INFECTION AND IMMUNITY, Aug. 1994, p. 3299–3304
0019-9567/94/$04.00+0

Vol. 62, No. 8

A Mutant Toxin of *Vibrio parahaemolyticus* Thermostable Direct Hemolysin Which Has Lost Hemolytic Activity but Retains Ability To Bind to Erythrocytes

GUANG-QING TANG, TETSUYA IIDA,* KOICHIRO YAMAMOTO, AND TAKESHI HONDA

Department of Bacteriology and Serology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565, Japan

Received 21 March 1994/Returned for modification 4 May 1994/Accepted 19 May 1994

A mutant toxin, R7, of thermostable direct hemolysin (TDH) with a single amino acid substitution at glycine 62 was analyzed. The hemolytic activity of R7 decreased to less than 1/1,000 of that of wild-type TDH, and its mouse lethality was undetectable. This mutant toxin, however, showed a marked inhibitory effect on hemolysis by wild-type TDH. Enzyme immunoassay and flow cytometric analysis demonstrated that R7 retained approximately 50% of the ability to bind to erythrocytes compared with that of wild-type TDH, suggesting that its inhibition of hemolysis by wild-type TDH might be due to blocking the binding sites on the erythrocyte membrane. Wild-type TDH affected the erythrocyte membrane by causing an influx of calcium and propidium iodide, while R7 showed no detectable effects of these kinds. These results suggest that hemolysis by TDH consists of at least two steps, binding and postbinding, and that R7 is likely to be a postbinding activity-deficient mutant toxin of TDH.

Vibrio parahaemolyticus is a bacterium that causes foodborne gastroenteritis (1, 14). Thermostable direct hemolysin (TDH) secreted from *V. parahaemolyticus* has been considered a major virulence factor for this disease (6). TDH is a homodimer protein with a molecular weight of 46,000 (6). Each peptide of the protein is composed of 165 amino acid residues (6).

TDH has a variety of biological activities, including hemolytic activity, cardiotoxicity, mouse lethality, and enterotoxicity (6). Although the precise mode of action of TDH in its various activities has not been well established, hemolysis by TDH has been thought to be caused by a serial process of first binding to the erythrocyte surface, then forming pores on the membrane, and finally causing colloidal osmotic lysis of erythrocytes (9). The receptor for TDH on the erythrocyte membrane was previously reported to be G_{T1} ganglioside (21, 22). However, recent studies have produced contradictory results (6); thus, the nature of the TDH receptor is still controversial.

Recently, we employed in vitro mutagenesis of the gene for TDH (*tdh*) to analyze the critical amino acid residues for hemolytic activity (12). As a result of these experiments, we obtained several mutant toxins of TDH that showed various deficiencies in hemolytic activity. Among these, R7, with a single amino acid substitution of serine for glycine 62, aroused our interest because it inhibited the hemolytic halo caused by wild-type TDH on a rabbit blood agar plate while its hemolytic activity decreased drastically. This made us speculate that R7 might retain the ability to bind to erythrocytes and compete for binding sites with wild-type TDH. Another mutant TDH, H17, which has an amino acid substitution at glycine 86, showed moderately decreased hemolytic activity. Probably because of its deficiency in binding to erythrocytes, H17 could not at all inhibit the hemolytic halo caused by wild-type TDH on rabbit blood agar. Both mutants were immunologically indistinguishable from wild-type TDH (12), suggesting that the conformations of these mutant TDHs are substantially similar to that of wild-type TDH.

In this study, by comparison with wild-type TDH and H17, we analyzed the properties of R7 throughout the hemolytic process, i.e., binding, permeabilization of the erythrocyte membrane, and hemolysis. The results showed that R7 had lost its postbinding activity, whereas it retained its ability to bind to erythrocytes.

MATERIALS AND METHODS

Bacterial strain and plasmid vector. *Escherichia coli* JM109 (24) was used for production of wild-type and mutant TDHs. Plasmid vector pKK223-3 (3) was used for the expression of the structural genes of wild-type and mutant TDHs.

