Reversion of recombinant toxoids: Mutations in diphtheria toxin that partially compensate for active-site deletions

KEVIN P. KILLEEN*, VINCENT ESCUYER*, JOHN J. MEKALANOS*, AND R. JOHN COLLIER§

*Department of Microbiology and Molecular Genetics, Harvard Medical School, and §the Shipley Institute of Medicine, 220 Longwood Avenue, Boston, MA 02115

Contributed by R. John Collier, December 26, 1991

ABSTRACT
Deleting an important active-site residue of diphtheria toxin, glutamic acid-148, reduces the toxin's ADP-ribosyltransferase activity by a factor of >10⁴. We considered using this mutation to construct a recombinant toxoid for expression by live attenuated vaccines and explored second-site mutations that might cause reversion. Activity was partially restored by substituting glutamic acid for valine-147 or by extending the deletion by five residues toward the NH₂ terminus, thereby placing glutamic acid-142 immediately adjacent to tyrosine-149. In both mutants the indicated glutamic acid may occupy a spatial locus similar to that of glutamic acid-148 in the unmutated protein. Simply deleting a crucial residue does not, therefore, provide confidence that a second-site mutation could not readily restore activity to a toxoid.

Immunity to a bacterial toxin may be acquired naturally during the course of an infection or artificially by injection of a detoxified form of the toxin (a toxoid) (1). Toxoids have traditionally been prepared by chemical modification of native toxins, with formaldehyde, for example (2), but molecularly cloned toxin genes now provide alternative genetic approaches to detoxification. Besides having the potential to replace classical toxoids, recombinant toxoids could be incorporated by genetic engineering into live attenuated bacterial or viral vaccines currently being developed. Such live vaccines offer the possibility to immunize against a broad array of diseases by administering only a single dose to the vaccinee (3, 4).

If the gene encoding a recombinant toxoid is to be included in the expression repertoire of a live vaccine, the mutation(s) that detoxifies the protein must be effectively nonrevertible while the strain remains viable within the vaccinee or in the environment. For toxins known to act as enzymes, one way to inactivate the molecule is to mutate a crucial active-site residue. Deleting such a residue might at first sight appear to be an ideal strategy, because the probability of reversion to the wild-type sequence, by insertion of an appropriate codon, should be vanishingly small. However, reversion might also occur by second-site mutations. Here we explore the effects of second-site mutations in sequences adjacent to a deletion in the active site of diphtheria toxin (DT).

RESULTS AND DISCUSSION

DT belongs to a class of toxins that act by ADP-ribosylation of target proteins within sensitive cells (10, 11). DT (193 residues) catalyzes transfer of the ADP-ribose moiety of NAD to elongation factor 2 (10, 11), thereby inactivating the factor. This blocks protein synthesis and causes death of the cell. Glu-148 was originally identified as an active-site residue by photoaffinity labeling (12-14). Substitution of aspartic acid, glutamine, or serine at this site diminishes enzymic and cytotoxic activities by two to three orders of magnitude, showing the exquisite dependence of activity on the spatial location and chemical nature of the Glu-148 side chain (8, 9, 14). Deletion of a functionally similar residue in Pseudomonas aeruginosa exotoxin A, Glu-553, also causes a drastic reduction in enzymic and cytotoxic activities (15).

Active-site deletions and substitutions were generated by oligonucleotide-directed mutagenesis of the DT gene. The mutant genes were expressed in E. coli, and cell lysates were assayed for NAD-elongation factor 2 ADP-ribosyltransferase activity and for DT-specific protein by Western blot analysis. After deletion of Glu-148 (Table 1, mutation 1) the specific NAD-elongation factor 2 ADP-ribosyltransferase activity of DT was undetectable (<10⁻⁴ that of wild-type DT). However, this deletion, when combined with the replacement of Val-147 with Glu, created a product with 6% wild-type activity (Table 1, mutation 7). In contrast, deletion of Glu-148 coupled with the Tyr-149 → Glu (Y149E) mutation yielded an inactive product (mutation 12).

