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Mutations in the A Subunit Affect Yield, Stability, and Protease Sensitivity of Nontoxic Derivatives of Heat-Labile Enterotoxin

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Heat-labile toxin (LT) is a protein related to cholera toxin, produced by enterotoxigenic *Escherichia coli* strains, that is organized as an AB_5 complex. A number of nontoxic derivatives of LT, useful for new or improved vaccines against diarrheal diseases or as mucosal adjuvants, have been constructed by site-directed mutagenesis. Here we have studied the biochemical properties of the nontoxic mutants LT-K7 (Arg-7→Lys), LT-D53 (Val-53→Asp), LT-K63 (Ser-63→Lys), LT-K97 (Val-97→Lys), LT-K104 (Tyr-104→Lys), LT-K114 (Ser-114→Lys), and LT-K7/K97 (Arg-7→Lys and Val-97→Lys). We have found that mutations in the A subunit may have profound effects on the ability to form the AB_5 structure and on the stability and trypsin sensitivity of the purified proteins. Unstable mutants, during long-term storage at 4°C, showed a decrease in the amount of the assembled protein in solution and a parallel appearance of soluble monomeric B subunit. This finding suggests that the stability of the B pentamer is influenced by the A subunit which is associated with it. Among the seven nontoxic mutants tested, LT-K63 was found to be efficient in AB_5 production, extremely stable during storage, resistant to proteolytic attack, and very immunogenic. In conclusion, LT-K63 is a good candidate for the development of antidiarrheal vaccines and mucosal adjuvants.

Heat-labile toxin (LT) is a bacterial protein produced by enterotoxigenic *Escherichia coli* strains (9, 24) that has 80% sequence homology with (4, 26, 31) and a three-dimensional structure similar to that of cholera toxin. Both toxins are organized in two domains, A and B, that are functionally different (17, 30). The B domain, which contains the GM_1 receptor-binding site (16, 29), is a pentamer composed of five identical B monomers of 11.8 kDa each, arranged in a ring-like structure with a central cavity that houses the carboxy-terminal part of the A subunit (23). The A subunit is an enzyme with ADP-ribosylating activity that is responsible for the toxicity of the toxin (15, 25).

The A subunit is formed by a single polypeptide chain of 27.2 kDa with a mostly globular structure (27). The carboxy-terminal end contains a loop followed by a long α-helix that departs from the globular structure and enters the central cavity of the B pentamer. Activation of the enzymatic activity requires proteolytic nicking of the loop and reduction of a disulfide bridge that links Cys-187 to Cys-199 (1, 2). Following proteolytic nicking and reduction, the A subunit is separated into two fragments: A_1 and A_2, of 21.8 and 5.4 kDa, respectively.

The A and B subunits are synthesized as precursors with signal peptides, and they are transported across the cytoplasmic membrane independently (7, 11, 13). Mature subunits are released into the periplasm by proteolysis of the signal peptides (3), where they assemble into the AB_5 heterohexameric structure (14). In the absence of the A subunit, the B subunits are still able to form the B_5 pentameric structure (12). However, the assembly process is threefold faster when the A subunit is present (10, 28).

This suggests that during assembly, the A subunit interacts with the B monomers or with assembly intermediates and speeds up the process. Additional evidence that the A subunit interacts mostly with B monomers or assembly intermediates is provided by the observation that in vitro, the A subunit can form the holotoxin if mixed with B monomers but cannot form AB_5 when mixed with the already assembled B pentamer (10).

Using computer modelling of the X-ray structure of LT (22, 23), we have identified some of the amino acids involved in its enzymatic activity and provided the rationale to probe their function by changing them by site-directed mutagenesis. Therefore, a number of LT and cholera toxin mutants were constructed and characterized (6, 19). Some of them were found to be devoid of enzymatic activity in the ADP-ribosylation assay, nontoxic in the Y1 cell assay at the highest concentration tested (Table 1), but still immunogenic, and they are presently being evaluated as candidates for antidiarrheal vaccines or mucosal adjuvants (5, 20).