Purification of wild-type and mutant TDHs from *E. coli*. Recombinant plasmids of pKK223-3 harboring the structural genes of wild-type TDH (pTI101), R7 (pTI102), and H17 (pTI103) (12) were introduced into *E. coli* JM109 by transformation. Transformants were cultivated in Luria-Bertani broth (1% Bacto tryptone, 0.5% yeast extract, and 0.5% NaCl) containing 100 μg of ampicillin per ml at 37°C for 16 h with rotary shaking. Synthesized proteins were purified by a previously reported method (25) with the modification that harvested cells were treated with 3,000 U of polymyxin B (Wako Pure Chemicals, Osaka, Japan) per ml in 10 mM phosphate-buffered saline (PBS) (pH 7.2) to lyse out periplasm substances for a subsequent series of column chromatography. Fractions containing wild-type TDH were assayed by hemolysis on a rabbit blood agar plate (13), and fractions containing mutant TDHs were assayed by the Ouchterlony double immunodiffusion test (18) with rabbit anti-TDH serum. The purity of samples was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (15).

Hemolytic activities of toxins. Fresh erythrocytes from a healthy human were washed in PBS three times and adjusted to a hematocrit of 4% in PBS. TDHs were serially diluted with

* Corresponding author. Mailing address: Department of Bacteriology and Serology, Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565, Japan. Phone: 81-6-879-8276. Fax: 81-6-879-8277.

PBS and mixed with the erythrocyte suspension described immediately above in equal volume (60 μl). Reaction mixtures were kept at 37°C for 1 h. After centrifugation at 2,000 × *g* for 2 min, the supernatant (100 μl) of the reaction mixture was taken for spectrophotometric measurement at 540 nm on a 96-well plate (8). As a control for 100% hemolysis, erythrocytes were treated with 2% (final concentration) Triton X-100 instead of toxins and the supernatant was measured as described above.

Mouse lethality. Various amounts of wild-type and mutant TDHs in PBS were injected intravenously into mice (4 weeks old; ddY strain), and survival or death was observed within 30 min. The 50% lethal dose (LD_{50}) was calculated according to the method described previously (2).

Inhibitory effect of R7 on hemolysis by wild-type TDH in test tubes. Washed human erythrocytes were adjusted to a hematocrit of 4%, mixed with an equal volume (60 μl) of serially diluted R7 in duplicate (experiments A and B), and incubated at 37°C for 1 h (preincubation). After centrifugation at 2,000 × *g* for 2 min, the supernatant of experiment A (containing unbound R7) was removed, washed once with 200 μl of PBS, and reconstituted with PBS to the original volume (120 μl). For experiment B, the same procedure as for experiment A was performed except that unbound R7 was not removed. Subsequently, wild-type TDH was applied to every tube to a final concentration of 2.3 μg/ml and the reaction mixtures were kept at 37°C for 1 h (postincubation). Then hemolytic activity was measured as described above.

Flow cytometric analysis of binding of wild-type and mutant TDHs to erythrocytes. Either wild-type or mutant TDH at a final concentration of 10 μg/ml was allowed to interact with human erythrocytes at a final hematocrit of 0.2% in 120 μl of PBS-dextran (PBS supplemented with 10 mM dextran [molecular weight {MW}, ~9,300] [Sigma, St. Louis, Mo.], a colloidal inhibitor, to prevent hemolysis) at 37°C for 1 h. After being washed with 500 μl of PBS-dextran three times by centrifugation, toxin-bound erythrocytes were allowed to interact with 1/100 diluted rabbit anti-TDH serum (see below) in PBS-dextran for 30 min at 37°C. After being washed as described above, 1/100 diluted fluorescein isothiocyanate (FITC)-labeled anti-rabbit second antibodies (anti-rabbit immunoglobulins G, A, and M [heavy and light chains]) (The Binding Site, Birmingham, United Kingdom) in PBS-dextran were added to the cell pellet and incubated for 30 min at 37°C. After being washed as described above, erythrocytes were suspended in 2 ml of PBS and loaded on FACScan (Becton Dickinson, San Jose, Calif.). Fluorescence intensity of FITC on 10,000 erythrocytes was recorded at 515 to 545 nm.

