Modulation of Protective Immunity, Eosinophilia, and Cytokine Responses by Selective Mutagenesis of a Recombinant G Protein Vaccine against Respiratory Syncytial Virus

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No vaccine is yet available for respiratory syncytial virus (RSV), which is the leading cause of lower respiratory tract infection (acute bronchiolitis and pneumonia) in early infancy (7, 14). RSV is also a significant risk factor for asthma (1). A formalin-inactivated RSV vaccine developed in the 1960s proved to be nonprotective and actually led to more-severe lung disease in vaccinated children during the subsequent RSV season (2, 6, 21, 22). Studies with BALB/c mice have demonstrated that formalin-inactivated RSV and some G-protein-encoding vaccinia recombinants prime for a harmful lung inflammatory response, notably involving eosinophils (3, 5, 8, 26, 38). Determinants of eosinophilia as well as protective immune responses recently have been mapped to a region of the RSV G protein located approximately between amino acids 183 and 197 (31, 35). Refined mapping of this region has indicated a putative Th-cell epitope containing a nine-amino-acid core from Ile185 to Lys193 (34, 37). Using synthetic peptides carrying alanine mutations in each of the amino acids between residues 183 and 195, Varga et al. (37) showed that amino acids Ile185 and Arg188 were particularly important for recognition of lung mononuclear cells from BALB/c mice immunized with the RSV G protein, expressed by a recombinant vaccinia virus. Work in our laboratory provided the first evidence that the amino acids Ile188 and Lys192 were important both for protective immunity to RSV and for induction of RSV-associated eosinophilia in BALB/c mice (16). In the present study, we confirm and extend these findings by performing a complete alanine scan mutagenesis of the 185-193 region and by examining the effects on protective immunity and eosinophilia following immunization and RSV challenge. Most significantly, we provide the first identification of individual amino acids in a recombinant, nonglycosylated RSV G-protein vaccine which may tilt the balance between protection and eosinophilia.

Sequences from the RSV (Long strain) G protein corresponding to amino acids 128 to 229 as well as the mutant 128-229 sequences described below were amplified from viral RNA by reverse transcription-PCR, and the resultant PCR products were cloned into the EcoRI and XhoI sites of a pET-32-LIC bacterial expression plasmid (Novagen, Madison, Wis.) modified and provided by P. Liu (Department of Biochemistry, Dalhousie University). For use as a control for immunoblotting, a portion of the dengue virus type 2 E protein sequence encompassing amino acids 304 to 404 was similarly inserted into the same modified pET-32-LIC plasmid. Site-directed mutagenesis of the RSV G128-229 protein sequence was performed according to the Stratagene QuikChange site-directed mutagenesis protocol. PCR was performed on modified template pET-32-LIC-G128-229 DNA (G128-229 sequence cloned into EcoRI and XhoI sites). The primer pairs designed for mutagenesis are shown in Table 1.

Thioredoxin (Trx) fusion proteins of RSV G or dengue virus type 2 E protein sequences were prepared as previously described (23). Briefly, the above-described RSV G or dengue virus type 2 E protein sequences contained in modified pET-32-LIC plasmids were expressed as Trx fusion proteins in transformed Escherichia coli BL21/DE3 cells after induction with isopropyl-D-thiogalactopyranoside. All Trx fusion proteins (which also contain a His tag for metal chelation affinity purification) were recovered from transformed cell pellets by extraction with 8 M urea, followed by affinity purification using TALON (Clontech, Palo Alto, Calif.) and dialysis against phosphate-buffered saline (PBS). Purified Trx-G128-229 or Trx-E proteins were freed from contaminating endotoxin by treatment with polymyxin B beads (Bio-Rad, Mississauga, Canada). All purified proteins showed a single protein band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

