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# Generation of Atypical Pulmonary Inflammatory Responses in BALB/c Mice after Immunization with the Native Attachment (G) Glycoprotein of Respiratory Syncytial Virus

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**The feasibility of using the highly purified native attachment (G) protein in a subunit vaccine against respiratory syncytial virus (RSV) was examined in a murine model with or without the fusion (F) protein of RSV and the adjuvant QS-21. The studies established that QS-21 was more potent than AlOH as an adjuvant for both F and G glycoproteins. Augmented antigen-dependent killer cell activity and complement-assisted serum neutralizing and anti-F and G protein immunoglobulin G2a antibody titers were observed. Immunization with G/QS-21 generated immune responses that were characterized by low levels of antigen-dependent killer cell activity, elevated levels of interleukin-5 (IL-5) and percentages of eosinophils in the bronchoalveolar lavage fluids after challenge, and splenic immunocytes that secreted IL-5 but not gamma interferon (IFN-γ) after in vitro stimulation with purified whole virus antigens. The pulmonary eosinophilia was similar to that induced by a facsimile of a formalin-inactivated vaccine used in previous clinical trials and was prevented by prior in vivo treatment with anti-IL-5 but not with control immunoglobulin G or anti-IFN-γ neutralizing monoclonal antibodies. Thus the data implied that vaccination with G/QS-21 generated helper T-cell immune responses that were type 2 in nature. Alternatively, the data suggested that the helper T-cell immune responses elicited by F/QS-21 were more type 1 in character. Neither eosinophilia nor elevated levels of IL-5 were observed in the lungs of mice after challenge. Noteworthy levels of antigen-dependent killer cell activity was observed, and splenic immunocytes secreted copious quantities of IFN-γ. Immunization with a combination vaccine composed of highly purified native F and G proteins plus QS-21 (F+G/QS-21) resulted in augmented complement-assisted serum neutralizing antibody titers compared with vaccination with either F/QS-21 or G/QS-21 alone. However, following vaccination with F+G/QS-21, the bronchoalveolar lavage fluids contained significant increases in IL-5 and percentages of eosinophils after challenge, the spleen cells appeared to secrete less IFN-γ after in vitro stimulation, and there was no evidence of increased numbers of antigen-dependent killer cell precursors. Taken together, the data imply that native G protein influences the nature of the immune responses elicited by F/QS-21. The results therefore suggest that G, not F, protein has more potential to bias the host for atypical pulmonary inflammatory responses.**

Pulmonary disease caused by respiratory syncytial virus (RSV) is the primary reason for the hospitalization of infants less than 6 months of age (5, 6, 46). In very young infants, or infants and children with underlying abnormalities (e.g., congenital heart disease, bronchopulmonary dysplasia, or cystic fibrosis), RSV is the major causative agent for bronchopneumonia, bronchiolitis, and pneumonia. Because of either low immunogenicity and/or for safety reasons, previous attempts to formulate a vaccine to prevent RSV-mediated disease in the lower respiratory tract have not been successful (3, 6, 25, 26, 48). The most noteworthy of these endeavors was the parenteral administration of virus inactivated by formalin treatment and adsorbed to alum adjuvant (23, 27). The experience with the formalin-inactivated RSV (FI-RSV) vaccine was of consequence primarily because atypical disease was observed in a significant number of seronegative infants on subsequent exposure to virus. The immunological mechanism(s) responsible for the aberrant disease were not entirely clear. Nonetheless, immunological analysis of sera (36) and peripheral blood mononuclear cells (28) from the FI-RSV vaccine recipients and studies in rodents (11, 12, 16) supported the notion that type 2 (TH2) helper T-cell (32) responses were involved. Hence, the prevailing hypothesis is that inactivated nonreplicating or subunit vaccines that induce primarily TH2 helper T-cell responses are contraindicated as vaccines for RSV (6, 35). Alternatively, a live attenuated RSV vaccine that elicits type 1 (TH1) helper T-cell (32) responses similar to those generated following natural infection is considered more endorsable (6, 35). However, the development of an attenuated vaccine for RSV also poses potential difficulties. First, in order to be a successful vaccine, the attenuated virus must be infectious and capable of limited replication in the upper respiratory tract without causing disease. Second, the vaccine must be highly immunogenic and induce protective immune responses that are not associated with atypical disease. The induction of protective immune responses may be difficult in view of the data which suggest that recovery from natural disease provides only limited protection (17). Finally, these criteria must be fulfilled in an immunologically immature host with circulating maternal antibodies against RSV (6, 35).

Several studies in humans (7, 17, 20, 33, 34, 50) indicate that the fusion (F) glycoprotein plays an important role in the generation of protective immune responses against RSV. The data further suggest that after infection, the immune responses directed against F protein are regulated by a predominance of TH1 helper T cells (2). However, after adsorption to the more

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conventional aluminum hydroxide (AlOH) adjuvant, immunization with the highly purified native F protein appears to generate immune responses that are TH2 in character (16, 19). Hence, after vaccination with F/AlOH, non-complement-fixing protein-specific immunoglobulin G1 (IgG1) antibodies predominate in the sera, little cytolytic T-cell activity is detected, and atypical pulmonary inflammatory responses are observed in the lungs of mice after challenge (19).

Recently we reported data on the ability of the highly purified saponin QS-21 (24) to improve both the quality and the quantity of the immune responses elicited by a subunit vaccine composed of F protein (19). After parenteral vaccination with F/QS-21, BALB/c mice possessed statistically heightened systemic humoral immune responses in comparison with cohort mice vaccinated with F/AlOH. Moreover and in contrast to F/AlOH, the humoral immune responses induced after vaccination with F/QS-21 consisted of augmented complement-assisted serum neutralizing antibody titers that were associated with an elevation in complement-fixing anti-F protein IgG antibody subclasses. The data further suggested that QS-21 had the potential to modify the nature of the pulmonary inflammatory response after challenge with infectious virus (19). The cellular constituents of the pulmonary infiltrates from mice immunized with F/QS-21 had a significantly greater percentage of lymphocytes and fewer neutrophils than those of mice vaccinated with F/AlOH. In addition, the lungs from the latter group contained heightened levels of cytolytic T-lymphocyte activity (19). Thus, the data indicated that the addition of QS-21 to the subunit vaccine served to broaden both the humoral and the cellular immune responses to levels characterized by infection.

Like the F protein, the large attachment (G) glycoprotein of RSV is located in the virus envelope and likely serves as an important antigen for the induction of protective immune responses (6, 17, 33, 34, 46). Following primary infection, human infants develop serum antibody titers to G protein (33). In addition, the levels of serum neutralizing antibody titers directed against the G protein have been correlated with protection of human volunteers from symptomatic disease after experimental infection with homologous virus (17). In rodent models, accelerated clearance of virus from the pulmonary tissues can be achieved via active immunization with a synthetic peptide of G protein (51) or passive immunization with monoclonal antibodies (MAbs) directed against G protein (47, 52). Hence, a reasonable hypothesis is that a subunit vaccine composed of highly purified native F and G proteins would generate augmented protective immune responses that are similar those elicited after natural infection.