Enzyme immunoassay of binding of wild-type and mutant TDHs to erythrocytes. Serially diluted wild-type or mutant TDHs were allowed to interact with 2% human erythrocytes (120 μl) in PBS-dextran at 37°C for 1 h. After being washed three times with 500 μl of PBS-dextran by centrifugation, toxin-bound erythrocytes were allowed to interact with 1/100 diluted anti-TDH serum (see below) in PBS-dextran at 37°C for 30 min. After being washed as described above, 1/500 diluted alkaline phosphatase-conjugated swine anti-rabbit immunoglobulins (DAKOPATTS a/s, Glostrup, Denmark) in PBS-dextran were added to the cell pellet and incubated for another 30 min at 37°C. Finally, after the erythrocytes had been washed as described above, 200 μl of the substrate solution, 1 mg of *p*-nitrophenyl phosphate (Wako, Tokyo, Japan) per ml in the substrate buffer (1 M diethanolamine—0.5 mM $MgCl_2$—0.2% NaN_3, adjusted to pH 9.8 by 1 N HCl) supplemented with 0.85% NaCl and 10 mM dextran, was added to the erythrocyte pellet in each tube. Enzyme reactions were performed at 37°C for 8 min. After centrifugation at 2,000 × *g* for 2 min, 150 μl of the supernatant was spectrophotometrically measured at 405 nm.

ELISA. The sandwich enzyme-linked immunosorbent assay (ELISA) was performed as described previously (10) to compare the immunological reactivities of wild-type and mutant TDHs with rabbit anti-TDH serum.

Calcium influx into erythrocytes. Washed human erythrocytes (2%) were loaded with the calcium indicator Fluo 3-acetoxymethyl ester (Fluo 3-AM) (Dojindo, Tokyo, Japan) at a final concentration of 5 μM in darkness at 37°C for 1 h. Fluo 3-loaded erythrocytes at a hematocrit of 2% were allowed to react with wild-type and mutant TDHs at serially diluted concentrations with either 2 μM $CaCl_2$ or 2 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] in 120 μl of PBS-dextran at 37°C for 1 h. After being washed once with 200 μl of PBS-dextran by centrifugation, erythrocytes were diluted, suspended in PBS, and loaded onto FACScan and Fluo 3 fluorescence intensity on 10,000 erythrocytes was recorded at 515 to 545 nm. The positive control experiment was done with 2 μM A23187 (Wako Pure Chemicals), a calcium-specific ionophore, in place of toxins. The negative control was Fluo 3-loaded erythrocytes with no treatment by toxins or ionophore.

Influx of PI into erythrocytes. Washed human erythrocytes with a final hematocrit of 2% were incubated with wild-type and mutant TDHs at serially diluted concentrations in the presence of propidium iodide (PI) (MW, 668; Sigma) at a final concentration of 500 μg/ml in 120 μl of PBS-dextran at 37°C for 1 h. After being washed with 200 μl of PBS-dextran by centrifugation, erythrocytes were diluted, suspended in PBS, and applied on FACScan and PI fluorescence intensity on 10,000 erythrocytes was recorded at 543 to 627 nm. The negative control was erythrocytes incubated only with PI.

Determination of protein content. Protein concentrations of toxins were determined by the method of Lowry et al. (17) with bovine serum albumin as the standard.

Preparation of anti-TDH serum. Anti-TDH serum from rabbits was prepared as described previously with wild-type TDH (5). Prior to being used, anti-TDH serum was diluted to 1/100 and absorbed by untreated human erythrocytes (final hematocrit, 2%) at 37°C for 1 h to remove nonspecific antibodies reacting to erythrocytes.

RESULTS

Purification of wild-type and mutant TDHs from *E. coli* to homogeneity. Wild-type and mutant TDHs (R7 and H17) were synthesized in *E. coli* JM109 and purified for characterization. The products purified from bacterial periplasm gave single bands on the SDS-PAGE gel (Fig. 1), suggesting that the toxins were purified to homogeneity. The mobilities of wild-type and mutant TDHs were indistinguishable (ca. 23 kDa). These purified samples were concentrated to 300 μg/ml by ultrafiltration through a PM10 membrane (Amicon, Danvers, Mass.) and stored in PBS at 4°C for the following analysis.

Biological activities of R7. To compare the biological activities of R7 with those of wild-type TDH and H17, we first analyzed the hemolytic activities of the toxins (Fig. 2). Wild-type TDH had dose-dependent hemolytic activity, and the 50% hemolytic dose was 0.55 μg/ml. For H17, hemolytic activity was also toxin dose dependent, with its 50% hemolytic dose increased to 12.8 μg/ml. R7, however, did not cause any hemolysis even at 150 μg/ml. Therefore, the hemolytic activity of R7 was estimated to have decreased to less than 1/1,000 of that of wild-type TDH.

