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Oral QS-21 Requires Early IL-4 Help for Induction of Mucosal and Systemic Immunity

Prosper N. Boyaka, Maria Rosaria Marino, Raymond J. Jackson, Frederik W. van Ginkel, Estelle Cornet-Boyaka, Kevin L. Kirk, Charlotte R. Kensil, and Jerry R. McGhee

The highly purified saponin derivative, QS-21, from the Quillaja saponaria Molina tree has been proved to be safe for parenteral administration and represents a potential alternative to bacterial enterotoxin derivatives as a mucosal adjuvant. Here we report that p.o. administration of QS-21 with the vaccine protein tetanus toxoid elicited strong serum IgM and IgG Ab responses, which were only slightly enhanced by further oral immunization. The IgG Ab subclass responses were predominantly IgG1 followed by IgG2a and IgG2b for the 50-µg p.o. dose of QS-21, whereas the 250-µg p.o. dose also induced IgG2a and IgG3 Abs. Low oral QS-21 doses induced transient IgE Ab responses 7 days after the primary immunization, whereas no IgE Ab responses were seen in mice given the higher QS-21 dose. Further, low but not high p.o. QS-21 doses triggered Ag-specific secretory IgA (S-IgA) Ab responses. Th1 cell responses showed higher IFN-γ (Th1-type) and lower IL-5, IL-6, and IL-10 (Th2-type) secretion after the high QS-21 p.o. dose than after low doses. Interestingly, the mucosal adjuvant activity of low oral QS-21 doses was diminished in IL-4−/− mice, suggesting a role for this cytokine in the initiation of mucosal immunity by oral QS-21. In summary, our results show that oral QS-21 enhances immunity to coadministered Ag and that different doses of QS-21 lead to distinct patterns of cytokine and serum Ab responses. We also show that an early IL-4 response is required for the induction of mucosal immunity by oral QS-21 as adjuvant. The Journal of Immunology, 2001, 166: 2283–2290.
Oral QS-21 also exhibits a dose-dependent mucosal adjuvancy which requires early IL-4 help. Finally, the dose-dependent pattern of immune responses induced by oral QS-21 as adjuvant is discussed with regard to the induction of targeted immune responses.

Materials and Methods

Mice

Specific pathogen-free C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA). IL-4-deficient (IL-4−/−) mice (28) were bred in the UAB Immunocompromised Mouse Facility of the Immunology Vaccine Center. All mice were maintained in horizontal laminar flow cabinets under specific pathogen free conditions. The mice were used at 8–12 wk of age in all experiments and were free of bacterial and viral pathogens as determined by routine Ab screening and by histological analysis of major organs and tissues of sentinel mice.

Immunization procedures

The saponin-derived QS-21 adjuvant was given together with vaccine grade tetanus toxoid (TT) (Connaught Laboratories, Swiftwater, PA) or with OVA (Sigma Chemical Co., St. Louis, MO) p.o. or by the parenteral route. Before oral immunization, groups of mice were deprived of food for 2 h and then given an isotonic bicarbonate solution (HBSS-7.5% sodium bicarbonate, 8.2) intragastrically to neutralize stomach acidity (24, 29). After 30 min, individual mice were gavaged with 0.25 ml PBS (pH 7.2) solution containing TT (250 μg/mouse) or OVA (1 mg/mouse) and increasing doses of QS-21. For parenteral immunization, mice received TT (50 μg/mouse) and QS-21 (20 μg/mouse) s.c. in a final volume of 200 μl. Groups of five to seven mice were immunized either p.o. or s.c. with TT (or OVA) plus QS-21 on days 0, 7, and 14, and fecal pellets and blood samples were collected as previously described (24, 29).

