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SEPARATION AND CHARACTERIZATION OF SAPONINS WITH ADJUVANT ACTIVITY FROM *Quillaja saponaria* MOLINA CORTEX

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Saponins were purified from *Quillaja saponaria* Molina bark by silica and reverse phase chromatography. The resulting purified saponins were tested for adjuvant activity in mice. Several distinct saponins, designated QS-7, QS-17, QS-18, and QS-21, were demonstrated to boost antibody levels by 100-fold or more when used in mouse immunizations with the Ag BSA and beef liver cytochrome b_5. These purified saponins increased titers in all major IgG subclasses. To determine optimal dose in mice for adjuvant response, QS-7 and QS-21 were tested in a dose-response study in intradermal immunization with BSA in mice; for both of these purified saponins, adjuvant response (determined by stimulation of ELISA titers to BSA) neared maximum at doses of 5 μg and was shown to plateau up to the highest dose tested, 80 μg. These purified saponins vary considerably in their toxicity, as assessed by lethality in mice; the main component, QS-18, being the most toxic. Saponins QS-7 and QS-21 showed no or very low toxicity in mice, respectively. None of these saponins stimulated production of reaginic antibodies. The monosaccharide composition of these saponins showed similar but distinct compositions with all four containing fucose, xylose, galactose, and glucuronic acid. Predominant differences were observed in the quantities of rhamnose, arabinose, and glucose. Monomer m.w. (determined by size exclusion HPLC) were determined to range from 1800 to 2200.

Formulation of effective vaccines requires not only the appropriate Ag, but also the appropriate adjuvant to optimize protective humoral and cell-mediated immune responses. The use of the same Ag with different adjuvants has been shown to elicit significantly different responses from the immune system. For example, comparison of immunization of mice with killed schistosomula from *Schistosoma mansoni* with the adjuvants bacillus Calmette-Guérin, pertussis, *Coryne bacterium parvum*, tetanus toxoid, *Escherichia coli* LPS, yeast glucan, aluminum hydroxide, and saponin showed that only the animals immunized with bacillus Calmette-Guérin or saponin were protected from challenge (1) despite the demonstration of significant humoral immunity by some of the ineffective adjuvants. In effect, Allison et al. have noted that adjuvants such as aluminum hydroxide and mineral oil produce primarily humoral immunity whereas adjuvants such as muramyl dipeptide are able to induce cell-mediated immunity as well as differences in the isotype of the antibodies elicited (2). A further consideration, in addition to the efficacy of the adjuvant for eliciting a protective immune response, is the issue of toxicity of the adjuvant. CFA, which is used widely in research vaccines, produces excellent humoral and cell-mediated immunity, but is unsuitable for use in human and veterinary vaccines because of the toxic side effects (3). Similarly LPS, which is also a strong adjuvant, is highly toxic (reviewed in Reference 4). Hence, there is a need for identification of adjuvants that are both safe and efficacious.

One such potential adjuvant system is a class of compounds extracted from plant sources, termed collectively as saponins because of the detergent properties associated with them. The detergent properties of saponins are caused by their amphipathic nature; they consist of a hydrophilic carbohydrate moiety and a hydrophobic steroid or triterpene moiety. The adjuvant effect of saponins was noted in 1951 by Espinet (5) who utilized a crude saponin mixture to increase the immune response to foot-and-mouth disease vaccine. Extracts of the bark of a South American tree, *Quillaja saponaria* Molina, have been shown to be potent adjuvants (6–8). Further studies by Dalsgaard showed that adjuvant activity in these extracts resides in the saponin fraction, which has been characterized as a mixture of triterpene glycosides (7). Crude preparations of *Quillaja* saponins have been used to boost the response to BSA (7), keyhole limpet hemocyanin (9), SRBC (8), as well as aluminum hydroxide-based vaccines (9, 10). In addition, partially purified *Quillaja* saponins have been reported to associate with hydrophobic or amphipathic proteins and lipids to form detergent/lipid/saponin complexes termed ISCOM[4] (11); these structures are typically prepared by solubilizing the Ag with non-ionic detergents and then exchanging the non-ionic for the saponin detergent by centrifugation through sucrose gradients containing saponins at a concentration higher than their critical micellar concentration. ISCOM, which have been prepared from surface Ag isolated from influenza virus, measles, toxoplasma, feline leukemia virus, EBV, and HIV-1 (11–13) induce

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[4] Abbreviations used in this paper: ISCOM, immunostimulating complexes; MDP, muramyl dipeptide; MPL, monophosphoryl lipid A; TDM, trehalose dimycolate; TFA, trifluoroacetic acid; I.D., inside diameter.

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serum antibody titers that are approximately 10-fold higher than immunization with protein micelles alone.