On the other hand, in order to use native G protein in a subunit vaccine, several issues must be addressed. For example, G protein is the major determinant of antigenic diversity between the subgroups of RSV (21, 45). Therefore, for the induction of immune responses that offer broad protection, a subunit vaccine may require highly purified native G proteins from both A and B subgroups. Most importantly, the subunit vaccine must be safe and not predispose recipients for atypical disease. Studies with rodents suggested that immunization with vaccinia virus-expressed recombinant G protein was associated with atypical pulmonary immune responses after challenge (2). Although not duplicated in a nonhuman primate model (22), enhanced histopathology was observed in the lungs of cotton rats vaccinated with an FG chimeric protein adsorbed to alum adjuvant after challenge (10). In addition, adoptive transfer of G-protein-specific TH2 helper T-cell lines was associated with atypical pulmonary inflammatory responses in BALB/c mice after challenge (1). Thus, to modify the effects of TH2 helper T cells in seronegative subjects, novel adjuvants and/or immune response modifiers may be required.

Since studies have suggested that recombinant G protein is an inducer of TH2 helper T-cell responses (2), we used a murine model to test the effects of highly purified native G protein on the immunogenicity of a combination vaccine composed of the adjuvant QS-21 and F protein, both inducers of immune responses associated with the induction of TH1 helper T-cell responses (1, 31, 37). In addition, we directly compared the atypical pulmonary inflammatory responses elicited by highly purified native G protein with those elicited by a facsimile of the original formalin-inactivated vaccine (23, 27). We report that QS-21 is a potent adjuvant for highly purified native G protein. However, the data suggest that after vaccination with G/QS-21, TH2 helper T-cell responses are dominant. Moreover, the results imply that highly purified native G protein transforms the capacity of F/QS-21 to induce TH1 helper T-cell responses. Finally, the results imply that G/QS-21 is similar to a facsimile of the original formalin-inactivated vaccine associated with atypical disease in human infants (23, 27) and predisposes mice for atypical pulmonary inflammatory responses after challenge.

## MATERIALS AND METHODS

**Animals.** Age-matched (8- to 10-week-old) female BALB/c were used in all experiments. All mice were housed in a facility accredited by the American Association for Accreditation of Laboratory Animal Care.

**Virus and cell lines.** The A2 strain of RSV was used throughout this study. Virus was produced in either Vero (ATCC CCL 81) or HEp-2 (ATCC CCL 23) cells for the production of purified F and G proteins and purified virus according to standard procedures. Purified virus was obtained by centrifugation over sorbitol density gradients and stored at −70°C until use. A HEp-2 cell antigen control was prepared with procedures identical to those described above, using mock-infected HEp-2 cell lysates as the starting material. A syngeneic uninfected BALB/c cell line (14a) was a gift of Bruce F. Fernie and used for the determination of antigen-dependent killer cell activity. The latter cell line was maintained in Dulbecco's modified Eagle's medium, (Gibco BRL, Gaithersburg, Md.) with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) (Hyclone Laboratories Inc., Logan, Utah).

**Preparation of antigens. (i) Purification of the F and G glycoproteins of RSV.** RSV F and G glycoproteins were obtained from Vero cells infected with the RSV. Following detergent lysis, F protein was purified by ion-exchange chromatography. G protein was purified by immunoaffinity chromatography using MAb L7 as described previously (53). The G protein was further purified by passage over immunoaffinity columns specific for RSV F (MAb L4) and N (MAb D14) proteins. These procedures yielded highly purified F and G proteins with a purity of >95%, as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and antigen capture enzyme-linked immunosorbent assay (ELISA). The Vero cell antigen control was prepared with procedures identical to those described above, using mock-infected Vero cell lysates as the starting material.

**(ii) FI-RSV.** The facsimile FI-RSV vaccine was produced in a manner similar to that previously described (27, 39). In brief, primary monkey kidney cells were grown (37°C, 5% $CO_2$) in tissue culture flasks, using basal medium Eagle (BME; Gibco BRL) with 10% FBS (Hyclone Laboratories) and supplemented with antibiotics. After infection (multiplicity of infection of 0.02) with the Bernett strain of RSV (donated by R. M. Hendry), the inoculum was removed and serum-free BME was added. When the cells showed greater than 80% cytopathic effect, the media were removed, clarified at 400 × *g* for 10 min, and passed through a 5-μm-pore-size SM filter (Millipore Corp., Bedford, Mass.). Formalin was added to clarified media at a final dilution of 1:4,000, and the media were incubated 3 days at 37°C with stirring. After formalin treatment, the virus suspension was ultracentrifuged, using a 45Ti rotor for 30 min at 32,000 × *g*. The supernatant was discarded, and pellets were resuspended to 1/25 the original volume with serum-free BME. The 25× material was compounded with 4 mg of aluminum hydroxide gel (Alu-Gel-S; Serva Fine Biochemicals, Inc., Westbury, N.Y.) per ml overnight at room temperature. The compounded material was centrifuged at 2,000 rpm for 10 min, and the pellet was resuspended in 1/4 volume with serum-free BME containing 200 μg each of neomycin, streptomycin, and polymyxin B per ml and 1:40,000 dilution of benzethonium chloride.

**(iii) FI-PIV3.** The formalin-inactivated parainfluenza virus type 3 (FI-PIV3) vaccine was produced by using the same conditions as described above with the C243 strain of PIV3 (ATCC VR93).

**Immunizations.** BALB/c mice were immunized at weeks 0 and 3 or 4 with either the purified F protein (5 or 3 μg per dose), G protein (2.5 to 0.3 μg per dose), a combination of F and G proteins (3 μg of F protein plus 1 μg of G protein per dose), an equal amount of mock-infected Vero cell antigen control,

adjuvant in phosphate-buffered saline (PBS) alone, infectious RSV A2 ($1 \times 10^6$ to $2 \times 10^6$ PFU), or an equal volume of HEp-2 cell antigen control. The purified proteins and the mock-infected Vero cell antigen control were mixed with either QS-21 (20 μg per dose; Cambridge Biotech Corporation, Worcester, Mass.), aluminum hydroxide gel (100 μg per dose; Alu-Gel-S; Serva Fine Biochemicals), or PBS alone. The F and/or G protein-based vaccines were prepared the day before immunization and nutated at 4°C overnight. For parenteral vaccination with purified proteins and control adjuvant, the mice were injected intramuscularly (0.1 ml). For experimental infection ($1 \times 10^6$ to $2 \times 10^6$ PFU of RSV A2) or intranasal instillation of mock-infected HEp-2 cell antigens, the mice were administered 0.05 ml of vaccine under injectable anesthesia (60 mg of ketamine [The Butler Co., Dublin, Ohio] per kg of body weight).

**Determination of neutralizing antibody titers and RSV infectivity.** The serum neutralizing antibody titers were determined as previously described by the plaque reduction neutralization test (18, 19). The titers were determined against RSV A2 and in the presence or absence of 5% (vol/vol) rabbit complement (GenTrak, Inc., Plymouth Meeting, Pa.) after visual enumeration of foci of infection. The neutralization titers were calculated as the reciprocal of the serum dilution which showed a 60% reduction (relative to the virus control) in the number of foci per well.

**BAL.** Bronchoalveolar lavage (BAL) was performed as previously described (19). In brief, the cells were pelleted from the BAL fluids, cytospun (Shanndon Cytofuge) onto glass slides, and stained (DifQik; Baxter). The relative percentage of eosinophils was determined after the examination of at least 400 cells per slide.