Analysis of Ab isotypes and IgG subclasses

An ELISA was used to titrate Abs levels in serum and mucosal secretions (24, 27, 29). Briefly, 96-well microtiter plates (Microtest III; Becton Dickinson, Oxnard, CA) were coated with a 100 μl solution of TT (5 μg/ml) 1.25 Limes flocculation U (LF U/ml), and serial 2-fold dilutions of serum or mucosal secretion were added to individual wells. Concentrations of IgM, IgG, or IgA Abs were determined by addition of a 1/30000 dilution of HRP-conjugated goat anti-mouse γ-, µ-, or α-heavy-chain-specific antisera (Southern Biotechnology Associates, Birmingham, AL). To determine IgG subclass titers, we used biotin-conjugated rat monoclonal anti-mouse γ1 (GI 7.3; 2 μg/ml), γ2a (R19-15; 1 μg/ml), γ2b (R12-3; 0.5 μg/ml) or γ3 (R40-82; 1 μg/ml) heavy-chain-specific Abs (Pharmingen, San Diego, CA), followed by HRP-conjugated streptavidin (Life Technologies, Gaithersburg, MD) (24, 30). The color was developed at room temperature for 15 min with ABTS substrate (Sigma). The color was developed at room temperature for 15 min with ABTS substrate (Sigma), and the absorbance was measured at 415 nm. Endpoint titers were determined as the last dilution exhibiting an OD of ≥0.1 when compared with negative controls.

Total serum IgE levels and Ag-specific IgE Abs were determined by a sensitive ELISA and a passive cutaneous anaphylaxis assay, respectively (24, 30). For total IgE measurements, Nunc Immuno-MaxiSorp plates were coated with 2 μg/ml rat monoclonal anti-mouse IgE Ab (PharMingen; R35-72). Serial dilutions of immune serum or standard mouse IgE (PharMingen) were then added followed by addition of 100 μl of a biotinylated rat monoclonal anti-mouse IgE Ab (PharMingen; R35-92). Streptavidin-conjugated HRP was used for detection as described above.

B cell enzyme-linked immunosorbent assay for IgA Ab-forming cells (AFC)

An enzyme-linked immunosorbent assay was used to quantitate numbers of IgA AFCs present in the lamina propria of the small intestine of mice immunized p.o. with TT (or OVA) and QS-21 as adjuvant (3, 24, 29). The lamina propria cells were isolated as previously described (3, 24, 29) and were >98% viable as determined by trypan blue dye exclusion. Isolated cells were resuspended in complete medium (RPMI 1640; Cellgro Medi-atech, Washington, DC) containing 10% FCS, 2 mM l-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin. For the evaluation of Ag-specific IgA AFCs, 96-well nitrocellulose-based plates were coated with a 100-μl solution of TT (5 μg/ml) or OVA (500 μg/ml) diluted in PBS and control wells received PBS. The wells were blocked with 1% BSA in PBS. Serial 5-fold dilutions (starting at 1 × 105 cells) were added to the wells in duplicate and incubated for 6 h at 37°C in 5% CO2 in air. Individual AFC were detected with peroxidase-labeled anti-mouse α-chain-specific Abs (1 μg/ml) (Southern Biotechnol-ogy Associates) and were visualized by adding the chromogenic substrate, 3-aminio-9-ethylcarbazole (Moss, Pasadena, MD). Individual AFCs were counted with the aid of a dissecting microscope (SZH Zoom Stereo Microscope System; Olympus, Lake Success, NY).

Assessment of Ag-specific CD4+ T cell responses

Single-cell suspensions of Peyer’s patches and spleens from immunized and control mice were obtained as previously described (3, 24, 27, 30). Splenic or Peyer’s patch CD4+ T cells were purified by using Mouse CD4 Collect Plus columns (Biotex Laboratories, Edmonton, Ontario, Canada) as described by the manufacturer. These CD4+ T cell preparations were >96% pure and contained ≤1% red blood cells and cells from a naive mouse. Brisk immunofluorescence staining using biotin-labeled anti-mouse IgG, IgM, and IgD Abs and OVA-adsorbed latex microspheres were analyzed by flow cytometry. For the direct analysis of Ag-specific T cell responses, 0.5 μg/ml phosphorylated (0.5 μg/ml) hen egg lysozyme (PharMingen), and rIL-4 (Endogen, Boston, MA) were added after 6 days of culture and 16 h before harvest. Approximately 10 TT-coated particles/cell optimally stimulated CD4+ T cell cultures based on the extent of cellular proliferation as well as levels of cytokines produced in culture supernatants. For the assessment of cytokine production, culture supernatants were harvested after a 6-day incubation period. Control wells contained cells only, cells incubated with unadsorbed beads, or cells incubated with beads coated with an irrelevant protein (i.e., hen egg lysozyme). All cell cultures were maintained at 37°C in 5% CO2 in air.