For vaccine and challenge experiments, groups of seven to nine BALB/c mice (6 to 8 weeks) were immunized twice subcutaneously, at 14-day intervals, with either PBS-alum, Trx-
TABLE 1. Primers used to generate mutant Trx-G proteins used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>I185A</td>
<td>CCTGCTGGGCTGCTGCAAAAGATAACCAAACAAAAACCCAGG</td>
</tr>
<tr>
<td>C186A</td>
<td>CTGCTGGGCTATCGCCAAAGTTTACCAAACAAAAACCCAGG</td>
</tr>
<tr>
<td>K187A</td>
<td>CCTGTTTTTTTTGATTTGTGTTTCTTTTTGCCATCCATGCAAGCCACCAGG</td>
</tr>
<tr>
<td>R188A</td>
<td>CTGCTTTTTTTTTGTGATTTTGATTTTGCATGAGCAAGCCACCAGG</td>
</tr>
<tr>
<td>I189A</td>
<td>CTGCTGGGCTATCGCCAAAGATTTGATTTTGCATGAGCAAGCCACCAGG</td>
</tr>
<tr>
<td>P190A</td>
<td>CTGCTGGGCTATCGCCAAAGATTTGATTTTGCATGAGCAAGCCACCAGG</td>
</tr>
<tr>
<td>N191A</td>
<td>CTGCAAAAAAGAATACCCAGCCAAAAACCGAGAAGAAGAAGCCACC</td>
</tr>
<tr>
<td>K192A</td>
<td>CTGGGTATTTTCTATTTTCTATTCTTTTGCAGATAGCCCAGCAGG</td>
</tr>
<tr>
<td>K193A</td>
<td>CTGGGTATTTTCTATTTTCTATTCTTTTGCAGATAGCCCAGCAGG</td>
</tr>
</tbody>
</table>

*For each indicated mutation, the forward and reverse primers are listed in that order. The mutations to alanine are underlined.

G128-229, or mutant Trx-G128-229 proteins in PBS-alum (10 μg of protein [38% of which is G128-229 sequence] in a volume of 50 μl). Alum was used as an adjuvant due to its known predisposition towards a Th2 response (30), including eosinophilia, in order to obtain a more sensitive readout of vaccine-associated eosinophilia as well as protection against RSV challenge. Alum has augmenting effects on RSV-associated immunopathology, both dependent on (12, 24) and independent of (11, 12, 20) the G protein. Fourteen days after the second dose, mice were challenged intranasally with RSV (2 × 10^6 PFU in 50 μl). Mice were sacrificed by using sodium pentobarbital 4 days later and assayed for titers of virus in lung and leukocyte infiltration in bronchoalveolar fluids as previously described (16, 23). Data were analyzed using the GraphPad (San Diego, Calif.) Instat software package, using analysis of variance by the Kruskal-Wallis test. Single comparisons between groups were done by using the Mann-Whitney test.

Effects of G-protein mutations on the induction of G-protein-specific antibodies following immunization with alum-adjuvanted Trx-G128-229. Immunoblot analysis (Fig. 1A) demonstrated the presence of serum antibodies specific for RSV G protein in mice immunized with wild-type or mutant Trx-G128-229 proteins. Strongest G-specific antibody (immunoglobulin G) responses were observed with the wild-type protein, followed by N191A, I189A, P190A, C186A, and R188A mutant proteins. Low but detectable levels of antibodies were found in mice immunized with either K192A or K193A mutant proteins. The lowest (in fact undetectable) levels of RSV G-protein antibodies were observed in sera from mice immunized with the I185A or K187A mutant protein. A control immunoblot showing serum antibody responses against the Trx portion of the various Trx-G-protein immunogens demonstrated comparable immunization efficiencies in all groups of immunized mice (Fig. 1B).

Analyses of RSV neutralization titers in sera from immunized mice (Table 2) similarly showed a strong dependence of neutralizing-antibody responses upon the amino acid sequence within the 185-193 region of the Trx-G128-229 protein used for immunization.
TABLE 2. Neutralization titers of sera from mice immunized with alum-adjuvanted Trx-G variant proteins

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>PRNT&lt;sub&gt;50&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Significant (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>From PBS</td>
<td>From wt&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PBS</td>
<td>7 ± 3</td>
<td>Not</td>
</tr>
<tr>
<td>Trx-G128-229 (wt)</td>
<td>144 ± 37</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Trx-G128-229 (I185A)</td>
<td>18 ± 5</td>
<td>Not</td>
</tr>
<tr>
<td>Trx-G128-229 (C186A)</td>
<td>81 ± 12</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Trx-G128-229 (K187A)</td>
<td>34 ± 11</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Trx-G128-229 (R188A)</td>
<td>39 ± 13</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Trx-G128-229 (I189A)</td>
<td>115 ± 38</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Trx-G128-229 (P190A)</td>
<td>98 ± 26</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Trx-G128-229 (N191A)</td>
<td>95 ± 21</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Trx-G128-229 (K192A)</td>
<td>27 ± 7</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Trx-G128-229 (K193A)</td>
<td>31 ± 12</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