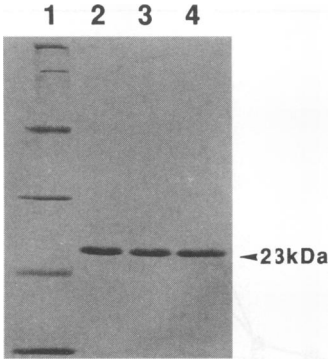

FIG. 1. Purity of wild-type and mutant TDHs examined by SDS-PAGE with a 12% polyacrylamide gel. Lanes: 1, molecular mass markers (Daiichi Pure Chemicals, Tokyo, Japan) (from top to bottom, 97.4, 66.3, 42.4, 30.0, 20.1, and 14.4 kDa); 2, wild-type TDH; 3, R7; 4, H17. Two micrograms of each toxin was loaded.

Another biological activity of TDH, mouse lethality, was also tested to compare R7 with wild-type TDH and H17. LD_{50}s of the toxins by intravenous injection were measured to be 1.1 μg per mouse (0.9 to 1.3 μg per mouse; $P = 0.1$) for wild-type TDH and 20 μg per mouse (6.6 to 61 μg per mouse; $P = 0.1$) for H17. However, even an R7 challenge of 50 μg per mouse was not lethal. Thus, the mouse lethality of R7 was experimentally undetectable.

These results demonstrate that R7 is deficient to a great extent in both hemolytic activity and mouse lethality and suggest that the glycine 62 amino acid residue in TDH is important for the expression of these two biological activities.

Inhibitory effect of R7 on hemolysis by wild-type TDH. At the beginning of isolation of this mutant toxin, we observed the inhibitory effect of R7 against hemolysis by wild-type TDH on a rabbit blood agar plate (12). This inhibitory effect was examined more quantitatively in test tubes.

Before interaction with wild-type TDH, erythrocytes were incubated with serially diluted R7 at 37°C for 1 h (preincubation with R7). Then wild-type TDH was added to a final concentration of 2.3 μg/ml, which was sufficient to cause 100% hemolysis, and incubated at 37°C (postincubation with wild-type TDH). After 1 h postincubation, hemolysis was measured as described above (experiment B). R7 showed a dose-dependent inhibition of hemolysis by wild-type TDH. At a concentration of 37.5 μg/ml, which was about 16 times that of wild-type TDH, hemolysis by wild-type TDH was inhibited almost completely (Fig. 3).

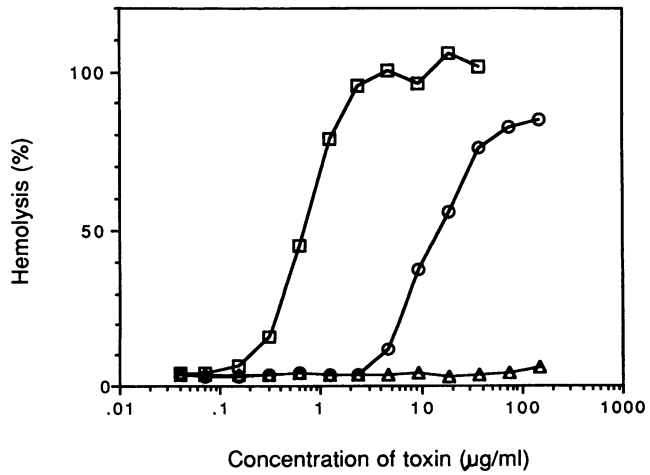

FIG. 2. Hemolytic activities of wild-type and mutant TDHs. □, wild-type TDH; ○, H17; △, R7. Hemolytic activity was determined as described in Materials and Methods. Data are representative of three independent experiments.

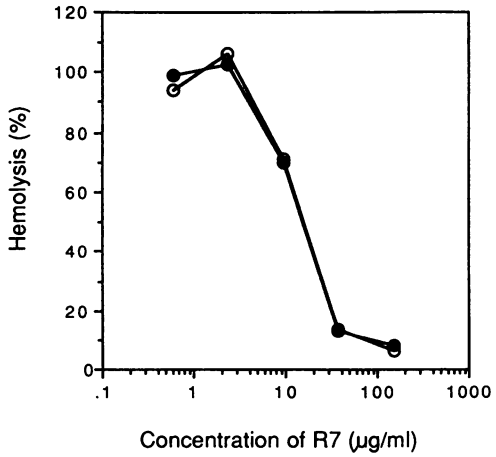

FIG. 3. Inhibitory effect of R7 on hemolysis by wild-type TDH. Erythrocytes were incubated with R7 (preincubation) at 37°C for 1 h and then incubated with 2.3 μg (final concentration) of wild-type TDH per ml (postincubation) at 37°C for 1 h. ○, experiment A, without removing unbound R7 after preincubation; ●, experiment B, with unbound R7 removed after preincubation. Data are representative of three independent experiments.