Cytokine-specific ELISA

Th1 and Th2 cytokine levels in culture supernatants were determined by ELISA using a cytokine-specific ELISA as previously described (3, 26, 27, 30). Briefly, Falcon Microtest III plates (Becton Dickinson, Oxnard, CA) were coated with 100 μl anti-cytokine mAb diluted in 0.1 M bicarbonate-TT or OVA-adsorbed latex microspheres or 1 mg/ml OVA as described previously (3, 24, 27, 30). CD4+ T cells (2 × 106 cells/ml) were cultured with rIL-2 (10 U/ml, PharMingen), and T cell-depleted, irradiated splenic feeder cells from naive mice in flat-bottom 96-well (200 μl/well) or 24-well (1 ml/well) tissue culture plates (Corning Glass Works, Corning, NY) for 4 days and then harvested for cytokine analysis. Supernatants were isolated, and the levels of cytokines in the supernatants were determined by using commercially available cytokine-specific ELISA kits.

Analysis of IL-4 by Western blotting

IL-4 production by Ag-specific splenic, Peyer’s patch, and mesenteric lymph node CD4+ T cells was also analyzed by Western blotting. Briefly, CD4+ T cells were isolated 5 days after one immunization and restimulated in vitro as described above. Cultures of CD4+ T cells were treated with monensin (GolgiStop; PharMingen) for the last 5 h before harvest. Cells were then washed three times in PBS and lysed in PBS containing 0.5% Triton X-100, 1 mM PMSE, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml apronin. Equal amounts of cell lysates (16 μg/lane) were subjected to electrophoresis in 15% SDS gel and Western blotting was performed on protein transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). IL-4 was detected by biotinylated rat anti-mouse IL-4 (clone BV6D6-24G2; PharMingen) followed by peroxidase-labeled anti-biotin Ab (Vector) and the reaction revealed with an ECL detection system (NEN Life Science Products, Boston, MA).

Tetanus toxin challenge

Tetanus toxin was obtained from Drs. Jean Halpern and William Habig (Division of Bacterial Products, Food and Drug Administration, Bethesda, MD). The toxin was diluted in gelatin-saline, and appropriate doses were injected s.c. into normal or immunized mice. The mice were monitored daily for paralysis and death.

The results are expressed as the mean ± SD. Statistical significance (p ≤ 0.05) was determined by Student’s t test and by ANOVA followed by the Fisher least significant difference test. For statistical analysis, cytokine levels below the detection limit were recorded as one-half the detection limit (e.g., IPN-γ = 7.5 pg/ml).
Results

Effects of oral QS-21 doses on serum Ab responses

We first determined the effects of increasing oral QS-21 doses on serum Ab responses to coadministered proteins. Mice immunized p.o. with 250 μg TT and QS-21 doses lower than 25 μg/dose did not enhance TT-specific serum Ab responses when compared with mice given the protein alone (Fig. 1A). When a QS-21 dose of 25 μg or higher was given p.o. with TT, significant increases in serum Ag-specific IgM and IgG Ab levels were seen 7 days later. By day 21, comparable serum IgG Ab response were seen in mice that received 25, 50, or 250 μg oral QS-21 (Fig. 1A). In contrast to IgM and IgG Ab responses, significant serum IgA Ab responses were not seen until day 21 and were highest in groups that received the 50-μg p.o. dose of QS-21 (Fig. 1B), suggesting that different oral doses of QS-21 promote distinct Ab isotype profiles to coadministered Ag. The IgE Ab responses were also differentially regulated by oral QS-21 doses. In this regard, enhanced total and Ag-specific IgE Abs were noted 7 days after the primary oral immunization with 50 μg QS-21. These IgE responses declined rapidly and were not detectable by day 14 or 21. (Table I). No IgE Ab responses were seen at days 7, 14, or 21 in serum of mice that received 250 μg QS-21 (Table I).