In addition to the potent adjuvant activity, the saponin fraction from *Quillaja* bark has strong hemolytic activity (7). This hemolytic activity has been suggested to be caused by the intercalation of saponins into cholesterol-containing membranes to form holes of approximately 80 Å, which can be observed with negative staining electron microscopy (14–16).

Despite the potential use of *Quillaja* saponins as adjuvants, their application has been limited because of the undesirable side effects of the commercially available preparations that are partially purified mixtures of saponins and other components (17–20). The adjuvant-active saponins have not been characterized because of the difficulty in purifying the active components to homogeneity. An adjuvant-active fraction was prepared from an aqueous extract of *Q. saponaria* bark by Dalsgaard (7) by using dialysis, anion exchange, and gel filtration chromatography in aqueous buffers; this fraction (designated Quil-A) was reported to be a single band by TLC on silica gel plates. However, we have found that this fraction is still a heterogeneous saponin mixture that can be resolved into multiple glycoside fractions by reverse phase HPLC. Higuchi et al. (21) have recently substantially purified a saponin from a methanolic extract of *Quillaja* bark and have characterized the glycoside moiety; however, this purified saponin was not tested for adjuvant effect. Hence, at present, there is no information on which components of the saponin fraction from *Quillaja* bark possess adjuvant activity. In this paper, we report a separation procedure for saponins extracted from the cortex of *Q. saponaria* Molina, identification of distinct saponin components with adjuvant activity and no apparent lethality in mice in an adjuvant-active dose range, identification of an adjuvant-saponin that is lethal at a lower dose than the original aqueous extract, and preliminary chemical characterization of these fractions.

MATERIALS AND METHODS

Purification of saponins. Coarsely chopped *Q. saponaria* bark (approximately 1 cm square, obtained from Hauser Chemicals, Boulder, CO) was stirred with 10 ml of water/g of bark at room temperature for 1 h. The extract was centrifuged and the supernatant containing the solubilized saponins was saved. The extraction step was repeated on the bark pellet and the two supernatants were pooled. To remove nonsaponin components, the supernatant pool was lyophilized, redissolved in 40 mM acetic acid in water at a concentration of 250 mg/ml (w/v) and either chromatographed through Sephadex G-50 (medium, Pharmacia, Piscataway, NJ) in 40 mM acetic acid with the hemolytic activity localized in the void volume fraction, or dialyzed against 40 mM acetic acid with the hemolytic activity retained by the dialysis membrane.

The hemolytic fraction was lyophilized and redissolved at a concentration of 200 mg/ml in 40 mM acetic acid in chloroform/methanol/water (62/32/6, v/v/v); 1 g of this fraction was applied to Silica Lichroprep (E. M. Science, Gibbston, NJ; 40 to 63 μm particle size, 2.5 cm I.D. × 20 cm height) and eluted isocratically in the solvent used to solubilize the saponins. The elution of saponins was monitored by carbohydrate assay (22). Fractions containing the saponins of interest were identified by reverse phase TLC with visualization with Bial's reagent (Sigma, St. Louis, MO) pooled individually, and rotavapped to dryness. The fractions from the silica chromatography were then redissolved in 40 mM acetic acid in 50% methanol and loaded on a semipreparative HPLC column (Vydac C_4, 5 μm particle size, 3000 nm pore size, 10 mm I.D. × 25 cm length). Saponin peaks, detected by absorbance at 214 nm, were eluted by using a methanol gradient at a flow rate of 4 ml/min, and individually rotavapped to dryness. Purity of saponins was assessed by analytic HPLC (Vydac C_4, 5 μm particle size, 3000 nm pore size, 4.6 mm I.D. × 25 cm length) with a gradient of 0.1% TFA in acetonitrile.

Immunologic procedures. CD-1 mice (8 to 10 wk of age) were immunized intradermally with a total volume of 0.2 ml injected at two sites per mouse. Each sample was tested in a group of five mice. The buffer used for all immunizations was PBS. The following proteins were used as Ag: BSA (Sigma) and purified cytochrome b_5 from beef liver, kindly provided by Dr. Philipp Strittmatter (University of Connecticut Health Center, Farmington, CT). CFA and IFA were obtained from Difco (Detroit, MI). MPL and TDM were obtained from Ribi Immunochemicals (Hamilton, MT). Squalene and Tween-20 were obtained from Sigma. Superfos Quil-A, a crudely enriched saponin preparation, and Alhydrogel (2% aluminum hydroxide) were obtained from Accurate Sciences, Westbury, NY.

The toxicities of Quil-A and purified saponins QS-7, 18, and 21, were tested in CD-1 mice by following procedures similar to those described above for immunizations. Varying doses of these compounds dissolved in sterile PBS were injected intradermally in mice. The mice were monitored for 72 h after injections and the results expressed in number of deaths per group.