In this study, we analyzed the properties of a panel of LT mutants with different efficiencies in assembly of the AB_5 structure and showed that single-amino-acid mutations in the A

TABLE 1. Enzymatic and toxic activities of the mutant molecules analyzed in this work

Name	Mutation(s)	Nontoxic concn (μg/ml)[a]	ADP-ribosylating activity[b]
LT		2.5×10^{-6}	+++
LT-K7	Arg→Lys	1.9	−
LT-D53	Val→Asp	9.6	ND
LT-K63	Ser→Lys	18.1	−
LT-K97	Val→Lys	38.8	−
LT-K104	Tyr→Lys	7.2	−
LT-K114	Ser→Lys	33.2	−
LT-K7/K97	Arg→Lys, Val→Lys	7.9	−

[a] Toxicity of the purified molecules on Y1 cells. Wild-type LT is toxic at 5 pg/ml; mutant molecules did not show toxicity at the highest concentration tested.

[b] In vitro ADP-ribosylation activity with polyarginine as the substrate. −, no activity; +++, high activity; ND, not determined.

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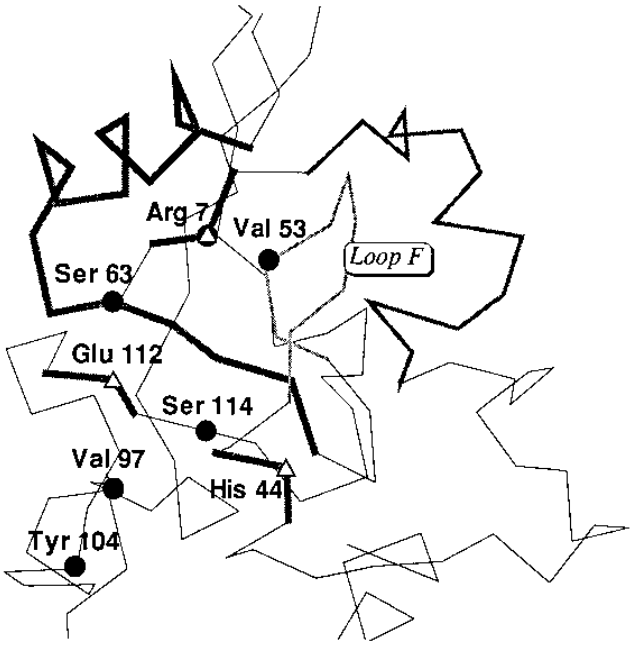

FIG. 1. α-Carbon trace of the NAD-binding and catalytic site of LT, which is part of the A subunit. Boldface lines indicate the structural motif common to other ADP-ribosylating toxins such as diphtheria toxin, exotoxin A of *Pseudomonas aeruginosa*, and pertussis toxin. This is made by a β-strand and then an α-helix that delimit the NAD-binding cavity, and it is flanked by β-strands carrying the catalytic residues, represented by triangles. Boldface lines on the top right show a loop whose structure is conserved among ADP-ribosylating toxins. Loop F, characteristic of LT and cholera toxin, is represented in the center of the figure as grey lines; it closes the catalytic site in the inactive configuration and is probably removed during the enzymatic reaction. The amino acids that have been mutated in the proteins described in this work are shown as solid circles.

subunit affected not only the enzymatic activity but also the protein yield, the sensitivity to proteases, and the stability in long-term storage of the assembled molecules.

Figure 1 shows the α-carbon trace of the structure of the region containing the active site and the positions of the amino acids changed in the nontoxic mutants analyzed in this study.

The mutant proteins were purified from the periplasm of the *E. coli* strains containing the mutated genes by using CPG 350 (Controlled Pore Glass), A5m agarose, and Sephacryl S-200 columns, as described by Pronk et al. (21). SDS-PAGE analysis of the purified material (Fig. 2b) derived from the elution from the Sephacryl S-200 column (Fig. 2a) showed that the end of the peak was enriched in B pentamer (B_5), suggesting that it contained mostly B subunits not assembled with the A subunit. Gel filtration of the same material on a HiLoad 16/60 Superdex 75 column confirmed that the purified material contained two molecular species that in this column could be easily separated into the two peaks, one containing the fully assembled holotoxoid (AB_5) and the other containing the free B_5 pentamer (Fig. 2c and d). By measuring the protein content of each of the two peaks shown in Fig. 2c, we were able to determine the ratio between the fully assembled molecule and the free pentamer and to calculate the percentage of the AB_5 molecule purified from each *E. coli* mutant strain. Surprisingly, we found that the percentage of fully assembled molecules varied considerably among the different mutants, ranging from 26% for LT-K7/K97, the mutant molecule containing the combination of two mutations, to 98% for LT-K63 (Fig. 2e). The finding that mutation of the A subunit affected the yield of the assembled molecule may be explained by an increased sensitivity of the A subunit to protease digestion. The degradation of the newly produced A subunit in the periplasm of an *E. coli* strain would make the amount of the A subunit not sufficient to assemble with the B monomers produced. This would lead the