The pattern of IgG subclass and IgE Ab responses have been shown to reflect the involvement of distinct T cell subsets and cytokine pathways in the development of humoral immunity (31). To determine patterns induced by oral QS-21, the TT-specific IgG subclass responses were assessed. Mice immunized p.o. with QS-21 doses of 10 μg developed only IgG1 Abs that were comparable to those induced by p.o. administration of protein Ag alone (Fig. 2). When oral QS-21 doses were increased to 25 or 50 μg, IgG1 Abs were significantly increased when compared with mice that received the Ag alone, and strong IgG2b Ab responses were also noted (Fig. 2). The pattern of IgG subclass response was different in mice that received 250 μg of QS-21. IgG1 Abs remained

Table I. Total and Ag-specific serum IgE responses after p.o. immunization with TT and increasing doses of QS-21

<table>
<thead>
<tr>
<th>Days After Initial Immunization</th>
<th>Dose of p.o. QS-21</th>
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<tbody>
<tr>
<td></td>
<td>0 μg</td>
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<tr>
<td></td>
<td>7</td>
</tr>
<tr>
<td>Total IgE (ng/ml)</td>
<td>&lt;150</td>
</tr>
<tr>
<td>TT-specific IgE (PCA titers)</td>
<td>&lt;3</td>
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*a Mice were immunized p.o. on days 0, 7, and 14 with TT and the indicated doses of QS-21 as adjuvant. Total and TT-specific serum IgE levels were determined by ELISA and passive cutaneous anaphylaxis assay (PCA), respectively, as indicated in Materials and Methods. Data are from four to five mice per group and are representative of three separate experiments.
predominant followed by comparable levels of IgG2a and IgG2b anti-TT Ab responses. The 250-µg oral dose of QS-21 also induced significant IgG3 Abs (Fig. 2). Taken together, these results imply that distinct T and B cell pathways may be involved in serum Ab responses induced by oral QS-21 doses. In particular, IgE and IgG1 Abs have been shown to be dependent on IL-4 and Th2-type cytokines, while IFN-γ promotes IgG2a Ab responses (31). Our results suggest that low QS-21 doses may induce Th2-type responses while higher may be associated with mixed Th1/Th2-type responses.

Effects of oral QS-21 doses on mucosal IgA Ab responses
Because orally administered QS-21 enhanced systemic Ab responses to coadministered proteins, we asked whether this adjuvant effect of QS-21 extended to the induction of S-IgA Abs. Significant Ag-specific S-IgA Ab responses were detected in fecal extracts of mice that received either 25 or 50 µg oral QS-21 per dose (Fig. 3A). No significant S-IgA Abs were detected in mucosal secretions of mice that received the 250-µg QS-21 p.o. dose. These results, which were further confirmed by the analysis of Ag-specific IgA-secreting cells in the lamina propria (Fig. 3B), indicate that orally administered QS-21 promotes mucosal immune responses and that this attribute decreases when high doses of QS-21 are administered. Because no histological signs of intestinal pathology were seen 24 or 48 h after p.o. administration of 500 or even 1000 µg QS-21 (data not shown), the difference in the mucosal adjuvanticity between low vs high QS-21 oral doses is unlikely due to a disruption of the epithelial barrier.