**In vivo administration of anticytokine antibodies.** Purified MAb TRFK.5 (8) was used to neutralize murine interleukin-5 (IL-5). The neutralizing MAb directed against murine gamma interferon (IFN-γ) (R4-6A2; ATCC HB 170) was purified from hybridoma culture supernatants over a recombinant protein G column (Pharmacia). Purified control rat IgG was purchased from Calbiochem (San Diego, Calif.). To neutralize the effects of IL-5 and IFN-γ on the induction of a pulmonary inflammatory response in vivo, naive mice and mice secondarily immunized 3 weeks earlier with purified F and/or G proteins were administered (intraperitoneally) 1 mg of purified neutralizing or control antibody. Two days later, the all mice were challenged with approximately $2 \times 10^6$ PFU of RSV A2.

**Serum antibody determinations.** The serum antibody titers were determined by ELISA as detailed earlier (18, 19). Briefly, 96-well plates were coated with the 20 ng of highly purified F or G protein per well. Serial threefold dilutions of serum prepared in PBS–0.3% Tween 20–0.01 M EDTA buffer (pH 7.0) were then added to the wells and incubated for 1 h at room temperature. After five washes with PBS–0.1% Tween 20, 100 μl of biotinylated goat anti-mouse IgG (1:8,000), IgG1 (1:11,000), or IgG2a (1:16,000) was added, and the plates were incubated 1 h at room temperature. All secondary antibodies were purchased from Brookwood Biomedical, Birmingham, Ala. Following another series of washes, 100 μl of streptavidin conjugated to horseradish peroxidase (1:10,000 dilution in PBS–0.3% Tween 20; Zymed) was added to the wells and incubated at room temperature for an additional 30 min. Peroxidase substrate (2,2′-azino-di[3-ethyl-benzthiazoline sulfonate [6]; Kirkegaard & Perry Laboratories, Gaithersburg, Md.) was added to the wells after washing and incubated at room temperature for 20 min, at which time the reaction was stopped with 100 μl of 1% sodium dodecyl sulfate (Pierce, Rockford, Ill.). Endpoint antibody titers were determined with a Dynatech ELISA reader (Dynatech Laboratories Inc., Chantilly, Va.) at 410 nm, with a reference of 630 nm.

**In vitro expansion of splenic immunocytes.** Single-cell suspensions were prepared from the spleens of five mice per group. After removal of erythrocytes by osmotic lysis ($NH_4Cl$), the splenocytes were cultured (37°C, 5% $CO_2$) in 96-well tissue culture plates (Corning Glass Works, Corning, N.Y.) at a concentration of $2.5 \times 10^7$ cells per ml of culture medium for the resolution of cytokines associated with helper T-cell subsets or in 6-well tissue culture plates (Costar, Cambridge, Mass.) at a concentration of $2 \times 10^6$ cells per ml of culture medium for the determination of antigen-dependent killer cell activity. The culture medium was composed of RPMI 1640 supplemented with 2 mM glutamine, 100 U of penicillin and 50 μg of streptomycin (Gibco BRL) per ml, $5 \times 10^{-4}$ M 2-mercaptoethanol (Sigma), 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer, and 1% normal mouse or 10% FBS for the generation of secreted cytokines or antigen-dependent killer cell activity, respectively.

**Cytokine determination.** To test for the presence of cytokines, triplicate wells of splenocytes were cultured in the presence of culture medium alone, concanavalin A (1 μg/ml; Sigma), a diphtheria toxoid cross-reactive protein ($CRM_{197}$; 10 μg/ml), or ascending doses of purified RSV A2 inactivated by UV irradiation (exposed to UV light for 45 s at 8,000 ergs/s). Thereafter, on days 2, 4, and 6 of culture, 100-μl aliquots of the supernatants from each triplicate well were pooled and stored at −20°C until assay. The spleen cell culture supernatants were assayed for IFN-γ and IL-5 by capture ELISA adapted from previously described procedures (13, 43). Briefly, enzyme immunoassay-radioimmunoassay plates (Costar) were coated overnight with 50 μl of the appropriate MAb per well. For the quantitation of IFN-γ and IL-5, R4-6A2 (3 μg/ml) and TRFK.5 (2.5 μg/ml), respectively, were used. The antibodies were obtained from PharMingen (San Diego, Calif.), diluted in carbonate-bicarbonate buffer (pH 9.6), and coated overnight at 37°C. The antibody solutions were removed, and nonspecific binding sites on the plastic were blocked by adding to each well 200 μl of blocking solution consisting of 5% FBS and 10% nonfat milk in Tris-buffered saline (TBS; 50 mM, pH 7.5) for 1 h at room temperature. The trays were then washed five times with TBS containing 0.1% Tween 20 (TBS-Tween).

The culture supernatants (50 μl) were diluted 1:1 with blocking solution, added to duplicate wells, and incubated for 90 min at room temperature. After the wells were washed five times, 50 μl of biotinylated detecting antibody diluted in blocking buffer was added to the wells for a further 90 min at room temperature. The biotinylated detecting antibodies, obtained from PharMingen, were XMG1.2 (1 μg/ml) for IFN-γ and TRFK.4 (2 μg/ml) for IL-5. After unbound detecting antibody was washed away, streptavidin-alkaline phosphatase (50 μl per well; Gibco BRL) diluted in TBS-Tween was added for 15 min, and the wells were then washed 10 times. Bound alkaline phosphatase was detected in a two-step amplified enzyme reaction described in detail elsewhere (44). Briefly, 50 μl of NADP (0.0002 M; Sigma) in 0.05 M diethanolamine buffer (pH 9.5) was added to each well for 10 min, followed by 50 μl of a solution containing 3% ethanol, 2.8 U of diaphorase (Worthington Biochemical Company) per ml, 45 U of alcohol dehydrogenase (Sigma) per ml, and the tetrazolium salt INT violet (0.001 M; Sigma) in 0.025 M phosphate buffer (pH 7.0). Ten minutes later, color development was stopped by adding 0.3 M sulfuric acid to the wells, and the optical density at 490 ($OD_{490}$) was measured with a Dynatech ELISA reader (Dynatech), with a reference of 630 nm.

Standard curves (log-log plot) were generated on each plate by using recombinant IFN-γ (Genzyme, Cambridge, Mass.) or IL-5 (PharMingen). The amount of cytokine in the supernatants was quantitated by interpolating on the linear portion of the standard curve. The limit of detection of these assays is defined as the amount of recombinant cytokine that gives an OD value equal to 3 standard deviations above the average $OD_{490}$ of eight wells containing no cytokine.

**Determination of percent cytotoxicity.** The presence of antigen-dependent killer cell precursors in the spleens of vaccinated mice was determined in a 4-h $^{51}Cr$ (Amersham Corp., Arlington Heights, Ill.) release assay (18, 19). Splenic precursor cells were cocultured for 6 days in the presence of syngeneic stimulator cells at the ratio of five responder splenocytes to one stimulator cell. The stimulator cells were prepared from naive bulk splenocytes after incubation (1 h, 37°C) with RSV A2 (multiplicity of infection of 1). The level of killer cell activity was determined against RSV-infected and control syngeneic targets. The targets were infected (1 h, 37°C, multiplicity of infection of 10) with RSV A2 and incubated overnight to allow optimal viral protein expression. Thereafter, the responder splenocytes were serially diluted threefold in RPMI 1640 containing 10% FBS and incubated with a standard number of $^{51}Cr$-labeled syngeneic target cells. Percent specific release was calculated as 100 × [(mean cpm experimental) − (mean cpm spontaneous release)]/[(mean cpm total release) − (mean cpm spontaneous release)].

**Statistical analysis.** The one-factor analysis of variance for repeated measures (Statview II; Abacus Concepts, Inc., Berkeley, Calif.) was used to determine significance.