Ag-specific antibody response was determined by ELISA. Immulon II plates were coated overnight at 4°C with 100 μl/well of coating solution, consisting of 10 μg/ml of the Ag in PBS. Plates were then washed twice with PBS and blocked in 10% normal goat serum (Hazelton, Rockville, MD) in PBS (150 μl/well for 1 h at room temperature). Plates were washed twice with 0.05% Tween 20 (Sigma) in water. Mouse serum was serially diluted 1/10 in 10% normal goat serum in PBS; 100 μl of each dilution was incubated on the plate for 1 h at room temperature. All dilutions were tested in duplicate on both Ag-coated and noncoated control wells. Plates were washed twice with 0.05% Tween 20. Goat anti-mouse IgG-horseradish peroxidase conjugate (H and L chain specific; Boehringer-Manneheim Indianapolis, IN), diluted in 10% normal goat serum in PBS, was incubated on the plate (100 μl/well for 30 min at room temperature). The plates were washed four times with 0.05% Tween 20 and then with water two times. The substrate for the reaction was tetramethylbenzidine (23). Titers were determined from the dilution resulting in an absorbance of 0.5. Relative titers of specific antibody isotypes were determined by titration of sera pools (prepared with equivolume ratios of individual mouse serum samples in a group) on Ag-coated plates with the use of goat anti-mouse alkaline phosphatase conjugates specific for IgM, IgG3, IgG1, $IgG2_a$, and $IgG2_b$, respectively (Southern Biotechnology Associates, Birmingham, AL) and a goat anti-mouse IgE-horseradish peroxidase conjugate (Nordic, El Toro, CA).

Hemolytic activity. Serial 1/2 dilutions of saponin in PBS were made in a round bottom microtiter plate. The final volume in each well was 100 μl. SRBC (40% sheep blood and 60% Alsever's solution; Whittaker Bioproducts, Walkersville, MD) were washed three times by low speed centrifugation of the blood followed by resuspension of the red cell pellet in PBS to the original volume. The red cell pellet was diluted to 2.5 × the original volume and then used in the hemolysis assay. Twenty-five microliters of the resuspended cells were added to each well in the microtiter plate and mixed by pipetting. After incubation at room temperature for 30 min, the plates were spun at 1000 rpm for 5 min in a Sorvall RT6000 in an H-1000 rotor to sediment unhemolyzed cells. Fifty microliters of the supernatant from each well were transferred to the same well of a flat bottom microtiter plate. Absorbance caused by released hemoglobin was determined at 570 nm with a Dynatech microtiter plate reader.

Carbohydrate analysis. Relative carbohydrate concentration was determined by the anthrone method of Scott and Melvin (22). The standard for the assay was glucose. Analysis of carbohydrate composition as trimethylglucosides was carried out under contract by the Complex Carbohydrate Corporation (Athens, GA).

Monomer size of saponins. Monomer size of the saponins was determined by HPLC gel permeation chromatography on a Zorbax PSM 60 Si column (6.2 mm I.D. × 25 cm height). Ginsenoside Rb_1 (m.w. = 1109; Waco Pure Chemicals, Dallas, TX) and 18-β-glycyrrhetinic acid (m.w. = 471; Fluka Chemicals, Everett, WA) were used as m.w. standards. Saponins and standards were solubilized in methanol at a concentration of 1 mg/ml. Twenty microliters were injected on the column and eluted in methanol at a flow rate of 1.0 ml/min. Absorbance at 214 nm was used to monitor the column.

RESULTS

Isolation and characterization of saponin adjuvants. Approximately 20 to 25% of the dry weight of *Q. saponaria* Molina bark is extractable in water. Dialysis of the aqueous extract resulted in retention of approximately 24% of the dry weight and 95% of the hemolytic activity of the extract, indicating that saponins present in the

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aqueous bark extract were retained by a dialysis membrane of 12,000 m.w. cutoff. Similar recoveries were achieved by chromatography of the aqueous extract on Sephadex G-50, with the saponin fraction localized in the void volume; reverse phase TLC showed that the identical components were isolated (not shown).

With the use of reverse phase HPLC, an unprocessed extract of *Q. saponaria* bark was shown to be a highly complex mixture. Treatment of this aqueous extract by ultrafiltration through a membrane with 10,000 m.w. cutoff removed almost all hydrophilic peaks from the retentate although multiple hydrophobic components were still present (Fig. 1*A*). Analysis of Quil-A, a commercial saponin that is commonly used in adjuvant studies, showed that this product contains all the peaks present in the ultrafiltrated aqueous bark extract shown in Figure 1*A*.