Th cell responses after oral administration of low vs high QS-21 doses
To address the nature of T cell help induced by different oral doses of QS-21, we analyzed the cytokine patterns in cultures of restimulated CD4+ T cells isolated at day 21 from Peyer’s patches or spleen of mice immunized p.o. with 50 or 250 µg QS-21. These two doses of QS-21 were selected as examples that either support or fail to induce S-IgA Abs, respectively. CD4+ T cells from mice immunized with 50 µg QS-21 secreted large amounts of IFN-γ (Th1-type cytokine) after in vitro restimulation with TT (Fig. 4A). Careful comparison of IFN-γ secretion by TT-specific CD4+ T cells revealed higher synthesis of IFN-γ by cells isolated from the Peyer’s patches when compared with TT-specific spleen CD4+ T cells (Fig. 4A). The TT-specific CD4+ T cells isolated from mice given 250 µg QS-21 p.o. also secreted large quantities of IFN-γ after in vitro restimulation with TT. Further, comparable IFN-γ levels were secreted by CD4+ T cells isolated from the mucosal (i.e., Peyer’s patches) or the systemic compartment (i.e., spleen) (Fig. 4A). We found no significant IL-4 secretion in culture supernatants of cells from mice that received the 50 or 250 µg QS-21 dose (Fig. 4A). When secretion of other Th2-type cytokines was analyzed, we noted enhanced IL-5 secretion by TT-specific spleen CD4+ T cells as well as enhanced IL-6 and IL-10 secretion by both Peyer’s patch and spleen CD4+ T cells from mice that received the 50 µg QS-21 p.o. dose. Secretion of IL-5, IL-6, and IL-10 by TT-specific Peyer’s patch and spleen CD4+ T cells from mice

FIGURE 2. Patterns of serum IgG subclass Ab responses in mice given increasing doses of oral QS-21 as adjuvant. Groups of mice received TT plus various doses of QS-21 and the IgG subclass Ab responses were determined by ELISA on day 21 after the initial immunization. The results are expressed as the mean ± SD of individual responses from five separate experiments.

FIGURE 3. Mucosal IgA anti-TT Ab responses in mice given oral TT plus increasing QS-21 doses. A, Fecal extracts were collected 21 days after the primary immunization for evaluation of S-IgA Ab responses. The results are expressed as the mean log2 titers ± SD and are from five separate experiments. B, Lamina propria cells were isolated 21 days after the primary immunization for evaluation of TT-specific IgA AFCs. The results are expressed as the mean ± SD and representative of four separate experiments.
given the 250-μg QS-21 p.o. dose was either slightly enhanced or unchanged when compared with control mice given TT only.

The IgE Ab response at day 7 and the pattern of IgG subclass responses measured at day 21 in mice that received low oral QS-21 doses strongly suggest that IL-4 was produced by Ag-specific CD4$^+$ T cells. However, we were also unable to detect IL-4 in culture supernatant of restimulated CD4$^+$ T cells isolated at days 3, 5, or 7 (data not shown). It is possible that IL-4 could be produced and subsequently bound immediately making its detection difficult. To test this possibility, cultures of CD4$^+$ T cells were treated with monensin, and IL-4 was analyzed in cell lysates. Using this method, we detected IL-4 in lysates of Peyer’s patch, mesenteric lymph node (MLN), and splenic (SP) CD4$^+$ T cells from mice given oral TT plus 50 or 250 μg QS-21 were isolated at days 5 or 15 after the initial immunization and restimulated in vitro. Cell cultures were treated with monensin (GolgiStop), and IL-4 was analyzed in cell lysates by Western blotting (16 μg/lane). Results are representative of three separate experiments.

The absence of endogenous IL-4 alters the mucosal adjuvant activity of oral QS-21

To confirm the role of IL-4 in the mucosal adjuvanticity of oral QS-21, IL-4 gene knockout (IL-4$^{-/-}$) mice and age-matched control (IL-4$^{+/+}$) littermates were immunized p.o. with 250 μg TT and 50 μg QS-21. An analysis of serum Ab responses at day 21 showed reduced TT-specific serum IgA and IgG in IL-4$^{-/-}$ mice when compared with control littermates (IL-4$^{+/+}$ mice) (Fig. 5A). The reduced Ag-specific IgG Ab responses were associated with reduced IgG1 and IgG2b responses, whereas IgG2a and IgG3 Abs were present (Fig. 5B).

