CONCISE COMMUNICATION

Serum Neutralizing Antibody Titers of Seropositive Chimpanzees Immunized with Vaccines Coformulated with Natural Fusion and Attachment Proteins of Respiratory Syncytial Virus

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Subunit vaccines formulated with purified fusion proteins from the A2 (PFP-2) or attenuated 248/404 (PFP-3) strains of respiratory syncytial virus (RSV) were evaluated, either alone or in combination with native attachment (G) protein, for their ability to augment serum neutralizing antibody titers in seropositive chimpanzees. The results suggested that combination vaccines enhanced serum neutralizing antibody titers against both laboratory strains and clinical isolates of RSV. When compared with PFP-2 alone, the resultant neutralizing antibody titers after vaccination with PFP-2+Ga protein were significantly elevated against 71% of A strains tested. In a confirmatory experiment, immunization with PFP-3+Ga+Gb proteins resulted in elevated serum neutralizing antibody titers against 86% of A and 50% of B strains tested versus injection with PFP-3 alone. The results suggest that subunit vaccines composed of both PFP and G proteins have more potential than PFP alone to augment neutralizing antibody titers in seropositive recipients.

Respiratory syncytial virus (RSV) is a negative-strand RNA virus. RSV is recognized as a major infectious agent associated with bronchopneumonia, bronchiolitis, and pneumonia in very young infants and in infants and children with congenital heart disease, bronchopulmonary dysplasia, or cystic fibrosis [1]. Asthma and atopy are also associated with RSV bronchiolitis early in life. RSV is also a major cause of respiratory tract disease in aged adults and patients with immunological abnormality [2]. Nonetheless, neither attenuated nor subunit vaccines have been developed. For subunit vaccines, immunization of naive subjects may be contraindicated, because exacerbated disease has been observed on subsequent infection of seronegative infants vaccinated with a formalin-inactivated vaccine [3]. We have, therefore, focused on the development of subunit vaccines for at-risk human populations previously infected with RSV, in whom there is no known potential for atypical disease. These subjects include the elderly, children with chronic pulmonary or cardiac diseases, and women in the third trimester of pregnancy. Several clinical trials with immunoaffinity (purified fusion protein [PFP-1])– or ion-exchange (PFP-2) chromatography–purified natural F protein showed it to be safe and immunogenic in institutionalized [4] or ambulatory [5] aged human volunteers, normal children >18 months of age [6], and children with cystic fibrosis [7] or bronchopulmonary dysplasia [8]. The clinical data, when taken together, suggest that ~50% of the recipients have >4-fold increases in serum neutralizing antibody titers.

To further improve immunogenicity of PFP-based vaccines, we have investigated formulations that incorporate novel adjuvants [9, 10] and/or additional antigens. With regard to antigens, we demonstrated recently [9] that serum-neutralizing antibody titers were significantly elevated in naive BALB/c mice when PFP was coformulated with highly purified natural G protein. Because our target populations were previously infected with RSV, the potential of vaccines composed of both PFP and G protein to augment serum-neutralizing antibody titers was investigated in seropositive chimpanzees. Our results show that coformulation with G protein significantly increases neutralizing antibodies against both A and B strains of RSV. Thus, combination vaccines have more potential to generate elevated humoral immune responses in seropositive populations.

Materials and Methods

Experimental groups. Two experiments were conducted at the New Iberia Research Center (New Iberia, LA); these studies used male and female chimpanzees 6–9 years old (22–45 kg body weight). Approximately 2 weeks before the start of each study, peripheral blood was collected for baseline serology. The chimpanzees were ranked according to anti–F protein IgG titers and placed into groups of 5, created by random selection from animals with the lowest titers. All chimpanzees were given a complete physical examination before entry into the study.
Results

As a prerequisite for conducting studies in seropositive chimpanzees, experiments were performed wherein vaccines coformulated with PFP-2 and Ga glycoproteins adsorbed to alum adjuvant were administered to BALB/c mice previously infected with the A2 strain of RSV. The results demonstrated that combination vaccines increased serum neutralizing antibody titers significantly more than did PFP-2 alone (data not shown). Moreover, challenge of seropositive mice vaccinated with PFP-2+Ga protein did not result in pulmonary eosinophilia (data not shown).

**Sero logical analyses.** Significant differences ($P<.05$) were determined after log transformation by the Student's $t$ test (JMP Statistical Discovery software; SAS Institute, Inc., Cary, NC).

### Table 1.  
The addition of natural Ga and Gb proteins to purified fusion protein (PFP) adsorbed to alum adjuvant increases serum neutralizing antibody titers against various strains of respiratory syncytial virus in seropositive chimpanzees.