Significant resolution of the saponin peaks in the ultrafiltration retentate was achieved by using a shallow gradient of methanol or acetonitrile on Vydac C_4 as described in *Materials and Methods* (Fig. 1*A*). All major peaks in this retentate fraction were reactive with anthrone, indicating the presence of carbohydrate, and caused foaminess in aqueous solution, indicating that they were saponin in nature. Different bark samples yielded qualitatively a similar pattern of peaks with the same retention times. However, some quantitative differences were observed between different bark samples, apparently as a result of differences between the bark samples because extractions from the same sample of bark yield consistent results. The saponin peaks isolated by HPLC were tested for adjuvant activity by using BSA as the test Ag. Adjuvant-active components were identified in 10 of the peaks tested including the major peaks (7, 17, 18, and 21) (data not shown). These peaks, particularly peak 18, predominate in most samples of bark or commercial *Quillaja* saponins tested.

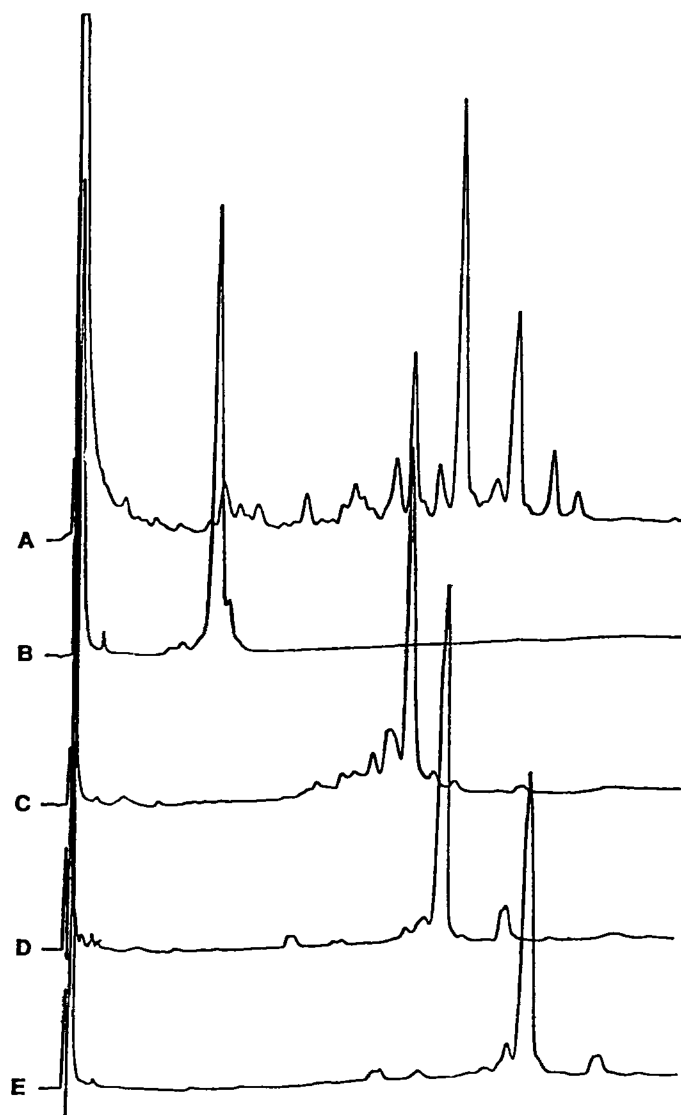

Figure 1. HPLC (Vydac C4, 4.6 mm × 25 cm, 5 μm particle size, 3000 nm pore size) of an aqueous bark extract treated by ultrafiltration (*A*), saponin QS-7 (*B*), saponin QS-17 (*C*), saponin QS-18 (*D*), and saponin QS-21 (*E*). Gradient was 30 to 40% 0.1% TFA/acetonitrile/30 min, 40%/15 min at a flow rate of 1 ml/min. A total of 100 μg of purified saponin or 200 μg bark extract (dry weight) was used per injection.

The major saponin peaks, purified as described in *Materials and Methods*, were further characterized for adjuvant activity as well as for physical and chemical properties. The purity of these samples is shown in Figure 1. The fractions, designated as saponins QS-7, 17, 18, and 21, with QS denoting the source to be *Q. saponaria*, are significantly pure in comparison with the starting extract, although several minor contaminants are evident in some fractions (Fig. 1 *B* to *E*).

Effect of dose on adjuvant effect in mice. To establish the range of effectiveness for purified saponins, dose response curves were carried out for two of the saponins, QS-7 and QS-21 (Fig. 2). These saponins were chosen because they represented the most hydrophilic (QS-7) and hydrophobic (QS-21) of the four saponins purified in this study. Hydrophobicity was assumed to be related to the retention time on reverse phase HPLC with the use of a hydrophobic resin. CD-1 mice were immunized intradermally twice with BSA plus the indicated dose of saponin at 2-wk intervals. Sera was analyzed for anti-BSA IgG by ELISA 1 wk after the second immunization. Anti-BSA IgG titers were considerably augmented by doses of saponin as low as 5 μg for both QS-7 and QS-21. The immune responses obtained with QS-7 and QS-21 were similar, reaching a plateau at doses between 10 and 80 μg. No significant differences were observed between QS-7 and QS-21.