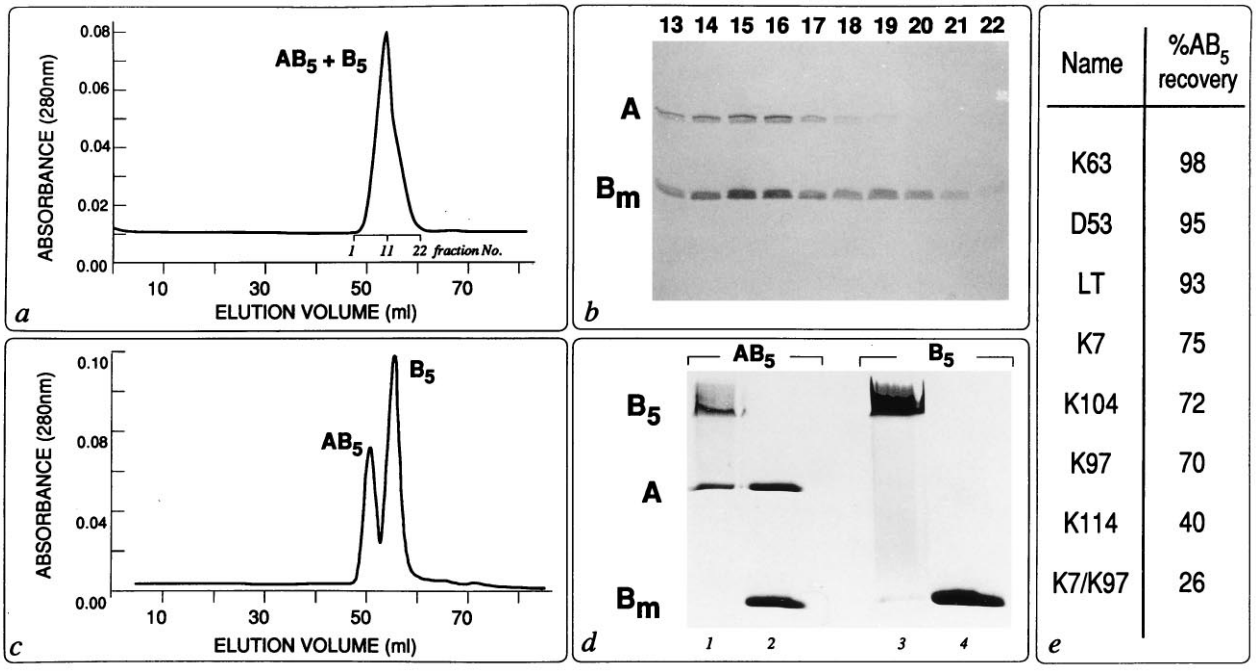

FIG. 2. Purification of LT molecules and recovery of the AB_5 complex. (a) Passage of LT-K114 containing material on a Sephacryl column resulted in a single peak that by SDS-PAGE was shown to contain the AB_5 complex (fractions 13 to 17 of panel b) and B pentamer (fractions 18 to 22 of panel b). (c) When the same material was run on a Superdex 75 column, the two peaks corresponding to the AB_5 and B_5 forms were separated. (d) SDS-PAGE analysis of the peaks containing the AB_5 and B_5 structures run under nondenaturing (lanes 1 and 3) and denaturing (lanes 2 and 4) conditions. (e) The value of AB_5 recovery for each molecule, expressed as $AB_5/(AB_5 + B_5)$ (as a percentage).