Longer deletions extending from Glu-148 NH₂-terminally as far as residue 144 (Table 1, mutations 2–5) yielded products

---

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: DT, diphtheria toxin; DTA, DT fragment A.
1Present address: Virus Research Institute, 61 Moulton Street, Cambridge, MA 02138.
2Present address: Faculté de Médecine Necker-Enfant Malades, Laboratoire de Bactériologie, 75730 Paris Cedex 15, France.
3To whom reprint requests should be addressed.
Table 1. ADP-ribosyltransferase activities of DTA with active-site mutations

<table>
<thead>
<tr>
<th>Mutation no.</th>
<th>Ala-141</th>
<th>Glu-142</th>
<th>Gly-143</th>
<th>Ser-144</th>
<th>Ser-145</th>
<th>Ser-146</th>
<th>Val-147</th>
<th>Glu-148</th>
<th>Tyr-149</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (wild type)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.6</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>6</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>9</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>Glu</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>6</td>
</tr>
<tr>
<td>10</td>
<td>Glu</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.6</td>
</tr>
<tr>
<td>11</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
</tbody>
</table>

- Deleted residue; Glu, Glu substitution; ND, <10^-4 wild-type activity.

with no detectable ADP-ribosylation activity. However, the next construct in this series (mutation 6), involving deletion of residues 143–148 inclusive, produced a protein with 0.6% wild-type activity. In mutation 6, unlike mutations 1–5, the NH2-proximal residue flanking the deletion is a glutamic acid (Glu-142). Activity ranging between 0.6% and 9% of wild-type DTA activity was observed when the NH2-proximal flanking residue of mutations 1–5 was replaced with glutamic acid creating mutations 7–11.

These results are consistent with a model in which the local polypeptide on the NH2-proximal flank of Glu-148 is more flexible and less firmly anchored than the local peptide on the COOH-proximal flank. The recently determined x-ray crystallographic structure of the DT dimer provides support for this model (16). Glu-148 residues within the antiparallel β-sheet bounding the active-site cleft and is only one residue removed from a large 10-residue loop (residues 137–146) that connects the Glu-148 β-strand to the adjacent NH2-proximal β-strand (Fig. 1). Presumably, when those residues contained within this flexible loop are either deleted or replaced (Table 1, mutations 3–6 and 8–11), the loop simply shortens in length, buffering the active site from structural distortion. The polypeptide backbone of the 4 residues immediately following Glu-148 (residues 149–152) is involved in H-bonding typical of antiparallel β-sheet, and this bonding and other packing interactions may firmly anchor this region of polypeptide within the protein. This may explain why the Tyr-149 → Glu mutation (Table 1, mutation 12) yielded a catalytically inactive product.

These results illustrate two discrete genetic changes that revert a DT active-site deletion mutant to a partially toxic state, one involving a substitution and the other an additional deletion. The levels of activity observed are in all cases <10% of wild-type but are clearly of concern if the protein is to be expressed in vivo by a live vaccine. The Val-147 → Glu substitution could occur by either of two 2-bp transversions of the valine codon, GTT, to the glutamic acid codons, GAA or GAG. Deletion of both the Val-147 and Glu-148 codons places Ser-146 next to Tyr-149 and would require a 3-bp mutation from the serine AGC codon to either of the glutamic acid codons, thereby reducing the risk of reversion.

It is impossible to predict how general the phenomena described will be among toxic proteins, but a conservative approach is clearly warranted if a modified toxin gene is to be incorporated into a live vaccine. Avoiding the incorporation of sequences encoding toxin active-site moieties altogether would be the most prudent approach. Where antibodies against epitopes on the receptor-binding moiety are known to be immunoprotective, expression of receptor-binding moieties (e.g., as subunits or fragments) alone may be sufficient and preferable. In toxins in which the enzymic moiety carries the most important neutralizing epitopes (e.g., pertussis toxin), multiple mutations, each of which independently detoxifies the molecule, are warranted. Assessment of risk ought not be made solely by focusing on the toxin alone, however, and should include consideration of other factors, including the properties of the vaccine strain itself (e.g., the extent of its multiplication in the vaccinee, its ability to persist outside the host, and its overall level of attenuation). Additional safeguards at all levels will be necessary to assure that live vaccines expressing recombinant toxoids remain innocuous and stable within the vaccinee and the environment.

We are grateful to Jeffrey Miller for helpful discussions and to David Eisenberg and Seunghyun Choe for sharing information on the crystallographic structure of diphtheria toxin prior to publication. This work was supported by Grants AI-22021 and AI-22848 from the National Institute of Allergy and Infectious Diseases. V.E. was a recipient of a fellowship from the European Molecular Biology Organization.