We also tested mucosal S-IgA Ab responses in IL-4$^{-/-}$ mice immunized p.o. with TT and 50 μg QS-21 as adjuvant. Interestingly, TT-specific S-IgA Ab responses were significantly reduced in fecal extracts of IL-4$^{-/-}$ mice when compared with control littermates. Further, whereas control mice orally immunized with this dose of QS-21 showed low levels of S-IgA Ab responses in vaginal washes, no TT-specific S-IgA Ab responses were detected in vaginal washes of IL-4$^{-/-}$ mice (Fig. 6).

Both low and high oral QS-21 doses induce protective immunity

Because different patterns of systemic immune responses were induced by low or high oral QS-21 doses, it was important to determine whether these Ab responses resulted in protective immunity. We addressed this question by challenging with tetanus toxin mice that had been immunized p.o. with TT and QS-21. Mice immunized with TT alone did not survive the challenge. However, mice immunized with 50 or 250 μg QS-21 were completely protected and survived the challenge (Table II).
FIGURE 5. Serum Ab responses in IL-4−/− mice immunized p.o. with QS-21 as adjuvant. Mice deficient in IL-4 (■) and control littersmates (□) were given oral TT and 50 μg QS-21. Ig isotype (A) and IgG subclass (B) responses were determined 21 days after the initial oral immunization. The results are expressed as the mean reciprocal log2 titers ± SD of individual responses from three separate experiments.

Discussion

The saponin derivative QS-21 has proved to be an effective adjuvant for enhanced serum Ab and CTL responses when given with a number of parenteral vaccine formulations (13, 32–34). However, parenterally administered vaccines do not stimulate mucosal IgA inductive sites for mucosal immunity. In addition, few formulations have been shown to exhibit mucosal adjuvanticity when administered p.o. A number of new findings regarding QS-21 as an adjuvant including its ability to induce both mucosal and systemic immunity when given orally have emanated from this study. We report that orally administered QS-21 enhances systemic immunity to coadministered protein Ag and that distinct Th cell subsets and cytokine pathways are involved in the adjuvant activity of low (25 or 50 μg) vs high (250 μg) oral QS-21 doses. We also report that low oral QS-21 doses induce S-IgA Ab responses, a feature that was dependent on early IL-4 help.

Extensive studies by our group have shown that multiple oral doses of CT as adjuvant were required for the induction of optimal serum IgG Ab responses to the protein vaccine TT (24, 29). The results reported here show that peak serum IgG Ab responses were induced by a single oral immunization with a low QS-21 dose and that further immunization only slightly enhanced these Ab responses. In separate experiments, we also found no significant differences in serum IgG Ab levels induced by a single oral QS-21 dose when compared with multiple delivery of this adjuvant; however, multiple oral QS-21 doses are needed for the induction of mucosal IgA responses (data not shown). Approximately 10-fold higher levels of CT are required as oral adjuvant when compared with the parenteral or nasal route (3, 8, 24, 29). Studies by others have shown that QS-21 doses of 10–20 μg were optimal for enhancing immune responses to parenteral vaccines in animal models (10, 12, 13, 15, 34). Interestingly, oral QS-21 enhanced systemic immunity to the coadministered Ag with doses only 1.5- to 3-fold higher than those required for systemic immunization. Further, these doses of oral QS-21 protected mice immunized with TT against systemic challenge with tetanus toxin (Table II).

To precisely characterize the nature of immune responses induced by oral QS-21 doses, we also analyzed the pattern of serum IgG subclasses. Interestingly, different doses of oral QS-21 elicited distinct IgG subclass responses, where low doses (i.e., 25 and 50 μg) induced IgG1 and IgG2b Abs, whereas high (i.e., 250 μg) doses resulted in IgG1, IgG2a, IgG2b, and IgG3 Ab responses. This finding has important implications for induction of immune responses that protect from either intracellular or extracellular pathogens. For example, in the mouse, IgG2a and IgG3 are opsonic and participate in complement-mediated killing, whereas IgG1 does not activate complement by the classical pathway but is effective in neutralization of exotoxins and extracellular bacteria. Both IgG1 and IgG2a Ab responses were consistently reported after parenteral immunization with protein or polysaccharide Ags given with QS-21 (11, 12, 33, 34). More variable results were reported for IgG2b Abs with either their presence (12) or absence after parenteral QS-21 delivery. Because no IgG2a Abs are induced by low p.o. QS-21 doses, this route of QS-21 delivery may provide a way to induce immune responses that differ from those achieved by parenteral vaccines.