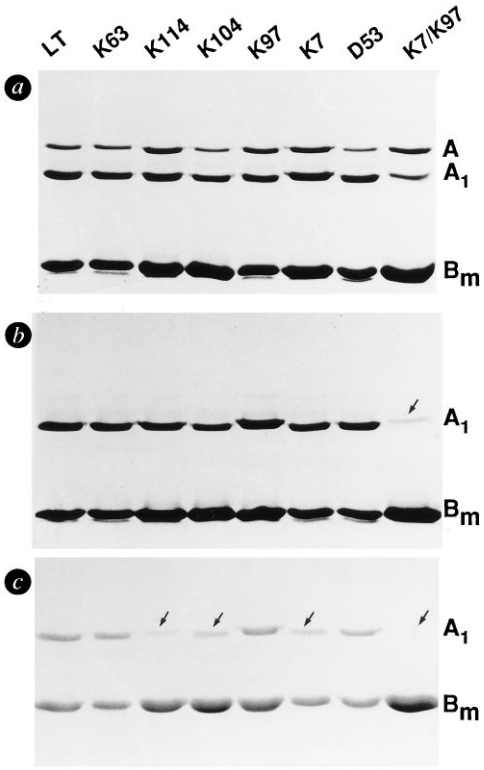

FIG. 3. Trypsin sensitivity of LT and LT mutants. SDS-PAGE analysis of the molecules treated with trypsin (molar ratio, 1:100, trypsin/protein) at the initial time (a), after 15 min (b), and after 85 min (c). Samples were boiled before the electrophoretic run to stop the enzymatic reaction, so the B subunit is present on the gel in the monomeric form (B_m). The A subunit is initially cleaved by trypsin in A_1 and A_2 fragments, of which A_1 is clearly recognizable on the gel; A_2 and small fragments deriving by degradation of A_1 are not identified under the conditions used.

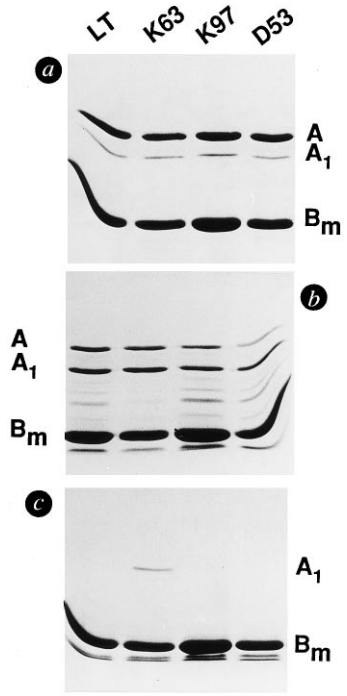

FIG. 4. Trypsin sensitivity of LT and LT mutants in denaturing conditions. SDS-PAGE analysis of LT, LT-K63, LT-K97, and LT-K53 treated with trypsin (molar ratio of trypsin/protein, 1:100) in presence of 3.5 M urea at the initial time (a), after 5 min (b), and after 30 min (c). The B subunit is present on the gel in the monomeric form because samples were boiled before electrophoresis.

B monomers to assemble into an A-subunit-free B pentamer. To see whether we could detect any difference among the mutants in terms of sensitivity of the A_1 polypeptide to proteolytic attack and whether there is any correlation with the yield in the AB_5 complex, the individual proteins were treated with minute amounts of trypsin and then incubated at 37°C. Samples were taken at different intervals of time and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). As shown in Fig. 3, trypsin treatment caused the almost immediate nicking of the A subunit into the A_1 and A_2 polypeptides (Fig. 3a). After 15 min of incubation, virtually all A subunits had been entirely converted to A_1 (Fig. 3b). However, by this time, the A_1 polypeptide of the double mutant LT-K7/K97 had also been largely degraded by the protease treatment. After 85 min of trypsin treatment, the A_1 subunit of LT-K114 had also been degraded almost completely, while LT-K7 and LT-K104 had been partially digested (Fig. 3c). The most resistant to proteolysis appeared to be wild-type LT, LT-D53, LT-K97, and LT-K63. To detect differences in trypsin sensitivity among these mutants, we repeated the experiment under denaturating conditions by adding 3.5 M urea to the solution and digesting the samples for 5 and 30 min. Figure 4 shows that under these conditions the mutant LT-K63 is the most resistant to trypsin digestion. In conclusion, the degree of trypsin sensitivity is as follows: LT-K7/K97 > LT-K114 > LT-K104 > LT-K7 > LT-D53 and LT-K97 > LT > LT-K63. Remarkably, the resistance to proteases follows in most cases the same order as the ability to assemble the AB_5 structure.

