunized with globo H–KLH or GD3–KLH mixed with botanical, saline or positive control immunological adjuvant, s.c. three times at 1 week intervals. Antibody responses were measured 1 and 2 weeks after the 3rd immunization. The following seven botanicals and fractions were tested: (1) H-48 (Honso USA Co.), (2) *Coriolus versicolor* raw water extract, purified polysaccharide-K (PSK) or purified polysaccharide-peptide (PSP) (Institute of Chinese Medicine (ICM)), (3) Maitake extract (Yukiguni Maitake Co. Ltd. and Tradeworks Group), (4) Echinacea lipophilic, neutral and acidic extracts (Gaia Herbs), (5) Astragalus water, 50% or 95% ethanol extracts (ICM), (6) Turmeric supercritical (SC) or hydro-ethanolic (HE) extracts (New Chapter) or 60% ethanol extract (ICM) and (7) yeast β-glucan (Biotec Pharmacon). Purified saponin extract QS-21 (Antigenics) and semisynthetic saponin GPI-0100 (Advanced BioTherapies) were used as positive control adjuvants. Sera were analyzed by ELISA against synthetic globo H ceramide or GD3 and KLH.
*Results:* Consistent significant adjuvant activity was observed after s.c. vaccination with the Coriolus extracts (especially PSK), a 95% ethanol extract of Astragalus and yeast β-glucan, and (to a lesser extent) Maitake. Antibodies against KLH in all cases and against globo H in most cases were induced by these botanicals. Little or no adjuvant activity was demonstrated with H-48 or Echinacea extracts or the Astragalus water extract. Experiments with GD3–KLH as immunogen confirmed the adjuvant activity of the Coriolus, yeast β-glucan and Astragalus extracts. While extraction with ethanol concentrated the active ingredients in Astragalus, it had no impact on Coriolus where the 90% ethanol precipitate and solute were equally active.
*Conclusions:* Some, but not all, botanicals purported to be immune stimulants had adjuvant activity in our model. PSK and Astragalus were surprisingly active and are being further fractionated to identify the most active adjuvant components.



## 1. Introduction

Many widely used botanicals are claimed to have immunostimulant effects, but clear evidence that they are able to augment immunological responses against defined antigens is lacking. Screening botanicals for immunomodulatory activity after oral ingestion is difficult due to unknowns such as bioavailability

*Abbreviations:* BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; HSA, human serum albumin; KLH, keyhole limpet hemocyanin; PBS, phosphate buffered saline.

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doi:10.1016/j.vaccine.2008.06.098