Adjuvant activity of purified saponins and research adjuvants. The purified *Quillaja* saponins (QS-7, 17, 18 and 21) were compared for effectiveness as adjuvants with various research adjuvants, such as aluminum hydroxide, CFA, and IFA, and a mixture of MPL and TDM. Saponins were used at a dose of 20 μg, an amount that falls in the plateau of maximum adjuvant effect observed with QS-7 and QS-21. Two immunizations with 10 μg of Ag cytochrome b_5 plus QS-7, 17, 18, or 21 in PBS resulted in an increase of approximately 10^3 in Ag-specific IgG ELISA titers when compared to a control group that received Ag alone. The titers observed in the groups receiving purified saponins were similar to those induced by the MPL/TDM mixture and CFA and IFA. However, purified saponins induced a higher response than aluminum hydroxide (Fig. 3).

Isotype of antibodies augmented by saponins. Adjuvants that augment similar IgG titers may differ considerably in boosting various IgG subclasses. Therefore, the

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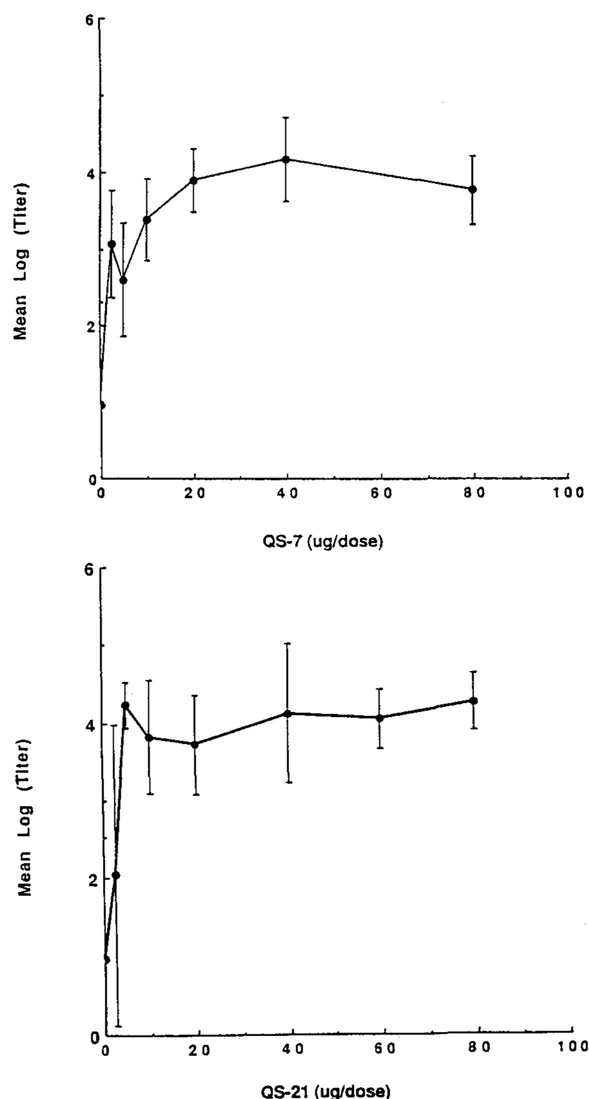

Figure 2. Ag-specific IgG ELISA titers induced in CD-1 mice by two intradermal immunizations with 5 μg BSA and the indicated dose of QS-7 and QS-21. Results are expressed as means ± SD.