To check the reversibility of the R7 inhibitory effect, unbound R7 was washed out after preincubation and wild-type TDH was applied postincubation (experiment A). The dose-dependent inhibition curve was almost the same as that described above (Fig. 3), indicating that the inhibitory effect of R7 is irreversible at least within the 1 h postincubation after unbound R7 is removed.

Thus, R7 was shown to have a strong inhibitory effect on hemolysis by wild-type TDH.

Binding of R7 to erythrocytes. One reasonable explanation for the inhibitory effect of R7 is that R7 retains the ability to bind to the erythrocyte surface and inhibits wild-type TDH hemolysis by blocking the binding sites of TDH. To examine this possibility, we attempted to compare the ability of R7 to bind to erythrocytes with those of wild-type TDH and H17.

We first analyzed the ability of R7 to bind to erythrocytes by the flow cytometric method and compared it with those of wild-type TDH and H17. Wild-type and mutant TDHs were allowed to bind to erythrocytes at the same final protein concentration of 10 μg/ml. Toxins bound to erythrocytes were detected by anti-TDH serum (rabbit) and FITC-labeled second antibodies (anti-rabbit). Erythrocytes untreated with toxins were analyzed in the same way as the negative control. Wild-type TDH and R7 caused obvious increases in the fluorescence intensity of erythrocytes while H17 did not (Fig. 4). The results of this binding analysis suggested that R7 retains the ability to bind to erythrocytes while H17 is deficient.

The ability of R7 to bind to erythrocytes was further confirmed and compared with those of wild-type TDH and H17 by enzyme immunoassay. R7 as well as wild-type TDH

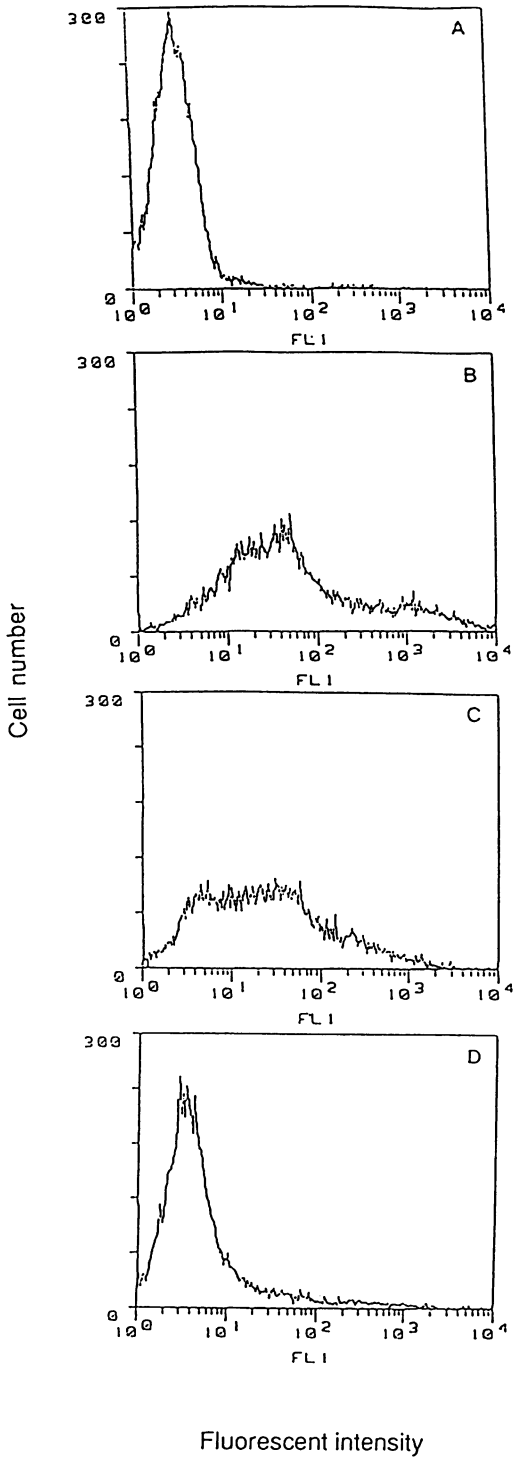

FIG. 4. Flow cytometric analysis of the binding of wild-type and mutant TDHs to erythrocytes. (A) Negative control, (B) wild-type TDH, (C) R7, (D) H17. Final concentrations (10 μg/ml) of toxins were used to interact with a final hematocrit of 0.2% of erythrocytes. Toxins bound to erythrocytes were detected with anti-wild-type TDH serum and FITC-labeled second antibodies as described in the text.