Cytokines produced by Th cell subsets control Ab isotypes and subclasses with Th1-type cytokines (i.e., IFN-γ) supporting IgG2a and IgG3, whereas IL-4 and other Th2-type cytokines provide help for IgG1 and IgE Abs (31, 35). Consistent with the IgG1 and IgG2b Ab subclass responses and Th2-type responses in mice that received low oral QS-21 doses, significant IgE Ab responses were detected at earlier time points in these mice. The generation of IgE Ab responses is generally associated with the production of IL-4. The transient nature of IgE responses with retention of a Th2-type IgG subclass profile suggested that low doses of QS-21 may stimulate a transient burst of IL-4. In this regard, in addition to CD4+ Th cells, NK cells (NK1.1) and mast cells also secrete IL-4 on stimulation (36, 37). Of interest, IL-4−/− mice immunized p.o. with 50 μg QS-21 showed reduced IgG1 and IgG2b but enhanced IgG2a and IgG3 Ab responses. This observation strongly suggests
that IL-4 was induced after the initial immunization and supported IgE as well as IgG1 and IgG2b Ab subclass responses.

QS-21 is a chemically defined triterpene glycoside (38). Various structure/function studies have been conducted with parenterally administered QS-21. The aldehyde group on the triterpene backbone is thought to be involved in the QS-21-enhancement of immune responses (10). QS-21 is esterified at C4 of fucose (38), and this ester bond has been shown to undergo a reversible intramolecular acyl migration under neutral pH conditions to form a minor isomer esterified at C3 (39). The esterification of QS-21 may be important for activity because QS-21 deesterified by alkaline hydrolysis abolishes adjuvanticity for both Ab and CTL responses (40). No study has investigated whether QS-21 could resist degradation in the gastrointestinal tract and retain adjuvant activity after oral administration. The addition of the less pure saponin derivative Quil A, which contains up to 23 different saponins (9), in immune-stimulating complexes was reported to enhance immune responses to Ag by the oral route (41). However, immune-stimulating complexes, which are cagelike particles, could protect the saponin derivative from degradation in the intestine. Our observation that orally administered QS-21 enhanced immune responses clearly suggests that this highly purified saponin resists degradation in the intestinal environment and can be incorporated into aqueous oral vaccine formulations.

Because mucosal immunization is critical in the induction of mucosal immunity, it was important to determine whether oral delivery of QS-21 resulted in S-IgA Ab responses. Low oral QS-21 doses supported Ag-specific S-IgA Ab levels comparable with those previously reported after oral immunization with the classical mucosal adjuvant CT (24, 29). In contrast, S-IgA Abs were not induced by high oral QS-21 doses. The reasons for this differential effect of low vs high oral QS-21 doses on mucosal immunity are discussed in more detail below. In separate studies, we noted that nasal administration of QS-21 induced Ag-specific IgA Ab responses in mucosal secretions (data not shown), adding support to the ability of mucosally administered QS-21 to trigger mucosal immunity. Further, histological examination of small intestine at 24 or 48 h after oral administration of 500 or 1000 μg of QS-21 showed no architectural changes or histopathological features, suggesting that the absence of mucosal immunity in mice that received 250 μg of QS-21 was not due to tissue necrosis or pathology (data not shown). Previous studies on the stability of the QS-21 formulation suggested that the molecule was stable at the pH of the intestinal environment (39). Thus, it is possible that the increased lipophilicity of high QS-21 doses results in absorption of this adjuvant at distant sites from mucosal inducive sites (i.e., Peyer’s patches). It is also possible that high QS-21 doses in the mucosal inducive site activates different pathway(s) than those resulting in S-IgA Ab responses. Alternatively, they may provide negative signals for S-IgA Ab responses.