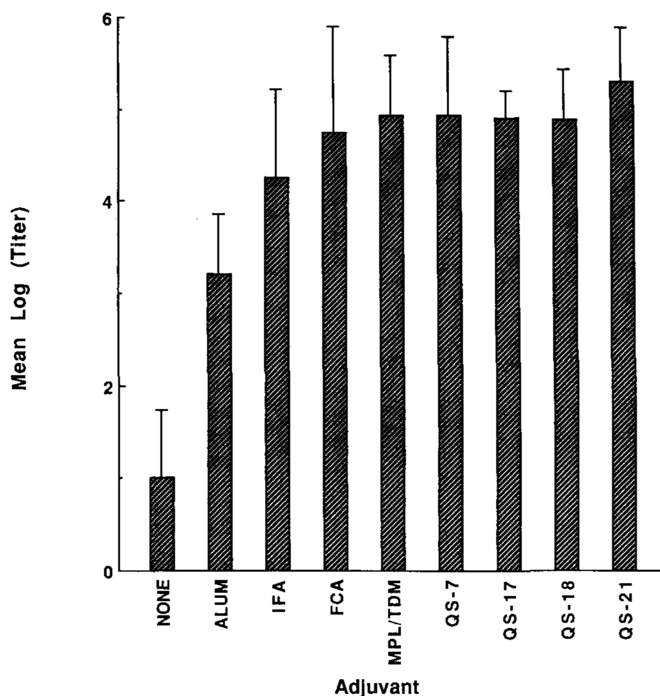

Figure 3. Ag-specific IgG ELISA titers induced in CD-1 mice by two intradermal immunizations with 10 μg cytochrome b_5 and the indicated adjuvant. Formulations adjuvanted with CFA and IFA were prepared by emulsification of 100 μl of Difco CFA or IFA with 100 μl of a PBS/Ag solution/dose. MPL/TDM formulations were prepared by homogenization of 50 μg MPL, 50 μg TDM, 2 μl Squalene, and 0.2 ml 0.2% Tween 20/PBS/Ag/dose. The alum preparation contained 400 μg aluminum hydroxide per dose. The saponin preparations, which were fully soluble in aqueous solution, contained 20 μg of the indicated saponin in 0.2 ml PBS/Ag per dose. Results are expressed as means ± SD.

TABLE I
Adjuvant effect on Ag-specific IgG Subclass

Adjuvant	Subclass Titer/Total IgG Titer[a]		
	G1	$G2_b$	$G2_a$
None	1.00	0	0
QS-7	0.35	0.21	0.44
QS-17	0.07	0.21	0.72
QS-18	0.10	0.06	0.84
QS-21	0.15	0.24	0.61
CFA	0.33	0.39	0.27
IFA	0.92	0.07	0.01
Aluminum hydroxide	0.91	0.09	0
MPL + TDM	0.24	0.38	0.38

[a] Sera were obtained at day 35 from the cytochrome b_5 immunization study described in Figure 3.

TABLE II
Lethality of saponins to CD-1 mice[a]

Dose (μg)	Quil-A	QS-7	QS-18	QS-21
125	1/5	0/5	4/5	0/5
250	2/5	0/5	5/5	0/5
500	4/5	0/5	5/5	1/5

[a] Results are expressed as number of deaths per group of five mice within 72 h after intradermal injection of saponins.

IgG subclass distribution of the IgG for the immunization experiment described in Figure 3 was determined. After two intradermal immunizations with cytochrome b_5 and saponins QS-7, 17, 18, and 21, antibodies were found in the three major IgG subclasses G1, $G2_b$, and $G2_a$ (Table I). With saponin fractions QS-17, 18 and 21, IgG2a antibodies predominated. In contrast, antibodies induced by Ag in PBS or on aluminum hydroxide were predominantly IgG1. CFA and MPL/TDM adjuvant augmented the production of isotypes IgG1, IgG2a, and IgG2b whereas IFA induced isotypes IgG1 and some IgG2b. In contrast to previous reports with the use of crude saponin preparations from *Q. saponaria* (2), no IgE antibodies were elicited by any of the purified saponins described here. Reaginic antibodies were not detectable at a 1/10 dilution for any of the adjuvants tested. The dose dependence of isotype distribution was not determined.

Purified saponins yielded consistent results in adjuvant effect. Five preparations of QS-21 that had been purified from different sources of *Q. saponaria* Molina bark were tested concurrently in an immunization study with BSA in mice; the mean and SD of the $\log_{10}$ ELISA titer of the five groups receiving three injections of 15 μg of QS-21 and 5 μg of BSA was 4.7 ± 0.13 in comparison with the control group which had a titer of 3.6.

Toxic and hemolytic activities. Toxicity (assessed by lethality) has been associated with the use of saponins as adjuvants (20). In effect, the commercial saponin preparation Quil-A was lethal to mice in the dose range of 100 to 125 μg (Table II), as determined with one preparation. The purified saponins described here exhibit a wide range of lethalities. QS-18, the predominant saponin species in

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the bark from *Q. saponaria* as well as in commercial preparations such as Quil-A, is the most lethal of those tested with deaths observed at doses as low as 25 μg (data not shown). In contrast, QS-7 is apparently nonlethal up to 500 μg and QS-21 is lethal only at 500 μg, with one mouse dead out of five mice receiving this dose (Table II). In mice, the minimum lethal dose/adjuvant-effective dose ratio is 50-fold for QS-21 and even higher for QS-7. However, the QS-18 adjuvant-effective dose is close to the lethal dose when assayed in mice. Apparently, the lethal effects of Quil-A can be explained in part by the large fraction of QS-18, which is the predominant component in its composition. The variability of QS-18 content in the bark used to prepare Quil-A and other commercial preparations will explain the differences in lethality observed with different preparations. From these results, we can state that there is no relationship between relative adjuvant activity and relative lethality.