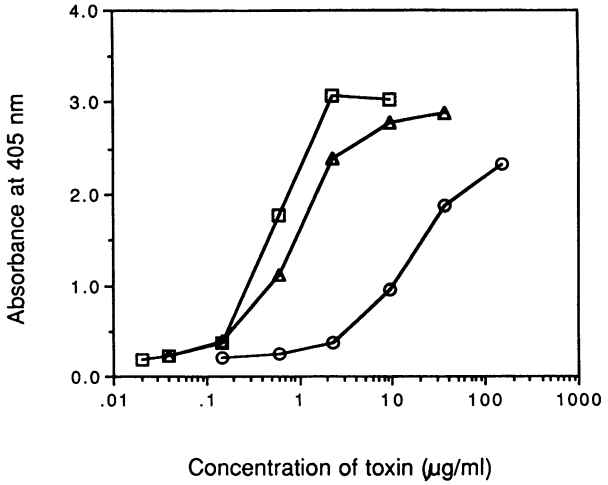

FIG. 5. Enzyme immunoassay of the binding of wild-type and mutant TDHs to erythrocytes. □, wild-type TDH; △, R7; ○, H17. Data are representative of five independent experiments.

and H17 was separately allowed to bind to erythrocytes. Toxins bound to erythrocytes were detected with anti-TDH serum (rabbit), alkaline phosphatase-conjugated second antibodies (anti-rabbit), and then the substrate for color development. As shown in Fig. 5, both wild-type TDH and R7 showed obvious binding to erythrocytes while H17 did not reach completely to the plateau even at 150 μg/ml. Since no difference in reactivity with anti-TDH serum was observed among wild-type and mutant TDHs by sandwich ELISA (data not shown), the 50% binding doses were estimated to be 0.4, 0.83, and 21.5 μg/ml for wild-type TDH, R7, and H17, respectively.

These results demonstrate that R7 retains approximately 50% of the ability of wild-type TDH to bind to erythrocytes, but that of H17 decreases to about 2% of the ability of wild-type TDH.

Effect of R7 on the permeability of the erythrocyte membrane. Since TDH is thought to cause colloidal osmotic hemolysis (9), we investigated its postbinding effect on erythrocytes, i.e., permeabilization of the erythrocyte membrane, and compared the effect of R7 with those of wild-type TDH and H17.

First, we analyzed the effects of wild-type and mutant TDHs on calcium influx into erythrocytes. Fluo 3, a calcium indicator, was loaded into erythrocytes to detect changes in intracellular calcium levels. Fluo 3-loaded erythrocytes were treated with various concentrations of wild-type and mutant TDHs in the presence or absence of extracellular calcium and incubated at 37°C for 1 h. Then erythrocytes were screened by flow cytometry. When the extracellular milieu of erythrocytes was free of calcium (possible trace calcium was eliminated by 2 mM EGTA), neither A23187 nor wild-type and mutant TDHs could induce any increase in the calcium level of the cytosol as shown by no change in fluorescence intensity (data not shown). When 2 μM calcium existed outside erythrocytes, calcium influx was induced by A23187 (95.5% of cells were observed to be positive with 2 μM A23187), wild-type TDH, and H17. This influx was toxin dose dependent (Fig. 6). After gating for the fluorescence intensity of the negative control was done, the doses at which 50% of erythrocytes became positive were 0.14 and 8.2 μg/ml for wild-type TDH and H17, respectively. On the contrary, R7 could induce no calcium influx even at 150 μg/ml. These results suggest that TDH increases calcium permeability