## RESULTS

**QS-21 as an adjuvant for G protein.** The data suggested that QS-21 was a potent adjuvant for highly purified native G protein (G/QS-21). Two weeks after secondary immunization, both serum anti-G protein IgG1 and IgG2a antibody titers were statistically higher than in the corresponding AlOH or PBS preparations (Table 1). For example, following secondary immunization with either G (1.2 μg)/QS-21 or G (0.3 μg)/QS-21, the IgG2a antibody titers were 2,000-fold higher than in comparable mice vaccinated with the PBS formulation. Compared with sera from mice vaccinated with the AlOH preparations, the G-protein-specific IgG2a antibody titers were statistically elevated 400 or 100 times, respectively (Table 1). That G protein prepared with QS-21 had greater potential to elicit augmented protective humoral immune responses was also supported by the elevated complement-assisted serum neutralizing antibody titers. Two weeks after secondary vaccination, the complement-assisted serum neutralizing antibody titers of mice immunized with G (1.2 μg)/QS-21 (Table 1) or G (1.0 μg)/QS-21 (Table 2) were significantly heightened 6- or 5-fold, respectively, compared with the AlOH preparation. The results suggested that vaccination with highly purified native G protein in PBS alone (G/PBS) elicited primarily serum antibodies of the IgG1 subclass (Table 1). Moreover, the data implied that the adsorption of native G protein to AlOH (G/AlOH) did little to further enhance anti-G protein IgG2a antibody titers. Compared with titers from mice immunized with G/PBS, statistically augmented serum IgG2a antibody titers were not observed. After challenge, infectious virus was not

TABLE 1. Secondary humoral immune responses of BALB/c mice vaccinated with the native G protein of RSV

| Expt no. | Antigen (amt [μg]) | Adjuvant | Antibody titer ($\log_{10}$)[a] | | | |
|---|---|---|---|---|---|---|
| | | | IgG | | Neutralizing | |
| | | | IgG1 | IgG2a | + | − |
| 1 | G (2.5) | QS-21 | 6.5 (0.2)[b] | 6.1 (0.4)[c] | 2.3 (0.8) | <1.3 |
| | G (2.5) | AlOH | 5.3 (0.2)[d] | 2.8 (0.8) | 1.9 (0.8) | <1.3 |
| | G (2.5) | PBS | 3.7 (0.8) | <1.7 | <1.3 | <1.3 |
| | PBS | QS-21 | NT | NT | <1.3 | <1.3 |
| 2 | G (1.2) | QS-21 | 5.8 (0.4)[b] | 5.4 (0.5)[c] | 2.2 (0.3)[e] | <1.3 |
| | G (1.2) | AlOH | 5.1 (0.2)[d] | <3.0 | 1.5 (0.4) | <1.3 |
| | G (1.2) | PBS | 4.1 (0.7) | 2.0 (0.6) | <1.3 | <1.3 |
| | G (0.3) | QS-21 | 5.7 (0.2)[d] | 5.3 (0.2)[c] | 1.6 (0.3) | <1.3 |
| | G (0.3) | AlOH | 5.5 (0.6)[d] | 2.4 (0.9) | 1.8 (0.4) | <1.3 |
| | G (0.3) | PBS | 3.2 (0.4) | <2.0 | <1.3 | <1.3 |

[a] The geometric mean endpoint IgG and neutralizing antibody titers were determined by ELISA and the plaque reduction neutralization test, respectively, on sera collected 2 weeks after secondary immunization. Neutralizing antibody titers were determined in the presence (+) or absence (−) of 5% complement. NT, not tested. The numbers in parentheses are 1 standard deviation of the mean. There were five mice per group.

[b] $P < 0.05$ compared with the corresponding IgG1 antibody titers elicited following vaccination with either the G/AlOH or G/PBS preparation.

[c] $P < 0.05$ compared with the corresponding IgG2a antibody titers elicited following vaccination with either the G/AlOH or G/PBS preparation.

[d] $P < 0.05$ compared with the corresponding IgG1 antibody titers elicited following vaccination with the G/PBS preparation.

[e] $P < 0.05$ compared with G (1.2 μg)/AlOH.

recovered from the lungs of mice twice immunized with either G/QS-21, G/AlOH, or G/PBS (data not shown).

**Effects of G protein on the humoral immune responses induced by F/QS-21.** Initial studies implied that a subunit vaccine composed of both F and G protein antigens was superior to one composed of either protein alone (data not shown). In a separate experiment, we tested the immunogenicity of a combination vaccine composed of both highly purified native F and G proteins mixed with QS-21. Similar to previous studies (reference 20 and Table 1), the results confirmed that immunization with QS-21 elicited augmented serum protein-specific IgG2a antibodies. Vaccination with either F/QS-21 or G/QS-21 alone induced anti-F or anti-G protein IgG2a antibody titers that were statistically greater (30- or 100-fold, respectively) than induced by the comparable AlOH-based vaccines (Table 2). The data further implied that the advantage of QS-21 over AlOH was maintained when the proteins were combined in the same preparation. The anti-F and anti-G-protein IgG2a antibody titers in the sera of mice 2 weeks after secondary vaccination with F+G/QS-21 were statistically higher than those from mice vaccinated with the comparable AlOH formulation (Table 2). Most significantly, the data suggested that the addition of highly purified native G protein to F/QS-21 facilitated the induction of augmented neutralizing antibody titers. Two weeks after secondary immunization, the complement-assisted neutralizing antibody titers in the sera of mice vaccinated with F+G/QS-21 were significantly greater than those generated after vaccination with F+G/AlOH and at least 48 or 24 times greater than those in comparable mice injected with either G/QS-21 or F/QS-21, respectively (Table 2). It was noteworthy that the serum anti-G-protein IgG1 antibody titers of mice vaccinated with either G/QS-21 alone or F+G/QS-21 were also statistically higher than that elicited by the corresponding AlOH-based vaccine.

**Effects of G protein on the cell-mediated immune responses induced by F/QS-21.** It was of consequence that the data suggested that compared with to the AlOH-based vaccines, that

TABLE 2. Effect of highly purified G protein on the systemic humoral immune responses of BALB/c mice vaccinated with F/QS-21[a]

| Vaccine | Adjuvant | Antibody titers ($\log_{10}$)[b] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | F protein | | | G protein | | | Neutralizing | |
| | | IgG | IgG1 | IgG2a | IgG | IgG1 | IgG2a | + | − |
| F + G | QS-21 | 6.7 (0.4)[c] | 6.0 (0.3)[d] | 5.7 (0.3)[e] | 6.8 (0.3)[f] | 6.5 (0.2)[g] | 5.9 (0.2)[h] | >4.0 (0.3)[i,j] | 3.0 (0.5)[i] |
| F + G | AlOH | 6.4 (0.2)[k] | 6.1 (0.2) | 4.7 (0.5) | 5.5 (1.1) | 5.1 (0.5) | 2.6 (0.9) | 2.9 (0.5)[i,l] | 2.0 (0.5) |
| G | QS-21 | <3.0[m] | NT | NT | 6.2 (0.5) | 6.3 (0.3)[g] | 5.4 (0.3)[h] | 2.3 (0.2)[n] | <1.3 |
| G | AlOH | <3.0[m] | NT | NT | 5.4 (0.3) | 5.6 (0.1) | 3.4 (1.0) | 1.6 (0.5) | <1.3 |
| F | QS-21 | 6.9 (0.3)[k] | 6.1 (0.2) | 6.1 (0.3)[e] | <3.0[m] | NT | NT | 2.6 (0.4)[i,o] | 1.7 (0.2) |
| F | AlOH | 5.7 (0.5) | 5.8 (0.3) | 4.6 (0.8) | <3.0[m] | NT | NT | 1.8 (0.4) | 1.5 (0.3) |
| PBS | QS-21 | <3.0[m] | NT | NT | NT | NT | NT | <1.3 | <1.3 |
| Mock | None | <3.0[m] | NT | NT | NT | NT | NT | <1.3 | <1.3 |
| RSV | None | 5.6 (0.4) | 4.4 (0.1) | 5.1 (0.1) | 5.5 (0.1) | 5.2 (0.6) | 4.9 (0.2) | 3.5[m] | 1.8[m] |