**Table 1**
Botanicals tested with Globo H-KLH vaccines, source, dose, and active ingredients

| Botanical extract | Source | Dose | Probable active ingredients |
|---|---|---|---|
| Coriolus | *Coriolus versicolor* water raw extract, ICM | 500 μg | β-Glucan protein linked, β-1,4 backbone, β-1,3 and β-1,6 side chains |
| PSK | *C. versicolor* protein bound polysaccharides, ICM | 0.25–2 mg | |
| PSK | 50% ethanol precipitate, ICM | 2 mg | |
| PSK | 80% ethanol precipitate, ICM | 2 mg | |
| PSK | 80% ethanol soluble, ICM | 2 mg | |
| PSP | *C. versicolor* protein bound polysaccharides, ICM | 250 μg | |
| PSK | *C. versicolor* protein bound polysaccharides (Krestin) Kureha | 250 μg | |
| Maitake | Maitake Gold 404 extract, Tradeworks Group, Inc. (Yukiguni) | 250 μg | β-Glucan, β-1,6 backbone, β-1,3 side chains |
| H-48 | All Inclusive Great Tonifying Formula, Honso Pharmaceutical Co., Ltd. | 250 μg, 1 mg | |
| Echinacea 1 | *Echinacea angustifolia* and *Echinacea purpurea* root 05090601, Gaia | 250 μg | Polysaccharides |
| Echinacea 2 | *E. purpurea* (neutral and week acidic polysaccharides) 05090602, Gaia | 250 μg | |
| Echinacea 3 | *E. purpurea* (strong acidic polysaccharides) 05090603, Gaia | 250 μg | |
| Turmeric 1 | Turmeric supercritical (SC) extract *Curcuma longa* D502401, New Chapter | 100 μg to 2 mg | Curcuminoids |
| Turmeric 2 | Turmeric Hydro-ethanolic (HE) extract *C. longa* D502402, New Chapter | 100 μg to 2 mg | |
| Turmeric 3 | Turmeric Hydro-ethanolic (HE) extract *C. longa*, ICM | 0.5–2 mg | |
| Astragalus 1 | *Astragalus membranaceus*, water extract 05 1105 01, ICM | 500 μg | Saponins |
| Astragalus 2 | *A. membranaceus*, ethanol 50% extract 05 1105 02, ICM | 500 μg | |
| Astragalus 3 | *A. membranaceus*, ethanol 95% extract 05 1105 03, ICM | 0.5–2 mg | |
| Yeast β-glucan | Yeast, Biotec Pharmacon | 100 μg, 0.25–2 mg | β-glucan, β-1,3 backbone β-1,6 side chains |
| Positive controls | | | |
| QS-21 | *Quillai Saponaria*, fraction 21, Antigenics Inc. | 10–20 μg | |
| GPI-0100 | *Q. Saponaria*, semisynthetic, Hawaii Biotech | 100 μg | |

(depending in part on issues such as formulation and concomitant food intake), the amount of active ingredient in the botanical selected, optimal dose and appropriate assay. As a prelude to testing popular botanical medicines as immune modulators after oral ingestion we have chosen to test their immunological activity as immunological adjuvants, mixed with antigens and injected subcutaneously, using resulting antibody titers as read-out. We hypothesized that this would permit us to identify active botanicals and botanical fractions and to identify the active ingredient(s). Our preliminary investigations testing this hypothesis are described here. As the antigenic targets for vaccines against infectious diseases and cancer have become better defined and as our capabilities for synthesizing or extracting these antigens have improved, the need for potent immunological adjuvants or other approaches to augmenting their immunogenicity has become more urgent. A second goal of these studies was to identify new more potent or less toxic adjuvants of value for use in vaccines.

Seven widely used botanicals have been selected for initial study based on prevalence of use and reports describing immunomodulatory activity. Multiple samples are tested for some of these seven due to variations in extraction methods and suppliers. The seven botanicals selected for initial study are Astragalus [1], Coriolus [2,3], Echinacea [3,4], H-48 (a formula consisting of 10 herbs) [5,6], Maitake [4,7], β-glucan of yeast origin [8] and Turmeric [4,9]. The preparations tested vary in complexity from multiple botanicals as in H-48 to the more purified Coriolus fractions PSK and PSP, and yeast β-glucan. Activity was tested in multiple experiments to limit sampling errors and, in addition, with or with out a suboptimal dose of the potent semisynthetic saponin adjuvant GPI-0100.

When the breast cancer carbohydrate antigen globo H [10] or neuroectodermal cancer ganglioside GD3 are conjugated to keyhole limpet hemocyanin (KLH), mixed with an immunological adjuvant and administered s.c., the magnitude of the antibody responses against globo H and GD3 and against KLH depend largely on the potency of the adjuvant [11]. We test here whether botanicals mixed with the vaccine and injected subcutaneously could function as immunological adjuvants, and if so, whether this could be enhanced by further extraction and purification. This mix of carbohydrate autoantigen and protein xenoantigen, weak immunogen and strong immunogen, reflects the range of antigens targeted in vaccines against cancer and infectious diseases, and the range of antigens encountered in life. We describe here results of our experiments with these seven botanicals mixed with globo H–KLH and GD3–KLH conjugates and administered s.c. to mice. We find that the Coriolus extracts, 95% ethanol extract of Astragalus and yeast β-glucan have potent adjuvant activity.