The only case in which the correlation is not perfect is LT-K97, which is an intermediate producer of AB_5 complex but stable under trypsin digestion. Interestingly, the X-ray structure of this molecule (18) showed that the newly introduced lysine in position 97 makes a hydrogen bond with Glu-112, suggesting that this structure is slow to form but stable once it has been formed.

The observations that the A subunit can become part of the holotoxin only if present during the B pentamer assembly and that it cannot assemble with the latter once the B pentamer is formed (10), combined with the observation that the A subunit is released from the AB_5 complex only when the B pentamer starts to unfold (8), suggest that the AB_5 complex is the result of strong A-B interactions, so that mutations affecting the stability of the A subunit could affect also the stability of the AB_5 complex. To verify this hypothesis, the mutant proteins were stored at 4°C and periodically analyzed by gel filtration on a Superdex 75 PC 3.2/30 column and SDS-PAGE. The gel filtration allowed the separation in a single run of the AB_5 complex from the free B pentamer (B_5), from the A subunit, and from the B monomer (B_m) (Fig. 5a). The B pentamers purified from the different mutant strains showed extreme stability under these conditions. Storage up to 1 year did not alter the elution profile, which remained identical to the one shown in Fig. 5b. Similarly, long-term storage of wild-type LT (not shown) or LT-K63 (Fig. 5c) did not cause any alteration of the elution profile, suggesting that the AB_5 complex remained intact during this period. Surprisingly, the profile of the other mutants changed considerably during storage. Figure 5d to f shows the pattern obtained with mutants LT-K97, LT-K114, and LT-K7/K97, respectively. As shown, in these proteins the peak of the AB_5 complex diminished in intensity and a new peak comigrating with the B monomer appeared. No peak