[a] BALB/c mice were vaccinated intramuscularly with either a combination of purified F (3 μg per dose) and G (1 μg per dose) proteins, G protein (1 μg per dose) alone, or F protein (3 μg per dose) alone. The vaccines were adjuvanted with either QS-21 (20 μg per dose) or AlOH (100 μg per dose). Control mice received either QS-21 (20 μg per dose) in PBS alone, were experimentally infected with RSV A2, or received an intranasal administration of mock-infected HEp-2 cell lysate (mock). Similar results were obtained in several independent studies. There were five mice per group.

[b] The geometric endpoint IgG and neutralizing antibody titers were determined by ELISA and the plaque reduction neutralization test, respectively, on sera collected 2 weeks after secondary immunization. The neutralizing antibody titers were determined in the presence (+) and absence (−) of complement. The numbers in parentheses are 1 standard deviation of the mean. NT, not tested.

[c] $P > 0.05$ compared with IgG antibody titers elicited after vaccination with F+G/AlOH or F/QS-21.

[d] $P > 0.05$ compared with IgG1 antibody titers elicited after vaccination with F+G/AlOH, F/QS-21, and F/AlOH.

[e] $P < 0.05$ compared with the corresponding anti-F protein IgG2a antibody titers elicited after vaccination with AlOH.

[f] $P < 0.05$ compared with IgG antibody titers elicited after vaccination with F+G/AlOH and G/AlOH.

[g] $P < 0.05$ compared with the corresponding anti-G protein IgG1 subclass antibody titers after vaccination with F+G/AlOH or G/AlOH.

[h] $P < 0.05$ compared with the corresponding anti-G protein IgG2a subclass antibody titers elicited by G/AlOH or F+G/AlOH.

[i] $P < 0.05$ compared with the neutralizing antibody titers observed in all groups immunized with F and/or G proteins.

[j] $P < 0.05$ compared with neutralizing antibody titers determined with the same sera in the absence of complement.

[k] $P < 0.05$ compared with anti-F protein IgG antibody titers elicited after vaccination with F/AlOH.

[l] $P < 0.05$ compared with the neutralizing antibody titers observed in groups vaccinated with either G/QS-21, G/AlOH, F/AlOH, or PBS/QS-21.

[m] Titers were determined on pooled serum samples.

[n] $P < 0.05$ compared with the neutralizing antibody titers observed in mice vaccinated with G/AlOH.

[o] $P < 0.05$ compared with the neutralizing antibody titers observed in in mice vaccinated with F/AlOH.

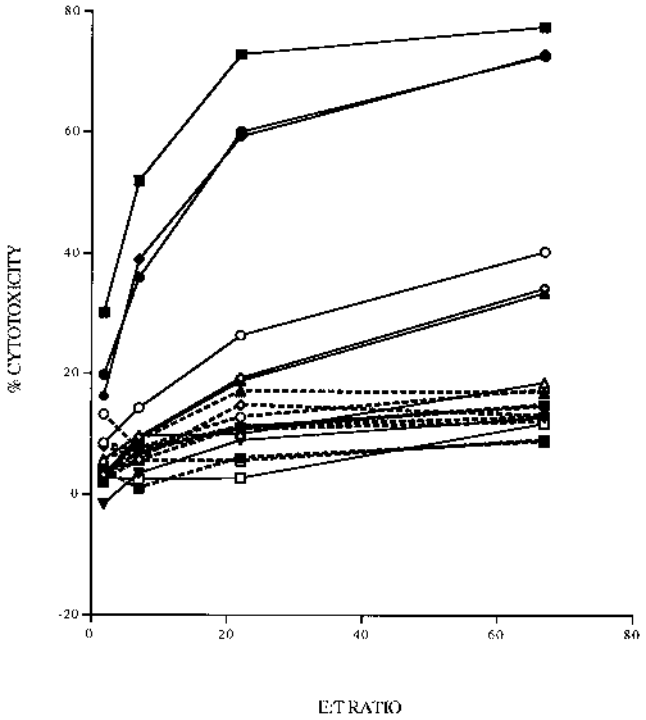


FIG. 1. Antigen-dependent killer cell activity in splenocytes of BALB/c mice vaccinated with purified native F and G proteins of RSV. BALB/c mice were vaccinated with either a combination of purified F (3 μg per dose) and G (1 μg per dose) proteins (diamonds), G protein (1 μg per dose) alone (triangles), or F protein (3 μg per dose) alone (circles). The vaccines were formulated with either QS-21 (20 μg per dose; filled symbols) or AlOH (100 μg per dose; open symbols). After 6 days of culture with RSV-infected stimulator cells, killer cell activity was assessed against RSV-infected (solid lines) and control (dashed lines) syngeneic targets. The results were compared with those obtained from the splenocytes of control mice injected with adjuvant alone (inverted triangles), mock-infected HEp-2 cell lysate (open squares), and infectious virus (filled squares). There were five mice per group. Similar results were obtained in a separate experiment of like design.