The hemolytic activities of the purified adjuvant-saponins were compared. Saponins QS-17, 18, and 21 caused hemolysis of SRBC at concentrations as low as 5 to 30 μg/ml, with concentrations resulting in 50% hemolysis being 25 ± 0 μg/ml, 15 ± 3 μg/ml, and 7 ± 2 μg/ml, respectively (mean and SD of purified preparations derived from two separate bark samples). However, no hemolysis was observed with QS-7 at concentrations up to 200 μg/ml (highest concentration tested). There is no correlation between hemolytic activity, lethality and adjuvant activity, i.e., QS-7, 18 and 21, have approximately the same adjuvant activity but are widely different in hemolytic activity and lethality.

Carbohydrate composition. Purification of saponins allowed a preliminary structural characterization. The analysis of the composition of the four saponins QS-7, 17, 18, and 21 demonstrated the presence of a highly complex glycoside component, consisting of seven or more monosaccharides in saponin QS-7 and eight or nine monosaccharides in saponin QS-17 (Table III). All four saponins contained components with the same linkage, including terminal rhamnose, xylose, galactose, and glucose residues as well as 3-xylose, 2,3-glucuronic acid, and 3,4-rhamnose (linkage data not shown). It appears that these saponins share a common glycoside structure although there are clear deviations in the carbohydrate composition and linkage of the saponins analyzed.

All saponins contain arabinose except for saponin QS-7. Saponin QS-21 contains a diminished amount of glucose, suggesting that this may be caused by a contaminant as it is present in a ratio significantly lower than 1:1 when normalized to galactose. Monomer size of the predominant saponins was estimated by size exclusion HPLC. For comparison, we used triterpene and triterpene glycoside standards of known m.w. This analysis was carried out in methanol to prevent micellization. The monomer size ranges from 1800 to 2200 and is consistent with the m.w. predicted for a triterpene with 8 to 10 monosaccharide residues. It is likely that monosaccharides galactose, glucose, and glucuronic acid are each present in a ratio of 1.0 mol of monosaccharide/mol of saponin as higher molar ratios would significantly increase the m.w.

TABLE III
Molar ratio of monosaccharide/saponin[a]

Monosaccharide	Saponin			
	QS-7	QS-17	QS-18	QS-21
Rhamnose	2.22	2.34	1.15	1.27
Fucose	0.90	0.96	0.88	0.91
Arabinose	Trace	0.98	0.74	0.77
Xylose	1.28	1.33	1.34	1.44
Galactose	1.00	1.00	1.00	1.00
Glucose	1.35	1.23	1.16	0.35
Glucuronic acid	0.65	0.64	0.72	0.74

[a] Determined as trimethylsilated methyl glycosides and normalized to galactose (assumed to be present at 1 mol/mol of saponin).

DISCUSSION

These results demonstrate that the saponin fraction obtained by aqueous extraction of *Q. saponaria* bark is actually a heterogeneous group of related glycosides. All previous attempts to purify adjuvant-active *Quillaja* saponins have been in aqueous solution by methods typically used to purify proteins, such as dialysis, ion exchange chromatography, and size exclusion chromatography (7). Although these methods are useful in partially separating saponins from nonsaponin components, they have been ineffective in separating individual saponins because of the tendency of saponins to form mixed micelles. Hence, effective separation requires the use of organic solvents or solvent/water systems that solubilize the amphiphilic saponins as monomers so that the formation of mixed micelles does not interfere with separation. In effect, adsorption and reverse phase chromatography in organic solvents as described in *Materials and Methods* has allowed the purification of individual saponins to a degree of homogeneity that is significantly higher than that achieved by earlier reports (7, 24).

Although previous reports suggested that exposure to organic solvents destroyed adjuvant activity (25), we were able to recover adjuvant activity by using organic solvents for silica and reverse phase chromatography. The carbohydrate analysis of the individual saponins described in this paper indicate that they consist predominantly of one component, although some heterogeneity is still present because multiple linkage forms of individual monosaccharides can be detected. The carbohydrate composition and linkage analysis of the purified *Quillaja* saponins are similar to that determined by Higuchi et al. for the hydrolytic breakdown products isolated from a partially purified *Quillaja* saponin preparation (26). Dalsgaard reported that the saponin fraction isolated by anion exchange and gel filtration (Quil-A) contained the monosaccharides xylose, arabinose, glucose, rhamnose, and fructose (25) in unspecified ratios. None of the saponins described in this study contain fructose. In addition, they contain monosaccharide residues not reported by Dalsgaard (fucose, galactose, and glucuronic acid).