<sup>a</sup> Individual sera were collected from mice 14 days after the second of two subcutaneous administrations of the indicated immunogen in alum. Sera were assayed for RSV-neutralizing antibodies by plaque reduction assay. The 50% plaque neutralization titer (PRNT<sub>50</sub>) is the reciprocal dilution of sera required to neutralize 50% of RSV plaques on HEp-2 cells. The results are expressed as means ± standard deviations. Data are from one of two experiments which showed close agreement with each other.

<sup>b</sup> wt, wild type.

Effects of G-protein mutations on protective efficacy of alum-adjuvanted Trx-G128-229. As shown in Fig. 2, wild-type Trx-G128-229 afforded mice the best protection against RSV infection, whereas the various mutant proteins varied markedly in their ability to protect mice against RSV challenge. N191A-mutated Trx-G128-229 was the next-best protective immunogen, followed by the P190A, R188A, and I189A proteins. The remaining Trx-G128-229 mutant proteins conferred low to intermediate levels of protection.

Effects of G-protein mutations on the eosinophilicpenity of alum-adjuvanted Trx-G128-229. Since we and others have utilized prokaryotically expressed fragments of the RSV G protein as vaccine candidates (16, 23, 27, 29), the ability of such fragments to protect against RSV challenge must be tempered with their potential to initiate harmful inflammatory responses. As we previously reported (16), wild-type Trx-G128-229 in fact sensitized mice to pulmonary eosinophilia following RSV challenge (Fig. 3). In contrast, parallel immunization with the various Trx-G128-229 mutants primed for distinctly different degrees of eosinophilic infiltration (Fig. 3). Certain mutant proteins, e.g., the I189A, K192A, and K193A proteins, primed for an even greater degree of eosinophilia than did the wild-type protein. Other mutant proteins generally tended towards a less eosinophilicigenic response.

Of particular potential interest to vaccine design are certain mutations, such as N191A and R188A, which gave rise to some degree of protection against RSV (Fig. 2) but with a dampened eosinophilia (Fig. 3).

Effects of G-protein mutations on lung cytokine mRNA responses. Analysis of lung RNA by RNase protection assay (RPA) illustrated striking differences among the mice immunized with wild-type or mutant Trx-G proteins and subsequently challenged with RSV. As shown in Fig. 4, the cytokines most prone to upregulation were interleukin 4 (IL-4), IL-10, IL-13, and to a lesser extent IL-5. Mice immunized with wild-type Trx-G prior to RSV challenge showed the greatest response in all four of these cytokine mRNAs. Nevertheless, the K193A, P190A, and I189A mutants were found to provoke dramatic IL-4, IL-10, and IL-13 responses, albeit to a lesser extent than did wild-type Trx-G. Weaker IL-4, IL-10, and IL-13 responses were observed with the N192A, N191A, R188A, K187A, and C186A mutants. Least effective was the I185A mutant.
FIG. 4. RPA of lung RNA, illustrating relative levels of cytokine mRNA in lungs of mice assayed 4 days after RSV challenge, having been previously immunized twice subcutaneously at 14-day intervals with PBS-alum or an alum-adjuvanted, wild-type (wt) or mutant Trx-G128-229 protein. RNA was isolated from individual mouse lungs by using the RNeasy minikit (QIAGEN, Mississauga, Canada) and then pooled per group of seven to nine mice. RNA was quantitated and subjected to RPA by using a transcription kit (BD-Pharmingen, Mississauga, Canada) to synthesize probe from a cytokine (MCK-1) template (BD-Pharmingen) and radiolabeled by using $[^32]P$UTP, followed by hybridization and RNase digestion, using an RPAIII kit (Ambion, Austin, Tex.). Reaction mixtures were resolved on a 5% polyacrylamide 8 M urea gel according to the manufacturer’s instructions, followed by drying and autoradiography at $-70^\circ$C, using an intensifying screen. Controls include yeast RNA, (similarly subjected to the entire RPA procedure), as well as $^{32}$P-labeled cytokine probe (not subjected to RNase digestion). Panels A and B show different regions of the polyacrylamide gel, which was autoradiographed for 3 days (A) or 1 h (B). Panel C shows relative levels of selected cytokines, IL-4, IL-5, IL-10, IL-13 and IFN-γ (normalized with respect to L-32 and glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) obtained by densitometric analysis of the autoradiograms. For each cytokine, relative levels are normalized to 100 for the wild type Trx-G128-229 protein.
The present study is the first amino acid by amino acid analysis of the major Th-cell epitope in the RSV G protein with reference to the crucial vaccine-relevant readouts of protection against RSV challenge and lung eosinophilic infiltration. Our results highlight a striking dependence of “beneficial” protective immunity versus “harmful” eosinophilia on specific amino acids within the 185-193 region of the RSV G protein. The present study complements the work of Varga et al. (37), who performed analyses of cytokine responses from peptide-stimulated Th cells taken from the lungs of mice primed with a G-protein-expressing recombinant vaccinia virus (37). This previous study identified a core epitope between amino acids 185 and 193 which was required for the production of both Th1 and Th2 cytokines by RSV G-protein-specific memory effector Th cells. The results of our own study demonstrate the importance of selected single-amino-acid substitutions on the crucial vaccine-relevant outcomes of protection against virus challenge and induction of eosinophilic inflammation. Our findings indicate certain substitutions which can either cripple or, more importantly, tailor the immune response towards either a more beneficial outcome or a more harmful outcome.

