INFECTION AND IMMUNITY, Feb. 1994, p. 757–758
0019-9567/94/$04.00+0

Vol. 62, No. 2

# Amino Acids in Pneumolysin Important for Hemolytic Activity Identified by Random Mutagenesis

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Received 26 August 1993/Returned for modification 20 September 1993/Accepted 15 November 1993

**The structural gene of pneumolysin was randomly mutagenized. A screen, developed to identify mutants with reduced hemolytic activity, identified substitutions within the cysteine-containing region and also at residues toward the N terminus of the toxin. These are the first reported changes within the N-terminal region of the toxin that affect cytolytic activity.**

The membrane-damaging toxin pneumolysin belongs to the thiol-activated toxin family (15) and is known to contribute to the virulence of *Streptococcus pneumoniae* (1, 2). The largest region of identity between members of this family is an 11-amino-acid cysteine-containing region (CCR) (6). Previously, we had used site-directed mutagenesis (SDM) to demonstrate the importance of the CCR for cytolytic activity (10, 14). Now, we report the random mutagenesis of the pneumolysin gene and the development of a hierarchical screening procedure to identify single amino acid substitutions that reduce the hemolytic activity of the toxin.

The pneumolysin gene, cloned into M13mp18, was used as a mutagenesis template. Random mutagenesis was used to introduce single base-specific substitutions throughout the gene (7). Plaques obtained after random mutagenesis were picked into 96-well microtiter plates containing 100 μl of TES buffer (10 mM Tris HCl [pH 8], 1 mM EDTA, 50 mM NaCl) and stored at 4°C for at least 4 h. An overnight culture of *Escherichia coli* JM101 (Stratagene, La Jolla, Calif.) was diluted 1:100 in LB medium (12), and 150 μl was added to each well of a fresh microtiter plate. Phage supernatants were transferred to each well by using a spiked replicator tool. The microtiter plates were incubated overnight at 37°C without shaking. A drop of chloroform was added to each well, using the replicator tool. Chloroform addition was repeated twice to ensure lysis of phage-infected cells, and the plates were left at room temperature for 15 min. Then 50 μl of supernatant was transferred to a fresh microtiter plate, and 50 μl of 2% sheep erythrocytes in phosphate-buffered saline (PBS) was added (14). Plates were then incubated at 37°C and examined by eye to determine the extent of hemolysis. The primary screen identified a number of clones that appeared to express pneumolysin with reduced hemolytic activity.

All nonhemolytic clones selected by the primary screen were subjected to a secondary screening procedure. They were plaque purified, and four plaques from each clone were amplified in JM101 (12). The harvested cells were sonicated and assayed for hemolytic activity. If all replicates of a clone were nonhemolytic, a standard dot immunoblot procedure was used to assay for toxin presence in sonicated extracts (12). The presence of pneumolysin was visualized by enhanced chemiluminescence (Amersham International, Amersham, United Kingdom). Clones that did not produce toxin were eliminated from further studies. Nonhemolytic clones that produced toxin were then analyzed by Western blotting (immunoblotting) (12), using enhanced chemiluminescence detection, to ensure that a full-length toxin was expressed.

Clones with reduced hemolytic activity were also subjected to a secondary screen. They were plaque purified and amplified in JM101. Twofold serial dilutions of sonicated extracts in PBS were made in microtiter plates, and an equal volume of 2% sheep erythrocytes was added to assess hemolytic activity. The activity of toxin from these clones was compared with that of M13-infected JM101 expressing wild-type pneumolysin. Approximately 94% of clones assayed at this stage were eliminated from subsequent analysis because they showed wild-type activity.

Single-stranded DNA of clones with less than wild-type activity was prepared and sequenced (13) with a Sequenase DNA sequencing kit (U.S. Biochemical Corp., Cleveland, Ohio). Selected pneumolysin mutants were subcloned into pUC18. Constructs were expressed in *E. coli* M15 (Qiagen, Chatsworth, Calif.), and toxin was purified as previously described (11). Toxin purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12). Quantitative hemolytic activity values were obtained as previously reported (5). Activity was measured as hemolytic units per microgram of toxin and expressed as a percentage of wild-type pneumolysin activity (10 hemolytic units/μg). Table 1 summarizes the mutations defined by DNA sequence analysis and indicates the specific activity of purified toxin expressed as a percentage of wild-type activity; some substitutions were identified during development of the screen. Table 1 shows that substitutions were made throughout the molecule by the random mutagenesis procedure.

The Trp-433→Arg substitution demonstrates that random mutagenesis, combined with our screen, will identify functionally important regions of pneumolysin. It also supports the importance of Trp-433 for hemolysis, previously suggested following the substitution Trp-433→Phe, which was shown to reduce hemolytic activity by 99% (10). The Trp-436→Arg substitution has the same activity as the Trp-436→Phe substitution (10). This finding indicates pneumolysin can tolerate radically different substitutions at position 436 and that this residue is less important for hemolysis than is Trp-433. These results conflict with work (9) on listeriolysin O. A Trp→Ala substitution at the equivalent residue within the CCR of listeriolysin O decreased activity by 99.9%.

Until now, all mutations affecting cytolytic activity were

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TABLE 1. Substitutions generated and in vitro hemolytic activities

| Substitution | % Hemolytic activity[a] |
|---|---|
| Arg-31→Cys[b] | 75 |
| Leu-75→Phe[b] | 100 |
| Val-127→Gly | 75 |
| His-156→Tyr[b] | 2 |
| Ala-432→Val[b] | 100 |
| Trp-433→Arg | <1 |
| Trp-436→Arg | 50 |
| Val-468→Leu[b] | 100 |

[a] Hemolytic activity expressed as a percentage of wild-type activity (10 hemolytic units/μg).
[b] Clone identified during development of the screen.

found in the C-terminal half of the molecule. The mutations at positions 31, 127, and 156 illustrate an advantage of a random approach to mutagenesis compared with SDM. Computer-based analysis (4) of the amino acid sequences of thiol-activated toxins identifies the CCR as the largest region of identity. Outside the CCR, smaller regions of homology are found within the family of thiol-activated toxins. There are no obvious reasons for predicting which, if any, of these regions are required for hemolysis. To investigate each by SDM would be costly in time and money. Three amino acid substitutions generated in this study lie within regions of pneumolysin possessing much lower homology than the CCR (8). In particular, the residue at position 156 lies within a region that would attract little attention on the basis of sequence analysis. Yet a loss of 98% of hemolytic activity after His-156→Tyr suggests that this residue is crucial for activity. What is curious about this observation is the fact that conversion of the histidine to tyrosine has such a dramatic effect. In all of the other thiol-activated toxins, the amino acid at this position is a tyrosine. However, histidines have been shown to be important in another membrane-damaging toxin, aerolysin (3). Our data suggest they also have a role in pneumolysin cytolytic activity.

Thus, random mutagenesis is particularly useful in situations in which functionally important amino acids cannot be predicted from amino acid sequence information. If combined with the type of screening procedure described here, this technique will be useful for studies of structure-function relationships of new hemolysins.

This work was supported by the British Medical Research Council. T.J.M. is a Royal Society Research Fellow.

We thank Neil Bennett for assistance with DNA sequencing and Steve Hooper, who was involved in screening. The assistance of D. B. Lowrie, NIMR, London, United Kingdom, in obtaining antipneumolysin antiserum is gratefully acknowledged also.

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