## 2. Methods

### 2.1. Botanicals (see Table 1)

*Astragalus* was provided by the Institute of Chinese Medicine (ICM), Chinese University of Hong Kong. Three extracts of *Astragalus membranaceus* were prepared using water (Astragalus 1), 50% ethanol (Astragalus 2), and 95% ethanol (Astragalus 3) as solvents.

*Coriolus* was obtained from the ICM in three different forms: *Coriolus versicolor* raw water extract, purified polysaccharide-K (PSK), and polysaccharide-peptide (PSP). PSK (Krestin) was also obtained from Kureha Corp. (Japan). In a final experiment, ICM PSK was fractionated in different concentrations of ethanol and the resulting precipitates (ppt) and soluble (S) fractions tested.

*Echinacea* was supplied by Gaia Herbs Inc. of Brevard, NC, in three different forms: lipophilic extract of *Echinacea angustifolia* and *Echinacea purpurea* roots (Echinacea 1), neutral and weak acidic polysaccharides from *E. purpurea* juice extract (Echinacea 2), and strong acidic polysaccharides from *E. purpurea* juice extract (Echinacea 3).

*H-48* is a combination of 10 herbal extracts supplied by the Honso Pharmaceutical Co. Ltd.

*Maitake* Gold 404 and Maitake (*Grifola frondosa*) mushroom extracts were produced by Yukiguni Maitake Co. Ltd., Japan and procured from Tradeworks Group of Brattleboro, VT.

*Turmeric* was obtained from New Chapter Inc. of Brattleboro, VT. It was extracted from the root of *Curcuma longa* in two forms, a supercritical (SC) extract (Turmeric 1) and a hydro-ethanolic (HE) extract (Turmeric 2). The ICM also provided a sample of Turmeric extracted using 60% ethanol (Turmeric 3).

*Yeast Beta glucan* (BBG500) was provided by Biotec Pharmacon.

**Table 2**
ELISA antibody titers after vaccination with Globo H-KLH plus or minus botanicals

| Globo H–KLH vaccine plus | Globo H IgM | KLH IgG |
|---|---|---|
| No adjuvant | 200, 400(2)[a], 800(6), 1600 | 400(2), 800(2), 1600, 3200(5) |
| Turmeric 2, 500 μg | 800(2), 1600(2), 6400 | **6400(3), 12,800, 51,200** |
| Turmeric 2, 2 mg | **1600(4), 3200** | **12,800, 25,600, 51,200(3)**[*] |
| PSK, 500 μg | 800(3), 1600, 3200(2) | **25,600(3), 51,200, 204,800** |
| PSK, 2 mg | **800, 1600(2), 3200(2)** | **51,200, 102,400(2), 204,800(2)** |
| Astragalus 3, 500 μg | 800(2), 1600, 6400, 12,800 | **6400, 12,800(2), 25,600, 51,200** |
| Astragalus 3, 2 mg | **800(2), 3200, 6400, 25,600** | **3200, 12,800, 25,600, 51,200(2)** |
| Yeast β-glucan, 500 μg | **1600(2), 3200, 12,800, 25,600** | **12,800, 102,400(3), 204,800** |
| Yeast β-glucan, 2 mg | **1600, 3200, 6400, 12,800, 25,600** | **51,200, 102,400, 204,800, 409,600(2)** |
| QS-21, 10 μg | **6400(2), 12,800(2), 25,600** | **409,600, 819,200(2), 1,638,400(2)** |

[a] Reciprocal antibody titer. Number in parentheses is the number of mice with that titer, if more than 1.
[*] Groups in bold are significantly ($p < 0.05$) higher than the no adjuvant control group.

In the case of extractions from raw botanicals, authenticity of botanicals was confirmed by tests described in specific pharmacopeial monographs and compared with an authenticated reference specimen. Voucher specimens of all botanicals studied here have been deposited either at the manufacturers' archive or at the Hong Kong Herbarium.