<table>
<thead>
<tr>
<th>Strain, group</th>
<th>Serum neutralizing antibody titers ($\log_{10}$)$^a$</th>
<th>% $&gt;4\times$ $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
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<tr>
<td>A2, A</td>
<td>1.2 ± 0.2</td>
<td>100/100</td>
</tr>
<tr>
<td>E10, A</td>
<td>1.2 ± 0.2</td>
<td>80/100</td>
</tr>
<tr>
<td>E21, A</td>
<td>1.2 ± 0.2</td>
<td>80/100</td>
</tr>
<tr>
<td>G2, A</td>
<td>1.2 ± 0.2</td>
<td>80/100</td>
</tr>
<tr>
<td>Long, A</td>
<td>1.2 ± 0.2</td>
<td>100/100</td>
</tr>
<tr>
<td>137, A</td>
<td>1.2 ± 0.2</td>
<td>80/100</td>
</tr>
<tr>
<td>148, A</td>
<td>1.2 ± 0.2</td>
<td>80/100</td>
</tr>
<tr>
<td>B1, B</td>
<td>1.2 ± 0.2</td>
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</tr>
<tr>
<td>35, B</td>
<td>1.2 ± 0.2</td>
<td>80/100</td>
</tr>
<tr>
<td>18537, B</td>
<td>1.2 ± 0.2</td>
<td>80/100</td>
</tr>
</tbody>
</table>

$^a$Seropositive chimpanzees were vaccinated on days 0, 28, and 118 with PFP-3 or PFP-3+Ga+Gb. The numbers are the geometric mean titers ($\pm 1$ SD) from sera collected on days 0 (Pre) and 132 (Post). There were 5 chimpanzees per group.

$^b$Denotes the percentage of chimpanzees with $>4$-fold increases in serum neutralizing antibodies compared with prevaccination titers on days 118 and 132 of the study. A titer of 5 was assigned to sera with titers $<5$.

$^c$Denotes significance ($P<.05$) vs. PFP-3 alone.

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**Purified natural proteins.** Fusion proteins were purified, as described elsewhere [9], by ion-exchange chromatography from Vero cells (ATCC No. CCL 81) infected with the A2 (PFP-2) or attenuated 248/404 (PFP-3) strains of RSV [11]. PFP-2 and PFP-3 were adsorbed to aluminum hydroxide or aluminum phosphate, respectively, and administered at 500 μg/dose. Natural G proteins were purified [9] by immunoaffinity chromatography from Vero cells infected with the A2 (Ga) or B1 (Gb [12]) strains. All proteins were $>$95% pure as estimated by SDS-PAGE and antigen-capture ELISA.

**Immunizations.** The chimpanzees were vaccinated intramuscularly (0.5 mL per injection). In the first experiment, vaccines were administered on days 0, 28, and 78 with 10 μg of PFP-2 alone or PFP-2 coformulated with 2 μg of Ga protein. The combination vaccine for tertiary injection on day 78 contained 10 μg of PFP-2 and 10 μg of Ga protein. In the second experiment, vaccines were administered on days 0, 28, and 118 with 20 μg of PFP-3 alone or PFP-3 coformulated with 20 μg of Ga and 20 μg of Gb proteins. There were no adverse events associated with the vaccines.

**Sero logical analyses.** Geometric mean end-point anti-F or Ga protein IgG and neutralizing antibody titers were determined, as described elsewhere [9], by ELISA and the plaque-reduction neutralization test, respectively. The neutralizing antibody titers were determined against 7 A and 4 B strains of RSV. The National Center for Biotechnology Information accession numbers for the A2, Long, B1, and 18537 laboratory strains are M11486, AF067125.1, AF013254, and D00736, respectively. The A strains designated as 137, 148, E10, E21, and G2 and the B strains designated as 6 and 35, obtained from patients with RSV disease, were a kind gift of Dr. C. B. Hall (University of Rochester School of Medicine and Dentistry, Rochester, NY). The neutralizing antibody titers were calculated as the reciprocal of the serum dilution that showed 60% reduction (relative to the virus control) in the number of foci per well. The ELISA plate microwells were coated with F or Ga proteins from the A2 strain of RSV. Insufficient amounts of protein from the B1 strain prevented determination of anti-Gb protein IgG titers. Bound IgG antibody was detected by using alkaline phosphatase-conjugated goat anti-human IgG antibodies (Jackson ImmunoReagents, West Grove, PA).

**Statistical analyses.** Significant differences ($P<.05$) were determined after log transformation by the Student’s $t$ test (JMP Statistical Discovery software; SAS Institute, Inc., Cary, NC).
the B1 strain (60%). After tertiary vaccination, 100% of the recipients had 4-fold increases against all strains tested. Compared with administration of PFP-3 alone, the titers generated after immunization with PFP-3 + Ga + Gb proteins were significantly elevated against 6 of 7 A and 2 of 4 B strains tested (table 1).

