A Single Mutation at PB1 Residue 319 Dramatically Increases the Safety of PR8 Live Attenuated Influenza Vaccine in a Murine Model without Compromising Vaccine Efficacy

Andrew Cox, Stephen Dewhurst
Department of Microbiology & Immunology, University of Rochester Medical Center, Rochester, New York, USA

The live attenuated influenza vaccine (LAIV) is preferentially recommended for use in most children yet remains unsafe for the groups most at risk. Here we have improved the safety of a mouse-adapted live attenuated influenza vaccine containing the same attenuating amino acid mutations as in human LAIV by adding an additional mutation at PB1 residue 319. This results in a vaccine with a 20-fold decrease in protective efficacy and a 10,000-fold increase in safety.

We replaced the natural (and universally conserved) leucine with glutamine at residue 319 of PB1 (Fig. 1A). This amino acid lies underneath the PA linker region and 30 Å from PB1 residue 391 (the nearest amino acid mutation of LAIV) (13). Mutating...
PB1 position 319 from leucine to glutamine in the context of the mutations of LAIV results in a dramatically altered polymerase activity profile as measured by the minigenome assay (Fig. 1B), as PR8 LAIV containing 319Q shows a sharp reduction in polymerase activity at temperatures as low as 37°C. Interestingly, the introduction of PB1 L319Q alone has little to no impact on temperature-sensitive polymerase activity.

We then rescued viruses containing either PB1 L319Q (PR8 319Q) alone or the attenuating mutations of LAIV and PB1 319Q (PR8 LAIV 319Q) and analyzed their growth in MDCK, A549, and Vero cells. A549 cells were selected because they are derived from the human airway, while Vero cells were chosen because they have a defective interferon response system (14–18).

Compared to wild-type (WT) viruses, PR8 containing only PB1 L319Q shows a slight reduction in replication at elevated temperatures in A549 cells, but not in MDCK or Vero cells (Fig. 1). PR8 LAIV shows impaired growth at 39°C in all cell types tested (12). In contrast, PR8 LAIV 319Q shows dramatically reduced replication at 37°C, and no virus was detected at 39°C in A549 and MDCK cells. Therefore, the addition of PB1 319Q dramatically increases the temperature sensitivity of viruses containing the attenuating mutation of LAIV. We also examined the stability of these viruses and saw that the viruses retained the attenuating mutations after 10 passages in tissue culture (data not shown).

We then sought to examine the safety of these viruses in mice. Five- to 7-week-old female B6 mice (Jackson Laboratory) were infected intranasally after light anesthetization with increasing doses of PR8 319Q and PR8 LAIV 319Q. Data from Cox et al. on the safety of LAIV in C57BL/6 mice is replicated for comparison (12). Addition of PB1 319Q to LAIV increased safety by 10,000-fold (from 10^2 focus-forming units [FFU] to 10^6 FFU), as determined by comparing maximum safe doses in mice (i.e., the maximum dose at which no weight loss was observed), as PR8 LAIV has a maximum safe dose of 100 FFU (Fig. 2 and Table 1). This 10,000-fold increase in safety over LAIV alone is accompanied by a slight (20-fold) increase in the vaccine dose needed for protection from lethal challenge (Fig. 2 and Table 1).

Virus replication was detected in the mouse lung with doses of 10^7 FFU seroconverted as measured by the HAI assay (Fig. 3B). One mouse infected with 10^7 FFU of PR8 LAIV 319Q seroconverted, but no mice infected with 10^6 FFU did. Notably, the maximum nonlethal dose of PR8 LAIV 319Q (10^6 FFU) elicited 4-fold-higher titers of serum HAI antibodies compared to the maximum nonlethal dose of PR8 LAIV (10^4 FFU) (Fig. 3B).

We also included an additional control for replication. We UV inactivated PR8 LAIV 319Q for 20 min (on ice) and confirmed inactivation by plaque assay (data not shown). We then infected mice with this UV-inactivated virus at 10^6 FFU (as determined before UV inactivation). Mice were bled, and sera were analyzed in parallel with the above sera. These mice did not seroconvert, as assessed by HAI—nor did they exhibit detectable levels of HA binding serum antibodies, as measured by enzyme-linked immunosorbent assay (ELISA) (in contrast, mice vaccinated with 10^6 FFU of live LAIV 319Q showed robust HA binding past a 1:10,000 dilution as did the sera of mice infected with a nonlethal dose of WT PR8). These data suggest that seroconversion requires a rep-

**TABLE 1**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Maximum safe dose (FFU)</th>
<th>PD-50 (FFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR8</td>
<td>1^c</td>
<td>ND</td>
</tr>
<tr>
<td>PR8 PB1 319Q</td>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>PR8 LAIV</td>
<td>10^6</td>
<td>20</td>
</tr>
<tr>
<td>PR8 LAIV PB1 319Q</td>
<td>1,000,000</td>
<td>400</td>
</tr>
</tbody>
</table>

^a Safety measurements for PR8 and PR8 LAIV are taken from previous work (12). We then examined the protective dose of PR8 LAIV and PR8 LAIV 319Q and found that the mean protective dose of PR8 LAIV to protect against lethal challenge was 20 and 30 FFU against H1N1 and H3N2, respectively. For PR8 LAIV 319Q, the mean protective dose against lethal challenge was 400 and 600 FFU against H1N1 and H3N2, respectively.