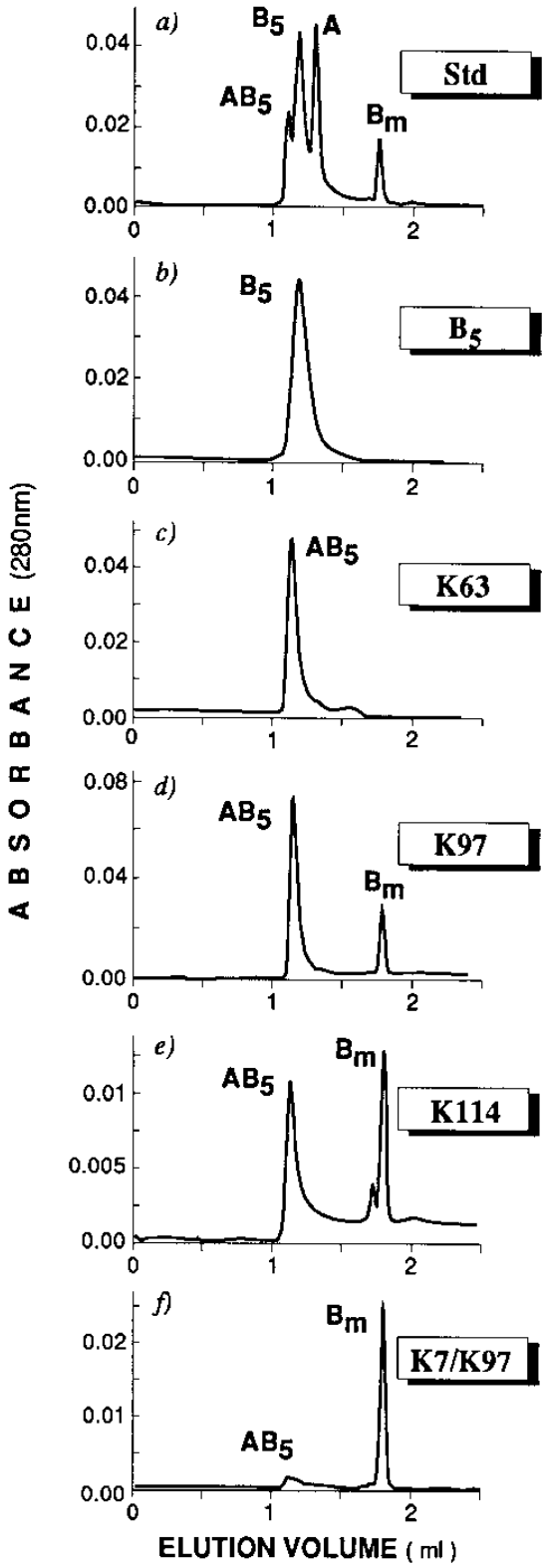

FIG. 5. Chromatographic profiles obtained by gel filtration of LT mutants after 1 year of storage at 4°C. (a) A standard plot showing the elution profiles of the AB_5 form of LT-K63 and of the molecular species derived from its experimental dissociation. A and B_5 was obtained by dissociation of AB_5 at pH 12.5; B_m was obtained by dissociation of B_5 at pH 1.9. After neutralization, the molecular forms were mixed and 10 μl of the sample was loaded on a Superdex 75 column (Smart System; Pharmacia). (b) Profile of purified B pentamer; (c to f) profiles of the mutants indicated.

TABLE 2. LT-neutralizing titers of total sera and of anti-A antibodies

LT mutant	Neutralizing titer of:		% Anti-A
	Total serum	Anti-A fraction	
LT-K63	1/4,096	1/1,024	25
LT-K97	1/3,584	1/244	6.25
LT-K7	1/4,352	1/136	3.12
LT-K114	1/4,096	1/64	1.56

corresponding to the A subunit was detected. (The A subunit could be digested by proteolytic attack or precipitate out of solution.) The decrease in intensity of the AB_5 peak was proportional to the B_m peak size increase. This phenomenon was minimal with mutants such as LT-K97, intermediate in LT-K114, and almost complete in the double mutant LT-K7/K97. These data suggested that some of the mutants, upon storage, disassembled the AB_5 structure and generated molecules that comigrate with the B monomers (in gel filtration and in SDS-PAGE) and that were recognized by anti-LTB antibodies in Western blotting (immunoblotting). The findings that mutations in the A subunit cause instability of the B pentamer and that the B subunit is found as a soluble B monomer in conditions which usually favor the B pentamer assembly were unexpected. Preliminary data from mass spectroscopy analysis showed that the molecular mass of the B_m was slightly higher than that of a standard B_m obtained by acid denaturation of the B pentamer (11,842.50 and 11,788.88 Da, respectively). This result suggests that the B monomer found in solution is a chemically modified form containing the covalent addition of new groups of approximately 54 Da, and this could explain the inability of the B_m to form the pentamer.

A previous study on the immunogenicity of the LT-K63 mutant showed that the A subunit plays an important role in the immunogenicity of the toxin and that this nontoxic mutant was able to induce neutralizing antibodies against the A subunit (20).

To verify whether the different stabilities of the mutants influenced their ability to induce neutralizing anti-A antibodies, rabbits were immunized systemically with four different mutants. The sera were collected, and the anti-A antibodies were purified and tested in a Y1 cell neutralization in vitro assay as previously described (20). The results are reported in Table 2. We found that the percentage of the anti-A neutralizing antibodies varied from 25% for the LT-K63 mutant to 1.54% for LT-K114.

We conclude that mutations introduced in the A subunit can have some effects on its ability to elicit an immune response: stable mutants induced more anti-A neutralizing antibodies than unstable mutants.

In conclusion, we have shown that nontoxic mutants of LT may have quite different properties, depending on the mutation used to eliminate the enzymatic activity and toxicity residing in the A subunit. The findings reported in this study allow a better understanding of the structure and function of LT and of the role of the A subunit in the assembly, stability, and immunogenicity of the holotoxin. Moreover, they are relevant for the development of new molecules to be used as vaccines and as mucosal adjuvants. In fact, the selection of the appropriate nontoxic mutant to be used for vaccination or as a mucosal adjuvant requires extensive characterization of the biochemical and immunological properties of the molecules proposed. In this work, we have shown that LT-K63 is a molecule produced with a high yield, resistant to proteases and to

long-term storage and very immunogenic. Therefore, we consider it a promising candidate for further studies.

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