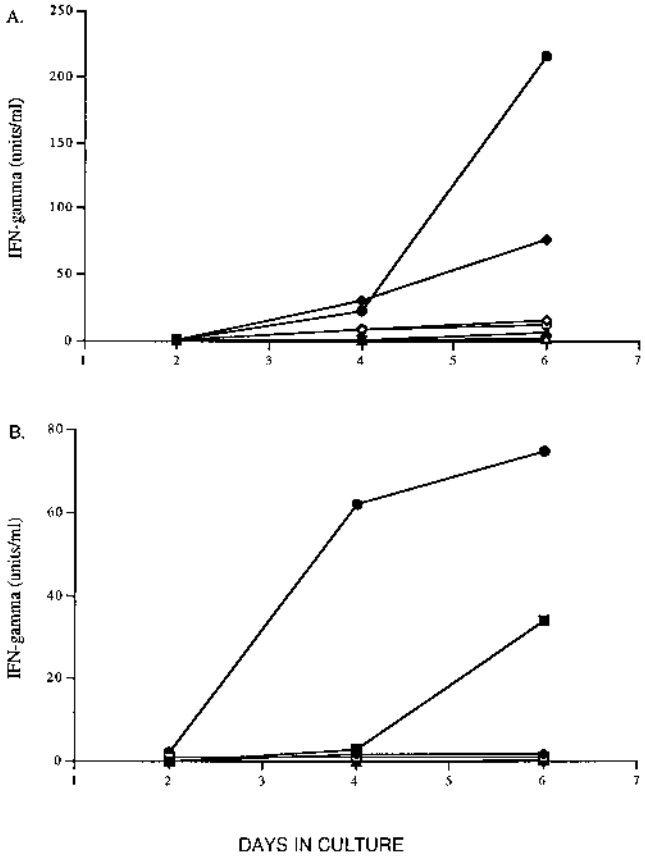


FIG. 2. Secretion of IFN-γ by the spleen cells from mice immunized with purified F and G glycoproteins of RSV formulated with QS-21. Panels A and B depict the results from two independent studies. BALB/c mice were vaccinated with either a combination of purified F (3 μg per dose) and G (1 μg per dose) proteins (diamonds), G protein (1 μg per dose) alone (triangles), or F protein (3 μg per dose) alone (circles). The vaccines were formulated with either QS-21 (20 μg per dose; filled symbols) or AlOH (100 μg per dose; open symbols). Control mice were injected with adjuvant alone (inverted triangles), mock-infected HEp-2 cell lysate (open squares), or infectious virus (filled squares). Two weeks after secondary vaccination, single-cell suspensions were prepared from spleens and stimulated in vitro for 6 days in the presence of $10^6$ PFU equivalents of purified RSV A2 per ml of culture medium. The supernatants from triplicate wells were pooled and tested for IFN-γ by capture ELISA. There were five mice per group.

vaccines prepared with QS-21 had more potential to generate memory antigen-dependent killer cells (Fig. 1). Splenocytes harvested 2 weeks after secondary immunization with F/QS-21 and cultured for 6 days with virally infected stimulator cells lysed syngeneic RSV-infected target cells in a dose- and antigen-dependent manner. It was noteworthy that the level of cytolysis in the effector cells was nearly identical to that obtained after in vitro stimulation of splenic immunocytes from mice immunized by experimental infection (Fig. 1). Control targets not infected with RSV were not lysed. The data further suggested that QS-21 transformed the capacity of highly purified native G protein to induce antigen-dependent killer cell responses (Fig. 1). After in vitro stimulation, the capacity of the effector cells from mice vaccinated with G/QS-21 to lyse RSV-infected syngeneic targets was nearly three times that of the corresponding cells from control mice injected with either mock-infected HEp-2 cell lysate or PBS/QS-21. Nonetheless, in comparison with F/QS-21, the lytic activity of effector cells from mice vaccinated with G/QS-21 was two to three times less (Fig. 1). The data further implied that in contrast to the dramatic elevations in complement-assisted neutralizing antibody titers (Table 2), the addition of G protein to F/QS-21 did not augment antigen-dependent killer cell activity (Fig. 1). Nearly identical levels of killer cell activity were detected after vaccination with either F+G/QS-21 or F/QS-21.

An examination of culture supernatants for cytokines demonstrated that vaccination with F/QS-21 generated splenic immunocytes which secreted IFN-γ after activation with purified UV-inactivated virus antigens (Fig. 2). The amount of IFN-γ in the supernatants was dependent on the presence and dose (data not shown) of RSV antigens in the culture medium and increased in quantity with time. The results further implied that the amount of IFN-γ in the supernatants of the mice vaccinated with F/QS-21 was similar to that secreted by the spleen cells of mice immunized by experimental infection. Moreover, after 6 days in vitro stimulation, the splenic immunocytes obtained 2 weeks after secondary immunization with F/QS-21 secreted nearly 200 times more IFN-γ than comparable immunocytes from mice administered F/AlOH (Fig. 2A). The splenocytes from mice vaccinated with either F/QS-21 or F/AlOH secreted noteworthy amounts of IL-5 (1,220 or 3,370 pg/ml, respectively) into the culture medium after 6 days of in vitro stimulation. IL-5 (680 pg/ml) was detected in the culture supernatants from the spleen cells of mice immunized by experimental infection.

TABLE 3. Effect of vaccination with purified G protein on the induction of eosinophilia in the lungs of BALB/c mice after challenge

| Antigen | Adjuvant | % Eosinophils[a] | IL-5[b] |
|---|---|---|---|
| F + G | QS-21 | 38.4 (19.9)[c] | 0.253 (0.034) |
| F + G | AlOH | 44.3 (11.3)[c] | 0.268 (0.141) |
| G | QS-21 | 40.8 (6.0)[c] | 0.335 (0.048)[d] |
| G | AlOH | 25.8 (14.4)[e] | 0.416 (0.228)[f] |
| F | QS-21 | 4.3 (3.5)[g] | 0.121 (0.006)[h] |
| F | AlOH | 19.1 (9.5)[i] | 0.212 (0.046)[j] |
| PBS | QS-21 | 0.9 (0.5) | 0.054 (0.003) |
| Mock | None | 1.3 (1.9) | 0.056 (0.004) |
| RSV | None | 0.7 (0.5) | 0.095 (0.006) |

[a] Mean percent eosinophils observed in BAL fluids 5 days after challenge. The numbers in parentheses are 1 standard deviation of the mean. There were five mice per group.
[b] Mean $OD_{490}$ detected in BAL fluid supernatants after testing in a cytokine ELISA. The numbers in parentheses are 1 standard deviation of the mean.
[c] $P < 0.05$ compared with F/QS-21, F/AlOH, PBS/QS-21, and RSV.
[d] $P > 0.05$ when compared with F+G/QS-21 and F+G/AlOH, and G/AlOH; $P < 0.05$ compared with F/QS-21, F/AlOH, and PBS/QS-21.
[e] $P < 0.05$ compared with F/QS-21, PBS/QS-21, and RSV; $P > 0.05$ compared with F/AlOH.
[f] $P < 0.05$ compared with all except G/QS-21.
[g] $P > 0.05$ compared with F/AlOH and RSV.
[h] $P < 0.05$ compared with F+G/QS-21.
[i] $P < 0.05$ compared with PBS/QS-21 and RSV.
[j] $P > 0.05$ compared with F+G/QS-21 and F+G/AlOH.

TABLE 4. Effect of anti-IL-5 antibody treatment on the eosinophila induced in the lungs of BALB/c mice vaccinated with the G protein of RSV[a]

| Antigen | Antibody[b] | % Eosinophils[c] |
|---|---|---|
| G protein | IL-5 | 7.2 (3.3)[d] |
| | IFN-γ | 49.1 (11.9)[e] |
| | Control | 50.1 (6.6)[f] |
| F protein | IL-5 | 0.8 (0.8)[g] |
| | IFN-γ | 11.2 (8.5) |
| | Control | 11.3 (5.9) |
| Vero cells | IL-5 | 1.0 (0.9)[h] |
| | IFN-γ | 18.1 (5.6) |
| | Control | 12.2 (4.7) |

[a] BALB/c mice were vaccinated with 1 or 3 μg of native G or F protein adjuvanted with QS-21 (20 μg per dose). Control mice received concentrated mock-infected Vero cell antigen (3 μg per dose). The results presented were obtained in a separate experiment of similar design.
[b] Two days before challenge, the mice were administered either 1 mg of anti-IL-5, anti-IFN-γ, or control rat IgG.
[c] Mean percent eosinophils observed in BAL fluids 5.5 days after challenge. The numbers in parentheses are 1 standard deviation of the mean. There were five mice per group.
[d] $P < 0.05$ compared with mice vaccinated with G/QS-21 and pretreated with either anti-IFN-γ or control IgG. $P > 0.05$ compared with all groups vaccinated with F/QS-21 or control mice immunized with Vero/QS-21 and pretreated with anti-IL-5.
[e] $P > 0.05$ compared with mice vaccinated with G/QS-21 and pretreated with control IgG. $P < 0.05$ compared with all groups vaccinated with F/QS-21 and control mice immunized with Vero/QS-21 and pretreated with anti-IL-5.
[f] $P < 0.05$ compared with all groups vaccinated with F/QS-21 or control mice immunized with Vero/QS-21 and pretreated with control IgG.
[g] $P > 0.05$ compared with control mice immunized with Vero/QS-21 and pretreated with anti-IL-5. $P < 0.05$ compared with mice vaccinated with F/QS-21 and pretreated with either anti-IFN-γ or control IgG.
[h] $P < 0.05$ compared with control mice vaccinated with Vero/QS-21 and pretreated with either anti-IFN-γ or control IgG.