^b The mean protective dose (PD-50) is shown. ND, not determined.

^c Data from reference 12.
licating virus and that this novel LAIV is not functioning like a virus-like particle but is replicating in the host and that this replication is required for immunogenicity.

We then sought to examine the protective efficacy of this novel vaccine candidate. Mice were challenged with 100 50% lethal doses (LD50) of either PR8 (homologous) or X31 (heterologous) virus 21 days after vaccination. Mice vaccinated with either phosphate-buffered saline (PBS) or 10^2 FFU of PR8 LAIV (the maximum safe dose) were included as negative and positive controls, respectively.

LAIV 319Q provided greater protection than LAIV at matched doses of priming virus (the highest dose of each virus that did not cause weight loss), suggesting that this safer vaccine candidate does not have compromised efficacy (Fig. 4). Additionally, LAIV 319Q protected mice against lethal challenge at vaccination doses of 10^3 or greater against both homologous and heterologous chal-

**FIG 3** The immunogenicity of PR8 LAIV plus PB1 L319Q is replication dependent. (A) PR8 LAIV plus PB1 L319Q replicates in vivo. Mice were inoculated as described above with 10^7 FFU of PR8 LAIV 319Q (n = 3 or 4). At 2 and 4 days postinfection, lung virus titers (TCID<sub>50</sub>/ml) were determined from total lung homogenates on MDCK cells using the method of Reed and Muench. The mean virus lung titers (longest lines) ± standard deviations (SD) (error bars) from individual mice are indicated. (B) Mice were bled 15 days postinfection, and sera were clarified by centrifugation and treated with receptor-destroying enzyme (BEI) for 18 h at 37°C and then neutralized by heat inactivation at 56°C for 30 min. PR8 (8 hemagglutinating units) was incubated with twofold serial dilutions of the indicated serum to determine the ability to neutralize virus binding to red blood cells (RBC); therefore, a 1/8 dilution is the limit of detection and indicated by the dotted line. Each symbol represents the value for an individual mouse, and the mean plus standard deviation (error bars) for the group of mice are shown. Statistical analysis was performed using one-way analysis of variance (ANOVA). Values that are statistically significantly different are indicated by the bar and asterisks as follows: **, P < 0.01.

**FIG 4** Homologous and heterologous protection are induced by PR8 LAIV 319Q. (A) Protection from lethal homologous challenge conferred by PR8 LAIV 319Q. Female 5- to 7-week-old B6 mice were inoculated intranasally with PBS (black line), 100 FFU of PR8 LAIV (blue line), or increasing doses of PR8 LAIV 319Q. Three weeks postpriming, mice were challenged intranasally with 100 LD<sub>50</sub> of a homologous virus (PR8 H1N1). For 2 weeks postchallenge, weight loss (top graph) (plotted data represent means ± standard errors of the means [SEM]) and survival (bottom graph) were monitored daily (n = 5). (B) Heterologous protection is induced by PR8 LAIV 319Q. Female 5- to 7-week-old B6 mice were inoculated as described above. Three weeks postpriming, mice were challenged intranasally with 100 LD<sub>50</sub> of a heterologous virus (X31 H3N2). For 2 weeks postchallenge, weight loss (top graph) (plotted data represent means ± SEM) and survival (bottom graph) were monitored daily (n = 5).
lenges. This implicates T cell-mediated immunity, as none of these mice displayed seroconversion to X31 at day 15 postvaccination (data not shown). Interestingly, mice that did not seroconvert to either virus were also protected against homologous and heterologous challenge. This echoes the work of Halliley et al. who showed that measuring vaccine responses to LAIV by HAI misses protective efficacy (19).

In summary, we have created a live candidate vaccine with 10,000-fold-increased safety that also retains robust immunogenicity in the murine model of influenza infection. The addition of PB1 L319Q may be considered for improving the safety of the current LAIV.

ACKNOWLEDGMENTS
A.C. was supported by a Technology Development Fund grant from University of Rochester URVentures and by the NIH (through grants T32AI118689 and T32GM007356). This research was funded by NIH grant R21 AI112717 (to S.D.).

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

FUNDING INFORMATION
HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID) provided funding to Stephen Dewhurst under grant numbers R21 AI112717-01 and HHSN2662007000008C. HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID) provided funding to Andrew Cox under grant number T32AI118689-01. HHS | NIH | National Institute of General Medical Sciences (NIGMS) provided funding to Andrew Cox under grant numbers T32-GM07356-35 and T32-GM068411-08. University of Rochester Technology Development Fund (TDF) provided funding to Andrew Cox.

REFERENCES

Downloaded from http://jvi.asm.org on October 20, 2017 by guest