It was noteworthy that 1 injection of PFP-2 or PFP-3, either alone or in combination with G proteins, significantly increased neutralizing antibodies when compared with preinjection titers. For example, 2 weeks after the first injection of PFP-3 alone, the titers of all animals were elevated 4-fold (6-118-fold) against the A2 (figure 1C) and B1 (4-97-fold; figure 1D) strains. Similarly, 2 weeks after the first injection of PFP-3 + Ga + Gb proteins, the neutralizing titers of 4 of 5 chimpanzees were elevated 30-221-fold and 3-135-fold against the A2 and B1 strains, respectively.

To further document the role of antibody responses to G protein in virus neutralization, we determined titers of anti–F and Ga protein IgG in the serum of chimpanzees vaccinated with PFP-3 + Ga + Gb protein or PFP-3 alone. Figure 1 (panels A and B) depicts the temporal development of anti–F and Ga protein IgG titers. No increases in anti–Ga protein titers were observed in chimpanzees vaccinated with PFP alone (data not shown). The effect of anti–Ga protein IgG (figure 1B) on neutralizing antibody titers was best exemplified by chimpanzee A93010 (figure 1, filled circles). This subject was seronegative to G protein on day 0 and consistently had the lowest anti–Ga protein IgG titers. Neutralizing antibodies (figure 1C, 1D) were first detected 4 weeks after the secondary injection and coincided with the appearance of anti–Ga protein IgG (figure 1B). Ninety days thereafter (day 118), the neutralizing titers against the B1 and A2 strains remained constant (figure 1D) or diminished to background (figure 1C). In comparison, anti–F protein IgG titers remained elevated between days 56 and 118 (figure 1A). Upon tertiary injection, the anti–F and Ga protein titers increased 10- and 316-fold, respectively, with resultant neutralizing titers dramatically enhanced 13-20-fold. Similar results were observed in the first study, wherein the neutralizing antibody titers of 2 animals were increased (5–6-fold) after tertiary vaccination without any significant increase in anti–F protein IgG titers (data not shown). In contrast, all chimpanzees’ anti–Ga protein titers were increased 4-899-fold. It is noteworthy that the addition of G proteins to PFP did not significantly affect anti–F protein antibody titers (figure 1A).

**Discussion**

The development of subunit vaccines for RSV has focused primarily on highly purified natural or recombinant fusion proteins alone or in combination with the attachment protein. Both proteins induce functional antibodies, which together are synergistic and neutralize virus infectivity in vitro [9, 13]. The purpose of the experiments described herein was to determine if the presence of G protein could enhance the ability of PFP-based vaccines to generate functional antibody titers in seropositive recipients. The results confirm those observed in BALB/c mice previously infected with RSV and substantiate in seropositive primates the potential of multicomponent subunit vaccines to augment neutralizing antibody titers beyond those achieved with PFP alone. When G protein was included in the vaccine, neutralizing titers were significantly increased against both laboratory strains and clinical isolates. Even against strains in which statistical differences were not observed, there was a strong trend toward elevated titers. That is, although diminished 2–25-fold 90 days after secondary vaccination, the neutralizing titers of most chimpanzees vaccinated
with PFP-3+Ga+Gb proteins were still 4 times greater than prevaccination titers. In contrast, fewer chimpanzees vaccinated with PFP alone had >4-fold increases in neutralizing titers at this time point.

Nonetheless, statistically elevated neutralizing titers versus PFP alone were not observed in the circulation until 2 weeks after tertiary vaccination. Thus, issues regarding the use of novel adjuvants or antigen dose should be addressed to successfully formulate combination vaccines that generate titers sufficient to neutralize virus infectivity throughout the RSV season. With regard to dose, the results imply that expansion in titers beyond that achieved with PFP alone is more dependent on previous exposure to RSV and preexisting anti-G protein titers than on the dose of antigen in the vaccine. In the studies presented herein, there was little evidence of a dosage effect with 2, 10, or 20 μg of Ga protein. An alternative explanation is that neutralizing titers were nearly maximum after vaccination with PFP alone, and, because the study population was small, the impact of G protein was difficult to detect. Thus, the potential advantage of combination vaccines to enhance serum neutralizing titers after 1 or 2 injections requires further testing in phase I clinical trials wherein the target population responds less effectively to PFP alone [4].

Finally, it is expected that expansion of neutralizing antibodies in seropositive humans is dependent on the conformational resemblance of vaccine antigens to F and G proteins in the virus envelope. A recent report [14] supports this notion. However, the report concludes that F protein, column-purified from epithelial cell lysates, is structurally immature and thus, for the most part, unable to augment neutralizing titers. This deduction must be tempered by data from several clinical studies [4–8] that clearly show the capacity of PFP-based vaccines to increase neutralizing antibody titers >4-fold in ~50% of recipients. When combined with results showing that PFP is recognized by a monoclonal antibody (L4) with potent neutralizing activity [15], the results presented herein strongly demonstrate that PFP is capable of enhancing the neutralizing titers of seropositive humans. The data presented herein also support the concept that the presence of G protein in the vaccine will expand these titers further.

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References