In comparison with F/QS-21, the results suggested that immunization with G/QS-21 induced a different set of regulatory cytokines. After in vitro stimulation with UV-inactivated virus, IFN-γ was not detected in the culture supernatants (Fig. 2). More importantly, the data from two separate studies implied that the addition of native G protein influenced the capacity of F/QS-21 to induce IFN-γ-secreting immunocytes. In the first experiment, the splenocytes from mice immunized with F+G/QS-21 appeared to secrete fourfold less IFN-γ than the splenocytes from mice vaccinated with F/QS-21 (Fig. 2A). In the second study (Fig. 2B), the results were more striking. The addition of native G protein to F/QS-21 resulted in a 70-fold reduction in IFN-γ levels 6 days after stimulation with purified UV-inactivated RSV. After in vitro culture, the effects of native G protein on the capacity of F/QS-21 to generate IL-5-secreting immunocytes were less conclusive (data not shown).

**Induction of TH2 helper T-cell cytokines and atypical pulmonary inflammatory responses after immunization with G protein.** A previous study (19) suggested that vaccination with F/QS-21 was not associated with a bias for atypical pulmonary inflammatory responses after challenge. In support of this conclusion, the relative percentage of eosinophils in the BAL fluids from mice vaccinated with F/QS-21 (4.3%) 5 days after challenge were not significantly different from that detected in the fluids of mice immunized by infection (0.7%) or of mice administered PBS/QS-21 (0.9%) or mock-infected HEp-2 cell lysates (1.3%) and undergoing primary infection (Table 3). In contrast, the data (Table 3) implied that vaccination with F/AlOH was associated with statistically heightened numbers of eosinophils (19.1%) after challenge. However, the most dramatic results were those which suggested that the addition of G protein to F/QS-21 predisposed BALB/c mice for atypical pulmonary inflammatory responses after challenge. Statistically significant elevations in the relative number of eosinophils were observed in the pulmonary tissues of mice vaccinated with either F+G/AlOH (44.3%) or F+G/QS-21 (38.4%) after challenge (Table 3). Indeed, eosinophilia was observed in the pulmonary tissues of all mice immunized with subunit vaccines containing highly purified native G protein, regardless of adjuvant.

Taken together, the results suggested that immunization with highly purified native G protein induced the cytokines associated with the activation of TH2 helper T-cell responses (2). In support of this hypothesis were the striking data that implied a relationship between the increased numbers of eosinophils in the pulmonary tissues and the presence of statistically elevated levels of IL-5 in the same BAL fluids 5 days after challenge (Table 3). Moreover, compared with the BAL fluids from mice vaccinated with F/QS-21 or infectious virus, IL-5 was significantly elevated in the fluids of mice vaccinated with either F+G/QS-21 or G/QS-21 after challenge. A relationship between IFN-γ and pulmonary eosinophilia was not observed (data not shown).

To further establish a role for TH2 helper T-cell cytokines in atypical pulmonary inflammatory responses, neutralizing anti-IL-5 or IFN-γ MAb or control IgG antibodies were administered to G/QS-21- and F/QS-21-vaccinated mice before challenge (Table 4). Compared with the BAL fluids from the lungs of mice immunized with F/QS-21 (11.3%) or Vero/QS-21 (12.2%) and pretreated with control IgG before challenge, the pulmonary tissues from comparable mice vaccinated with G/QS-21 contained significantly elevated percentages of eosinophils (50.1%). However, the prior administration of neutralizing anti-IL-5 MAb strongly influenced atypical pulmonary inflammatory responses. G/QS-21-vaccinated mice administered anti-IL-5 MAb 2 days before challenge had a significantly lower level of eosinophils 5 days after infection (Table 4). Prior treatment with either anti-IFN-γ MAb or control antibodies had no effect on pulmonary eosinophilia.

TABLE 5. Inflammatory responses in the lungs of BALB/c mice vaccinated with either FI-RSV or highly purified G glycoprotein of RSV after challenge

| Antigen | Adjuvant | % eosinophils[a] |
|---|---|---|
| FI-RSV | AlOH | 33.3 (10.6)[b] |
| FI-PIV3 | AlOH | 2.5 (1.6) |
| G protein | QS-21 | 52.1 (5.2)[c] |
| F protein | QS-21 | 9.1 (4.2)[d] |
| PBS | QS-21 | 0.04 (0.09) |
| Mock | None | 7.3 (9.0) |
| RSV | None | 0.2 (0.4) |

[a] BALB/c mice were vaccinated on weeks 0 and 4. Two weeks later, the mice were challenged with the A2 strain of RSV. The numbers are the mean percent eosinophils in BAL fluids 5 days after challenge. There were five mice per group. The numbers in parentheses are 1 standard deviation of the mean. Similar results were also obtained in two other separate experiments.

[b] $P < 0.05$ compared with mice vaccinated with FI-PIV3 or infected with RSV.

[c] $P < 0.05$ compared with mice vaccinated with PBS/QS-21 or infected with RSV.

[d] $P < 0.05$ compared with mice vaccinated with either G/QS-21 and FI-RSV.

Thus, the data implied that immunization with G protein was associated with local inflammatory responses in the lungs after challenge that were distinct from those observed in mice immunized with either F/QS-21 or infectious virus. The most dramatic observation from the differential analyses were the data which suggested that vaccination with G/QS-21 was similar to vaccination with FI-RSV and biased the local inflammatory responses for a relative increase in the number of eosinophils after challenge (Table 5). The differential analyses of the cellular constituents of the BAL fluids 5 days after challenge revealed that mice vaccinated with either G/QS-21 (52.1%) or FI-RSV (33.3%) had significantly greater percentages of eosinophils compared with the BAL fluids from mice vaccinated with either F/QS-21 or infectious virus or from control mice injected with either PBS/QS-21 or FI-PIV3 and undergoing primary infection (Table 5).

## DISCUSSION

The results presented herein authenticate previous studies (19) of BALB/c mice and demonstrate that QS-21 is a potent adjuvant for the F protein of RSV. The results establish that QS-21 facilitates the induction of heightened humoral and cell-mediated immune responses. Compared with subunit vaccines either adsorbed to AlOH or prepared in PBS alone, the F-protein-specific total IgG and IgG2a antibody titers in the circulation are all dramatically augmented after secondary vaccination. Consistent with previous studies (19), the results also show that immunization with F/QS-21 elicits elevated antigen-dependent killer cell activities. Moreover, the data in this report extend our earlier observations and further suggest that the capacity of the adjuvant to enhance both the quality and the quantity of the protective immune responses is a consequence of its ability to induce distinct helper T-cell subsets. That is, the results suggest that QS-21 enables the induction of immune responses to F protein that are TH1-like in nature, and unlike the case for AlOH, more similar to those observed after natural infection. Hence, the data imply that QS-21 is a most promising adjuvant for future RSV subunit vaccines.