### 2.2. Vaccine production

Globo H hexasaccharide (molecular weight 1055 Da) was synthesized and conjugated to KLH ($8 \times 10^6$ Da) essentially as previously described [9]. Globo H/KLH molar ratios in the conjugate ranging between 500/1 and 800/1 were used in these studies. The globo H–KLH used here was purchased from Optimer Pharmaceuticals (San Diego, CA) who synthesized it under contract. It was provided as globo H ceramide for use as target in ELISA assays and as globo H–KLH conjugate for vaccine production. GD3 was extracted from bovine buttermilk and purchased from Matreya Inc. (Pleasant Gap, PA), and conjugated to KLH as previously described [13]. The GD3/KLH molar ratio in the conjugate was 950/1. KLH for vaccine production and serological target was purchased from Sigma. GPI-0100 and QS-21 were used as positives controls. GPI-0100 was provided by Galenica Pharmaceuticals, Inc. (now Hawaii Biotech, Inc., Aiea, HI) and used at a dose of 50–100 μg as positive control immunological adjuvant or 10 μg when used in combination with other botanicals. QS-21 was provided by Aquila Biopharmaceuticals, Inc. (now Antigenics Inc., New York, NY) and used as positive control at a dose of 10 μg. The botanicals were tested at doses between 200 μg and 2 mg in individual experiments with all tested at a dose of at least 500 μg.

### 2.3. Vaccine administration

Six-week-old female C57Bl/6 mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). Groups of five mice were immunized s.c. three times at 1 week intervals with globo H–KLH or GD3-KLH containing 3–5 μg of globo H mixed with either various botanicals (see Table 2) in 0.1 ml saline, 10 μg or GD3 QS-21 or 50–100 μg GPI-0100 as positive controls or with a 10 μg dose of GPI-0100 plus various botanicals.

### 2.4. Serological assays

Mice are bled from the retro-orbital sinus under general anesthesia 7 days after the third immunization for ELISA, and sera frozen for future testing.

*ELISA*: Enzyme linked immunosorbent assays are performed as described previously [12,13]. The target antigens are globo H ceramide, GD3 or KLH. To determine the titers of antibodies, ELISA plates are coated with antigen, generally at 0.1 μg/well. Serially diluted sera in 1% HSA in PBS are added to wells of the coated plate and incubated for 1 h at room temperature. Goat anti-mouse IgM or IgG conjugated with alkaline phosphatase (Southern Biotechnology, Birmingham, AL) serve as second antibodies. The antibody titer is defined as the highest serum dilution showing an absorbance 0.1 or greater over that of normal sera. A response is considered positive by ELISA if the titer of reactivity increased from undetectable pretreatment to at least 1:40 after vaccination, or if detectable pretreatment, by 8-fold.

*Statistical analysis*: For each experimental run, results in serological assays for treated mice were compared to the no adjuvant or low dose GPI-0100 controls using the Mann–Whitney test. The mean and standard error of the difference between ranks from different experimental runs were then combined using fixed effect meta-analysis. All statistical analyses were conducted using Stata 9.2 (Stata Corp., College Station, TX).

## 3. Results

### 3.1. ELISA results after immunizations

Eight separate experiments were conducted with the globo H–KLH vaccine, with all extracts tested in at least two experiments (see Table 1 for botanicals tested, sources and dose range). Antibodies induced against KLH were almost exclusively IgG while those induced against globo H were almost exclusively IgM, as expected. In addition, as expected, antibodies induced against KLH were more sensitive indicators of adjuvant activity than those induced against globo H. Relevant serologic titers from one of the eight experiments using the globo H–KLH vaccine is shown in Table 2 and the results of all eight individual experiments and a meta-analysis of the pooled data are summarized in Table 3.