To address potential mechanisms involved in the ability of low but not high oral QS-21 doses to mediate mucosal immunity, we analyzed the pattern of cytokines produced by Ag-specific CD4⁺ T cells isolated from mice immunized with low or high oral doses of QS-21. Both low and high oral QS-21 doses induced Ag-specific CD4⁺ T cells secreting IFN-γ (i.e., Th1-type); however, no significant Th2-type cytokines were noted in culture supernatants from high dose QS-21-induced Ag-specific CD4⁺ T cells when compared with unstimulated control cultures. This pattern of Th cell subsets is consistent with the reported ability of parenterally administered QS-21 to induce CTLs (10, 13, 33, 34, 42) and the pattern of IgG subclass responses induced by high oral QS-21 doses. One likely explanation for our results is that the high QS-21 doses induce hyperresponsive Th1-type responses, which are characterized by exaggerated DTH responses. Thus, high Th1 responses and IFN-γ synthesis provides less effective help for B cell responses, including the B cell subsets that give rise to IgA responses. In this regard, S-IgA Ab responses were reported after immunization with vaccine formulations that promote either Th1- (26) or Th2-type responses (23–25, 30). However, regimens that induce exaggerated DTH responses are not optimal for provision of help for B cells and IgA Ab responses.

It was of interest that both a Th2-type-associated IgG subclass pattern and a mixed Th1- and Th2-type cytokine profile were induced by the low oral doses of QS-21 that supported mucosal S-IgA Ab responses. We have shown that Ag-specific CD4⁺ T cells produce IL-4 after one low oral QS-21 dose, a feature that was lost after additional oral QS-21 doses. Our results in IL-4−/− mice clearly show that IL-4 controlled the Th2-type-associated IgG subclass responses induced by low oral doses of QS-21. Taken together, our findings suggest that the IgG subclass pattern that occurs at day 21 results from an initial IL-4 response. Further, IL-4 was required for the mucosal adjuvant activity of oral QS-21 because low oral QS-21 doses failed to induce S-IgA responses in IL-4−/− mice. These observations are also consistent with the absence of mucosal S-IgA after oral immunization with the high dose of QS-21 that induced strong Th1-type responses. Studies

![Image](http://www.jimmunol.org/DownloadedFrom/2017/11/17/2289)

**FIGURE 6.** Mucosal S-IgA Ab responses in IL-4−/− mice orally immunized with QS-21 as adjuvant. Mice deficient in IL-4 (■) and control littermates (□) were given oral TT and 50 μg QS-21. Three weeks after the initial immunization, S-IgA Ab responses were measured in mucosal secretions. The results are expressed as the mean reciprocal log₂ titers ± SD and are from three separate experiments.

<table>
<thead>
<tr>
<th>Dose of p.o. QS-21</th>
<th>Serum IgG (reciprocal log₂ titers)</th>
<th>Tetanus toxin (μg/mouse)</th>
<th>No. of survivors/group</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 μg</td>
<td>11 ± 0.7 11 ± 1.0 18 ± 1.0 19 ± 0.5</td>
<td>1 100 100 100</td>
<td>0/5 0/3 5/5 3/3</td>
</tr>
<tr>
<td>50 μg</td>
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<tr>
<td>250 μg</td>
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</table>
are under way to determine the mechanisms that control the induction of distinct Th cell-derived cytokine responses by low or high oral doses of QS-21.

We have shown that oral administration of the saponin derivative QS-21 can induce distinct patterns of serum Ab systemic immune responses which are determined by the dose of QS-21 administered. We have also shown that appropriate oral QS-21 doses result in S-IgA Ab responses that are comparable with those induced by the well-characterized mucosal adjuvant CT. The targeted immune responses induced by low vs high oral QS-21 doses and the fact that QS-21 is not immunogenic (32) have important implications for the design of future oral vaccine formulations.

References