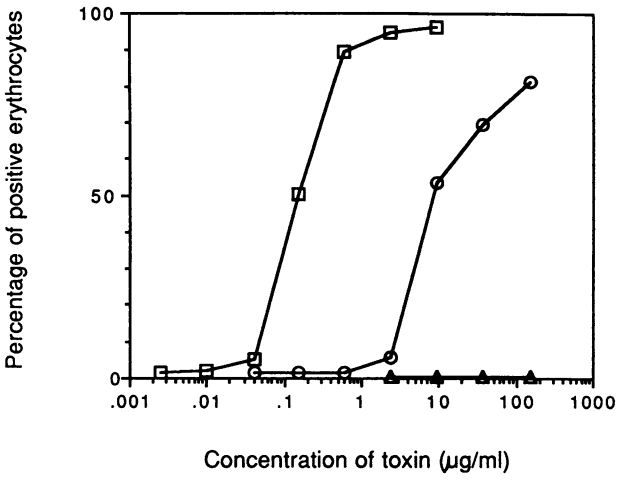

FIG. 6. Calcium influx into erythrocytes induced by wild-type and mutant TDHs. □, wild-type TDH; ○, H17; △, R7. Fluo 3-loaded erythrocytes were used as the negative control for gating. Erythrocytes with fluorescence intensities of >10 were defined as positive cells. The ratios of positive cells among 10,000 recorded erythrocytes are shown as percentages of positive cells. Data are representative of five independent experiments.

through the erythrocyte membrane in a toxin dose-dependent manner. On the other hand, though it retains more than 50% of the ability of wild-type TDH to bind to erythrocytes , R7 could not increase the permeability of the erythrocyte membrane to calcium at all.

PI, known as a membrane integrity indicator, was further exploited to analyze the effects of toxins on permeabilization of the erythrocyte membrane. Erythrocytes were treated with toxins in the presence of extracellular PI, and the influx of PI into erythrocytes was analyzed by flow cytometry. Similar to the influx of calcium, PI influx was induced by wild-type TDH and H17. This influx was also toxin dose dependent (Fig. 7).

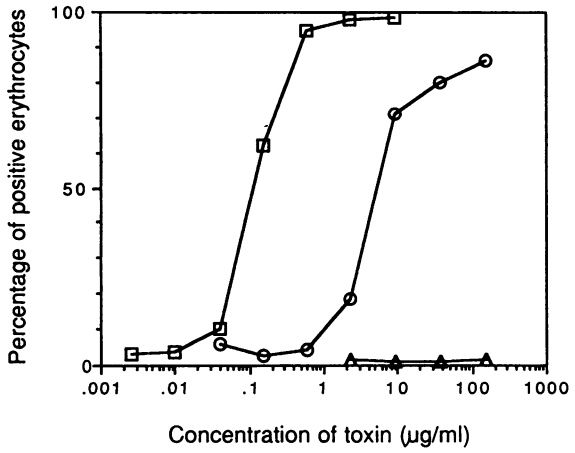

FIG. 7. PI influx into erythrocytes induced by wild-type and mutant TDHs. □, wild-type TDH; ○, H17; △, R7. Erythrocytes without toxin treatment were used as the negative control for gating. Erythrocytes with fluorescence intensities of >10 were defined as positive cells. The ratios of positive cells among 10,000 recorded erythrocytes were shown as percentages of positive cells. Data are representative of five independent experiments.

After gating for the fluorescence intensity of the negative control was done, the doses at which 50% of erythrocytes became positive were 0.13 and 6.2 μg/ml for wild-type TDH and H17, respectively. On the contrary, R7 did not induce any PI influx even at 150 μg/ml.

These results suggest that wild-type TDH permeabilized the erythrocyte membrane, resulting in PI influx. In the case of R7, this effect did not seem to occur in spite of its ability to bind to erythrocytes.

DISCUSSION

R7 is a mutant toxin of TDH with a single amino acid substitution at glycine 62. In this study, we purified and characterized R7 synthesized in *E. coli.*

R7 has lost biological activities of TDH such as hemolytic activity and mouse lethality (Fig. 2). On the other hand, R7 strongly inhibits hemolysis by wild-type TDH (Fig. 3). Moreover, R7 retains the ability to bind to erythrocytes (approximately 50% of the ability of wild-type TDH) (Fig. 4 and 5). The most probable interpretation for the inhibitory effect of R7 against wild-type TDH is that R7 inhibits hemolysis by wild-type TDH through blocking binding sites on the erythrocyte surface from the action of wild-type TDH, although other interpretations might be possible. In contrast to R7, H17 is a mutant toxin of TDH which is deficient in its ability to bind to erythrocytes. This might explain why H17 is not able to inhibit the hemolytic halo caused by wild-type TDH on rabbit blood agar.