Coriolus extracts induced significantly elevated antibody titers against KLH, with PSK having the most activity, both as adjuvant alone and when added to a low dose of GPI-0100. PSK also induced significantly elevated antibody titers against globo H in three of five experiments. Vaccines containing 95% ethanol extract of Astragalus induced consistent antibodies against KLH (overall $p < 0.001$) alone and in one of three experiments against globo H ($p < 0.05$), with the 50% extract showing intermediate activity against KLH ($p = 0.03$ in one experiment). Yeast β-glucan alone or with GPI-0100 resulted in significantly increased antibody titers against KLH in all experiments (overall $p < 0.001$) and against globo H in two of four experiments (overall $p = 0.002$ and <0.001, respectively). Turmeric 2 and 3 each induced increased antibody titers against KLH ($p = 0.05$ and 0.002) or globo H ($p = 0.05$) in one experiment but Turmeric of ICM origin also resulted in significantly decreased antibody titer in

**Table 3**
Summary of ELISA results of individual experiments and of meta-analysis

| Botanical extract | Proportion of positive experiments* compared to respective no adjuvant negative controls, and meta-analysis *p*-value | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | No GPI-0100 | | | | Plus GPI-0100 (10 μg) | | | |
| | Globo H | | KLH | | Globo H | | KLH | |
| | | | | | 3/5 | **0.008**[a] | 4/5 | **<0.001**[a] |
| Coriolus | 0/1 | 0.9 | 1/1 | **0.002** | 0/1 | 0.7 | 0/1 | 0.2 |
| PSK | 3/5 | **<0.001** | 4/5 | **<0.001** | 0/1 | 0.3 | 1/1 | **0.004** |
| PSP | 0/1 | 0.6 | 1/1 | **0.002** | 0/1 | 0.5 | 0/1 | 0.3 |
| Maitake | 1/4 | 0.6 | 1/4 | **0.03** | 0/1 | 0.15 | 1/1 | **0.04** |
| H-48 | 0/1 | 0.6 | 0/1 | 0.7 | 0/1 | 0.7 | 0/1 | 0.7 |
| Echinacea 1 | 0/1 | 0.09 | 0/1 | 0.4 | 0/1 | 0.5 | 0/1 | 0.02** |
| Echinacea 2 | 0/1 | 0.02** | 0/1 | 0.8 | 0/1 | 0.2 | 0/1 | 0.1 |
| Echinacea 3 | 0/1 | 0.4 | 0/1 | 0.9 | 0/1 | 0.2 | 0/1 | 0.9 |
| Turmeric 1 | 0/1 | 0.3 | 0/1 | 0.07 | 0/1 | 0.9 | 0/1 | 0.5 |
| Turmeric 2 | 1/2 | 0.06 | 1/2 | 0.4 | 0/1 | 0.052 | 0/1 | 0.08 |
| Turmeric 3 | 0/1 | 0.04** | 1/1 | **0.002** | 0/1 | 0.4 | 0/1 | 0.02** |
| Astragalus 1 | 0/1 | 0.9 | 0/1 | 0.4 | 0/1 | 0.15 | 0/1 | 0.4 |
| Astragalus 2 | 0/1 | 0.7 | 1/1 | **0.03** | 0/2 | 0.9 | 0/2 | 0.9 |
| Astragalus 3 | 1/3 | 0.9 | 3/3 | **<0.001** | 0/2 | 0.16 | 1/2 | **0.012** |
| Yeast β-glucan | 2/4 | **0.002** | 4/4 | **<0.001** | 2/4 | **<0.001** | 4/4 | **<0.001** |
| GPI-0100 (100 μg) | 5/5 | **<0.0001** | 5/5 | **<0.001** | | | | |
| QS-21 (10 μg) | 1/1 | **<0.002** | 1/1 | **<0.002** | | | | |

[a] Values in bold are compared to mice receiving no adjuvant.
* Positive experiment = *p*-value for higher titers induced by indicated botanical compared to no adjuvant or low dose GPI-0100 controls <0.05.
** Botanical-induced antibody titers that were significantly lower than the controls in these individual experiments.

one experiment ($p = 0.04$). None of the other study extracts (Echinacea, Maitake, H-48, or Astragalus water extract) demonstrated consistent adjuvant activity against KLH or globo H in individual experiments, though in the meta-analysis shown in Table 3 Maitake significantly increased antibody titers against KLH. The positive control extract GPI-0100 was strongly positive in all experiments for induction of antibodies against KLH and globo H. Mice were weighed and inspected at 24 and 48 h and 7 days after vaccinations. Loss of vigor or coat grooming or more than 5% of weight were considered evidence of toxicity. Despite doses ranging between 200 μg and 2 mg per vaccination, no obvious toxicity was detected with any of these botanicals. The dose administered was limited only by solubility and the volume limitation for subcutaneous vaccinations in mice, 0.2 ml.