Adjuvant activity was demonstrated to be associated with several of the saponins, including those that appear to be most predominant, QS-7, 17, 18, and 21. Hence, the adjuvant activity of *Quillaja* bark extracts is associated with several distinct saponins rather than a single component, although the carbohydrate analysis indicates that these saponins may be structurally related. Not all peaks contained components that could serve as adjuvants in our test system.

Saponins QS-7, 17, 18, and 21 were tested more extensively because they were the predominant peaks in most bark samples analyzed. These fractions typically induced an increase in Ag-specific IgG titers when used at doses

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ranging from 10 to 20 μg in intradermal immunization in mice. The adjuvant effect of these saponins was observed with both BSA and cytochrome b_5. Evidence that close proximity of Ag and saponin are important for the response was shown by our observation that saponin and BSA injected separately into different flanks of the mice did not induce a boost of Ag-specific IgG titers (data not shown), indicating no apparent systemic response. A similar result has been observed by Bomford (8). The strong antibody response elicited by ISCOM, which are reported to be a complex of saponin, Ag, and lipid (11, 12), are consistent with a close association of Ag and saponin being necessary for the adjuvant response. However, the adjuvant effects of saponins cannot be attributed simply to their detergent properties, i.e., saponin QS-7, which is a poor detergent as revealed by its nonhemolytic properties, has adjuvant characteristics similar to QS-17, 18, or 21, which are highly hemolytic.

Purified saponin adjuvants stimulate an equivalent or higher secondary immune response than that obtained by using aluminum hydroxide, CFA and IFA, or MPL/TDM adjuvants. ELISA titers measured via the end point dilution method, as was done in this study, are thought to be proportional to the concentration of high and medium avidity antibodies (27). Therefore, if it is assumed that the ELISA titers reported here reflect the concentration of these populations, then the purified saponins induce quantities of medium and high avidity IgG comparable with CFA, IFA, and MPL/TDM, and higher than those induced by aluminum hydroxide. However, differences in the concentrations of low avidity antibodies cannot be ruled out. Saponins also influence the Ag-specific isotype profile. A comparison of isotypes produced by mice in response to immunization with purified saponin showed induction of the three major IgG subclasses, G1, $G2_b$, and $G2_a$. The isotype profile observed with these purified saponins differs from that reported by Allison and Byars with a crude saponin (2) in which they found predominantly an IgG1 response to immunization of mice with Ag and crude saponin mixture, a response similar to that elicited by aluminum hydroxide. Under the immunization conditions utilized in this study, saponins induced significant levels of IgG2a and IgG2b as well as G1 antibodies; for some saponins, IgG2a predominated. Ag-specific IgE was not detected, even with the highly toxic QS-18, indicating that other components in crude preparations are responsible for the production of reaginic antibodies.

The high level of protection observed with the use of saponins with vaccines in mice (1) may in part be caused by the ability of saponins to induce an isotype profile similar to that observed in natural immunity arising from a viral or bacterial infection. Viral infections in mice induce an IgG response in which IgG2a accounts for 65 to 92% of total specific antibody (28). IgG2a has also been shown to be protective against protozoal infections (29). Both C fixation and antibody-dependent cellular cytotoxicity in mice can be mediated by IgG2a antibodies (30).

Commercially available saponin preparations are highly heterogeneous mixtures of adjuvant-active and inactive components. The relative concentrations of these components will vary according to the source of the bark, leading to difficulty in preparation of batches with a consistent composition. Substantial variation has been noted between different sources of commercially available saponins (31, 32). Purified saponins can be readily standardized, and this property allows preparation of vaccines with known proportions of a given active saponin or saponins.

The use of purified saponins for immunization allows selection of saponins with the optimal combination of adjuvant activity and negligible lethality. Preliminary studies indicate that some adjuvant-active saponins are significantly more lethal than others when tested at doses over the range of 25 to 500 μg in mice. It may be possible to select an adjuvant-active saponin for use in a vaccine that provides a wider safety margin between adjuvant-active and lethal doses than that in crude saponin extracts (which contains a larger fraction of lethal saponin adjuvants such as QS-18).

No attempt was made to correlate saponin structure with the biologic effects, adjuvant activity, and lethality associated with *Quillaja* saponins. A complete structural determination will involve sequencing of the glycoside moieties, identification of the triterpene, and identification of the point of linkage of the glycoside moieties onto the triterpene backbone. Comparison of the complete structures of naturally occurring variants such as those described here will provide information on what parts of the structure are involved in specific biologic activities. Further information on the minimal structure involved in these activities can be gained by analysis of less complex saponins produced by specific chemical or enzymatic hydrolysis of saponins of known structure. These studies are ongoing.

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