Th2 cytokine mRNA levels in the lung were dramatically elevated following immunization with the wild type or one of several Trx-G mutants, followed by RSV challenge. The strongest response was seen with IL-13, which has been recently implicated in asthma (10) as well as in RSV vaccine-induced disease (18). In our study, Th2-type cytokines, IL-13 along with IL-10, IL-4, and IL-5, showed distinct responses dependent on the particular Trx-G protein variant used for immunization. High levels of IL-13 and IL-10 correlated well with high levels of eosinophilia observed in RSV-challenged mice which had been immunized with the wild type or with the I189A, P190A, K192A, or K193A mutant. In contrast, the K187A and R188A mutants were poor inducers of IL-13, IL-10, and eosinophilia despite being good inducers of IL-4. Differential production of IL-4 and IL-13 has been previously reported between mice immunized with recombinant vaccinia virus expressing the RSV G protein and those immunized with formalin-inactivated RSV (19).

In contrast to Th2 cytokines, the prototype Th1 cytokine, gamma interferon (IFN-γ), was elevated for all experimental mouse groups. This likely reflects the expression of IFN-γ from NK cells as well as Th1 cells (36), the rapid induction of IFN-γ upon infection with RSV (17), and the prevalent nature of IFN-γ expression even in immune processes in which a Th2 response appears to predominate (32, 33, 38).

It should be emphasized that the RSV G protein represents only one of many complex immunological determinants of RSV which have potential relevance to vaccine design. For example, recent work has shown that Th2-associated eosinophilia can also be primed in mice immunized with alum-adjuncted formalin-inactivated RSV lacking the G protein (20, 28). Nevertheless, the G protein contains the only RSV determinant so far linked to defined Th-cell responses (34, 37) and thus offers a unique opportunity to study Th1/Th2 regulation by a single viral epitope at the amino acid level.

The search for a safe and effective RSV vaccine remains elusive due to an unknown number of beneficial as well as adverse immunological determinants located on both the G protein (13, 26, 31, 34, 35, 37) and other viral constituents (20). Additional complicating factors include evidence that vaccine-relevant epitopes identified in animal models, such as the mouse, may vary from those in humans (4). Nevertheless, there is considerable evidence that vaccine-enhanced RSV immunopathology in both mice (3, 8) and humans (reviewed in references 9 and 25) is the consequence of an imbalanced Th2/Th1 response which may therefore be amenable to modulation by selective mutagenic approaches of relevant epitopes, as illustrated by the present study.

In the design of safer and more effective RSV vaccines, the benefits of amino acid mutagenesis, such as those described here, may be further enhanced with improved vaccine delivery systems. Encapsulation of Trx-G proteins within liposomes, for example, increases protection against RSV while suppressing eosinophilia (15, 23). Clearly, there are potential opportunities for new developments in molecular engineering as well as immunization delivery in the realization of a successful RSV vaccine.

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