To confirm that the immune response against a different vaccine would also be enhanced by these botanicals, a final experiment using a GD3–KLH vaccine was conducted (see Table 4). Here the natural glycolipid GD3 ganglioside was substituted for the synthetic carbohydrate globo H conjugated to KLH. Astragalus and especially PSK were again active. PSK was also fractionated in the same general way as Astragalus had been, but unlike Astragalus where activity was greatly enhanced by extraction with 95% alcohol, alcohol extraction of PSK had no clear impact on the adjuvant potency of the resulting PSK fractions.

## 4. Discussion

We have screened seven separate botanicals all claimed to have immune enhancing activity. Adjuvant activity in our model was found for some, but not all extracts. Our most striking finding was the potency of the four *C. versicolor* extracts. These extracts are known to be rich in β-glucans, the presumed most active ingredient. While all four Coriolus extracts had activity over a wide range of doses, PSK supplied by ICM was the most active. In some experiments, this equaled or surpassed reactivity seen with an equal weight of purified β-glucan of yeast origin. The potency of PSK as an adjuvant was confirmed with a second conjugate vaccine as well, GD3–KLH. Since PSK contains less than 25% β-glucan polysaccharides by weight, this suggests that β-(1,4) backbone with β-(1,3) and β-(1,6) glucocytic linkages characteristic of *C. versicolor* β-glucans (as apposed to the β-(1,3) backbone with β-(1,6) linkages characteristic of yeast β-glucans) may have unique potency. This could also be a consequence of the protein core in the protein bound polysaccharides which characterize PSK and PSP [14]. Both PSK and PSP have been described to have wide ranging impact on WBC count, phagocytic functions, T-helper cell activation, T-cell function and cytokine production when tested either *in vitro* or administered *in vivo* [15,16]. Neither have been used as immunological adjuvant mixed with vaccine and there has been no attempt

**Table 4**
ELISA antibody titers after vaccination with GD3–KLH plus or minus botanicals

| GD3–KLH Vaccine Plus | GD3 IgM[a] | KLH IgG |
|---|---|---|
| No adjuvant | 0(3), 20(2) | 0(2), 6400(3) |
| PSK, 2 mg | 0(2), 40, 80(2) | **6400**, **25,600**, **102,400(2)**, **409,600** |
| PSK 50% ethanol ppt, 2 mg[b] | **40(2)**, **80(3)*** | **6400**, **25,600**, **102,400(2)**, **409,600** |
| PSK 80% ethanol ppt, 2 mg | 0(2), 20(2), 160 | **6400(2)**, **25,600**, **102,400(2)** |
| PSK 80% ethanol sol, 2 mg | 0(4), 20 | **1600**, **6400**, **25,600(2)**, **102,400** |
| Astragalus 95% ethanol sol, 2 mg | 0(5) | **6400**, **25,600(3)**, **102,400** |
| GPI-0100, 50 μg | **40**, **160**, **320(2)**, **640** | **25,600**, **102,400**, **409,600**, **1,638,400(2)** |

[a] Reciprocal antibody titer. Number in parentheses is the number of mice with that titer, if more than 1.
[b] Extraction in the indicated percent ethanol was performed and the precipitate (ppt) and solute (sol) tested.
* Groups in bold are significantly ($p < 0.05$) higher than the no adjuvant control group.

to link particular structures in PSK or PSP with adjuvant activity. Our findings suggest the possibility that β-glucans in Coriolous extracts are uniquely potent as immunological adjuvants, a possibility we are pursuing initially by further fractionating PSK and testing the individual fractions. An initial attempt at fractionating PSK demonstrated that ethanol extraction, the method that worked well for Astragalus, had no detectable impact on PSK.