Several studies in rodents (4, 47, 51, 52), nonhuman primates (22), and humans (17, 33, 34) indicate that the immune responses directed against the attachment (G) glycoprotein may also play an important role in the clearance of virus from the pulmonary tissues. Thus, the addition of highly purified native G protein may be of substantial benefit to an F-protein-based subunit vaccine and generate augmented protective immune responses. However, recent studies with rodents showed that immunization with recombinant G protein was accompanied by atypical pulmonary immune responses after challenge (1). These atypical immune responses were associated with the generation of G-protein-dependent TH2 helper T cells and characterized by pulmonary eosinophilia and hemorrhagic pneumonitis. Because QS-21 is a potent adjuvant for F protein (19) and appears to have dramatic effects on the induction of TH1-like immune responses (37), we investigated whether this saponin had similar effects on the generation of immune responses to G protein. Our initial studies supported this scenario and suggested that QS-21 was also a potent adjuvant for the highly purified native G protein. Indeed, the data suggested that vaccination with the highly purified G/QS-21 enabled the induction of antigen-dependent killer cell activity. In comparison with G/AlOH, G/QS-21 generated increased levels of protein-specific total IgG, IgG1, IgG2a, and complement-enhanced neutralizing antibody titers in the circulation 2 weeks after secondary vaccination. The data further suggested that vaccination with F+G/QS-21 generated complement-enhanced neutralizing antibody titers that were dramatically higher than those induced after vaccination with either F/QS-21 or G/QS-21 alone. Even after adsorption to AlOH, the combination of F and G proteins appeared to elicit significantly elevated neutralizing antibody titers.

Nonetheless, the cytokines secreted by the splenocytes cultured with purified whole virus antigens suggested that QS-21 was not able to modify the predominance of native G protein-induced TH2-like immune responses. In support of this conclusion, only IL-5 was detected in the spleen cell culture supernatants. IFN-γ was not detected. In addition, G/QS-21 generated heightened serum G-protein-specific IgG1 antibody titers (relative to G/AlOH) 2 weeks after secondary immunization. Regardless, the most solid testament for the potency of G protein in generating TH2-like immune responses was the morphology of the local pulmonary inflammatory responses after challenge. Statistically significant elevations in the relative percentage of eosinophils in the pulmonary tissues of either G/QS-21- or F+G/QS-21-vaccinated mice were observed after challenge. More convincing evidence was the presence of the cytokine most associated with eosinophilia (8), IL-5, in the BAL fluids of mice immunized with F+G/QS-21 after challenge and the ability of neutralizing anti-IL-5 MAb to abrogate the atypical pulmonary inflammatory response. Prior in vivo treatment with either control or anti-IFN-γ MAb had no effect on pulmonary eosinophilia. Atypical pulmonary eosinophilia was not detected in the BAL fluids of mice vaccinated with F/QS-21 alone.

These studies do not identify the phenotype of the immunocytes responsible for the secretion of IL-5 and/or IFN-γ. Since anti-IL-5 neutralizing antibodies prohibit the induction of pulmonary eosinophilia, the data imply that the cytokine secreting cells are G-protein-activated T cells of the CD4 subset as previously found after vaccination with recombinant G protein (1, 2). Alternatively, T cells bearing CD8 surface determinants have been shown to secrete the cytokines associated with TH2 helper cells (38, 40, 41) and could have an important role in the generation of atypical pulmonary inflammatory responses. In addition, recent reports have indicated that natural killer cells (54) and eosinophils (14) themselves may be a source of IL-5. The experiments presented herein also do not address the immunological mechanism(s) responsible for the disparate secretion of cytokines after vaccination

with either native F protein or native G protein. The cumulative data suggest that the physical chemistry of the individual glycoprotein may determine the phenotype of the helper T-cell subset induced. Specifically, native G protein has a novel structure among paramyxoviruses that is distinguished by the extent of glycosylation (approximately 60%) (42). Therefore, the hypothesis is that processing of intensely glycosylated antigens and presentation to T-cell clones is somewhat distinct from that of glycoproteins with fewer carbohydrate moieties. Thus, the induction of distinct helper T-cell subsets may reflect discrete differences in the capacities of antigen-presenting cells to process heavily glycosylated antigens.

The potential impact of these observations on future subunit vaccine development is considerable. The results emphasize that proper formulation of the vaccine is essential. That is, compared with AlOH, QS-21 clearly augments complement-fixing IgG2 antibody subclasses, complement-enhanced neutralizing antibody titers, and antigen-dependent killer cell activities, all associated with the generation of TH1 helper T-cell responses. However, the data also point out the limitations of QS-21 as an adjuvant for RSV F and G glycoproteins. One cannot assume that the TH1 helper T-cell responses facilitated by formulation with QS-21 will dominant TH2 helper T-cell responses. TH2-like immune responses may also be heightened and, depending on the nature of the antigen, predominate. Hence, when taken together and viewed in the context of the propensities of F and G proteins to generate diverse immunoregulatory pathways (1, 2), the conclusion is that the primary consequence of QS-21 is amplification, not transformation, of the immune responses.

The data further imply that the goal of formulating a combination vaccine composed of both native F and G proteins for seronegative infants requires reevaluation. If G protein contributes to atypical pulmonary inflammatory responses, its addition to a subunit vaccine is contraindicated. This is not to imply, however, that the concept of using G protein should be summarily discarded without further investigation. It has been reported that BALB/c mice may be particularly susceptible to eosinophila (30). Hence, the percentage of eosinophils observed in the pulmonary tissues of mice may be an aberration and not accurately reflect the true potential for human vaccinees. Furthermore, if the extent of glycosylation (42) is important for the determination of helper T-cell phenotype, it may be possible to construct an immunogenic nonglycosylated G protein via genetic or biochemical means. Peptides of G protein comprising neutralizing and/or cytolytic T-cell epitopes may also be useful in future formulations (51). In addition, novel adjuvants and/or immunomodulators such as IL-12 (49) may have the capacity to circumvent the potent TH2 properties of G protein.

The results presented herein are most propitious for the development of future subunit vaccines. The data are in agreement with and support those of other investigators (1, 2) and strongly suggest that native G protein has a propensity for inducing IL-5-secreting, TH2-like immune responses. This conclusion is supported by the pulmonary eosinophilia observed in mice vaccinated with G protein 5 days after challenge. Alternatively, formulation of the native F protein with QS-21 is not associated with atypical pulmonary inflammatory responses and in that regard appears similar to experimental infection with RSV or vaccination with recombinant F protein (2). This is despite the observation that immunization with F/QS-21, in addition to IFN-γ-secreting TH1-like immune responses, generates noteworthy serum IgG1 antibody titers, IL-5-secreting splenocytes, and modest amounts of IL-5 in the lungs after challenge. Taken together, the results imply that the TH1-like immune responses are dominant in vivo. Most noteworthy, pulmonary eosinophilia was primarily observed after immunization with either FI-RSV or vaccines containing native G protein. Thus, when viewed in the context of the eosinophilia associated with the human FI-RSV experience (23, 27), the eosinophil cationic protein associated with bronchiolitis in human infants (9, 15, 29), and the atypical pulmonary inflammatory responses in rodents following immunization with either recombinant (1) or highly purified native G/QS-21 or a facsimile FI-RSV vaccine, it is tempting to speculate that the G, not F, protein carries the antigenic determinants with the most potential to elicit atypical pulmonary disease in seronegative infants.

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