We have identified saponins and in particular the saponin fraction QS-21 and the semisynthetic saponin mix GPI-0100 as uniquely potent immunological adjuvants when mixed with conjugate vaccines containing glycolipids or peptides chemically conjugated to KLH [11,17]. The maximal doses of QS-21 used in mice (20 μg) and patients (100 μg) were selected for minimal weight loss in mice and acceptable local erythema/induration and systemic flu-like symptoms in patients [18]. The quest continues for immunological adjuvants with more potent adjuvant activity and more limited local and systemic toxicities. While most studies have been focused on *Quillaja saponaria* saponins, there are many additional botanicals expressing other saponins. A possible case in point is *A. membranaceus* which we demonstrate here to have significant adjuvant activity in the 95% ethanol fraction.

*A. membranaceus* is a well known traditional Chinese medicinal plant used widely for a variety of indications. The main constituents of the *A. membranaceus* root are polysaccharides, saponins and flavonoids. The cyclolanostane-type saponins have been identified as the most active ingredient with significant lymphocyte proliferation and immunostimulatory activities [19]. Using the hemolytic activity of saponins from various botanicals as a surrogate for toxicity, the hemolytic and immunological adjuvant activities of a series of saponin-rich botanicals have been compared [20,21]. Saponins were extracted using 70% ethanol, ether and *n*-butanol. Fifty, 100 and 200 μg had comparable activity, augmenting antibody titers against ovalbumin by approximately 10-fold. Saponins of *A. membranaceus* were identified as having lower hemolytic activity and stronger adjuvant activity than saponins in the other botanicals tested. We demonstrate here that the 95% ethanol extract of *A. membranaceus* (the saponin-rich fraction) also augments antibody responses against weak glycolipid autoantigens such as globo H and strong xenoantigens such as KLH. The 95% ethanol extract tested here consists of approximately 40% saponins. At least 15 such separate saponins have been identified in *A. membranaceus* saponins and the chemical structures defined [21]. Four of these (astragalosides I–IV) are commercially available. While purifying the other 11 individual saponins from extracted saponins for use as adjuvants would be difficult, the recent description of the total chemical synthesis of QS-21 [22] raises the possibility that these remaining individual *A. membranaceus* saponins could be synthesized. The described low hemolytic activity of these saponins and potent adjuvant activity suggest that this would be fruitful. It is expected that even among this family of saponins some would have greater or lesser toxicity and that this might be distinct from their adjuvant activity.

It has been claimed by proponents of botanical medicine that crude or simple extracts of botanicals or mixtures of botanicals have unique potencies that cannot be replicated or exceeded by any of the individual chemical constituents. Such a claim is a testable hypothesis, at least with regard to the saponins in the 95% ethanol extract of Astragalus and β-glucans in PSK. This is especially relevant in the case of immunological adjuvants where efficacy frequently varies with dose but dose administered is limited by toxicity. The clear superiority of QS-21 over cruder fractions or unfractionated *Q. saponaria* saponins was largely a consequence of the superior immunogenicity/toxicity ratio. It may be that saponins are not the only immunologically active components in the 95% ethanol fraction or that a mixture of these saponins will prove superior to any one, but again this should be testable. The same applies to the unexpected potency of the β-glucans in PSK. In addition, if the goal is optimizing the adjuvant activity of botanicals, identification of the most active components is a necessary first step to developing relevant markers for improved methods of extraction and for confirming relevant batch to batch consistency. The studies described here are our first steps in these directions.

## Acknowledgments

This project was supported by Grant Number 1 P50 AT002779-01 from the National Center for Complementary and Alternative Medicine (NCCAM) and the Office of Dietary Supplements (ODS). Mr. William H. and Mrs. Alice Goodwin and the Commonwealth Foundation for Cancer Research and The Experimental Therapeutics Center (ETC) of Memorial Sloan-Kettering Cancer Center. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NCCAM, ODS or the National Institute of Health and the Commonwealth Foundation for Cancer Research or the ETC.

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