TDH is believed to work as a pore-forming toxin and to cause colloidal osmotic hemolysis (9). Although the detailed processes of TDH action are not clear, we may postulate that TDH has an initial effect of permeabilization of the erythrocyte membrane prior to lysis of cells. Huntley et al. (11) showed that erythrocytes became permeable to monovalent cations through the action of TDH. Goshima et al. (4) reported that TDH caused an influx of extracellular calcium into mouse myocardial cells and that this influx always accompanied degradation of the cell shape and full contraction of myofibrils. In the present work, we used erythrocytes as target cells to analyze the effect of TDH on membrane permeabilization to one of the bivalent cations, calcium. The calcium ion is known to have a variety of physiological effects on many kinds of cells. Thus, it is possible that the calcium ion might affect TDH action on erythrocytes; a report implying such a possibility has been published (20). However, since neither the absence nor presence of extracellular calcium at the concentration used in this study had any significant effect on TDH hemolysis (23), the change of intracellular level of calcium was considered to be simply an indicator of the permeability of the erythrocyte membrane in this study. Calcium influx was induced by wild-type TDH and H17 (Fig. 6). On the contrary, R7 could not permeabilize the erythrocyte membrane to extracellular calcium at all. In addition to calcium, we used PI, a membrane integrity indicator (19), to analyze permeabilization of the erythrocyte membrane by TDH. PI influx was induced by wild-type TDH and H17 (Fig. 7). Nevertheless, R7 could not permeabilize the erythrocyte membrane to allow the influx of PI into erythrocytes. Thus, R7 was shown to have lost the membrane permeabilizing effect as well as the biological activities of TDH in spite of its considerable retention of the ability to bind to erythrocytes.

Glycine is an apolar and uncharged amino acid with the simplest R group (—H) among all the 20 amino acids. Glycine plays an important role in environment-dependent helix modulation and provides, in part, the structural basis for confor-

mational transitions of protein molecules, such as those accompanying membrane insertion (16). In the case of R7, with the substitution of serine for glycine 62, the molecule might have lost this kind of structural basis for the events after binding. If this is the case, hemolytic processes must be interrupted accordingly.

Considering the biological activities of TDH, the parallel losses of the mutant TDHs (R7 and H17) in terms of hemolytic activity and mouse lethality imply that TDH has a common active site for these activities, as suggested in a previous study (7). The amino acid residue glycine 62 may play an important role in the expression of the biological activities of TDH but not in binding. The toxicity of R7 decreased drastically while it was immunologically indistinguishable from wild-type TDH. These properties of R7 might make it a favorable candidate as a toxoid vaccine for prophylaxis of the enteritis caused by *V. parahaemolyticus*. An investigation along this line should be the focus of further study. Moreover, since R7 possesses the ability to bind to erythrocytes, it might have promise as a probe for revealing the hitherto unknown nature of a receptor for TDH on the erythrocyte surface.

ACKNOWLEDGMENT

This study was supported by a grant-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1. **Blake, P. A., R. E. Weaver, and D. G. Hollis.** 1980. Diseases of humans (other than cholera) caused by vibrios. Annu. Rev. Microbiol. **34:**341–367.
2. **Dixon, W. J.** 1965. The up-and-down method for small samples. J. Am. Stat. Assoc. **60:**967–978.
3. **Egon, A., and J. Brousius.** 1985. "ATG vectors" for regulated high-level expression of cloned genes in *Escherichia coli*. Gene **40:**183–190.
4. **Goshima, K., K. Owaribe, H. Yamanaka, and S. Yoshino.** 1978. Requirement of calcium ions for cell degeneration with a toxin (vibriolysin) from *Vibrio parahaemolyticus*. Infect. Immun. **22:**821–832.
5. **Honda, T., S. Chearskul, Y. Takeda, and T. Miwatani.** 1980. Immunological methods for detection of Kanagawa phenomenon of *Vibrio parahaemolyticus*. J. Clin. Microbiol. **11:**600–603.
6. **Honda, T., and T. Iida.** 1993. The pathogenicity of *Vibrio parahaemolyticus* and the role of the thermostable direct haemolysin and related haemolysins. Rev. Med. Microbiol. **4:**106–113.
7. **Honda, T., Y. Ni, S. Hori, H. Takakura, S. Tsunasawa, F. Sakiyama, and T. Miwatani.** 1989. A mutant hemolysin with lower biological activity produced by a mutant *Vibrio parahaemolyticus*. FEMS Microbiol. Lett. **61:**95–100.
8. **Honda, T., Y. Ni, and T. Miwatani.** 1988. Purification and characterization of a hemolysin produced by a clinical isolate of Kanagawa phenomenon-negative *Vibrio parahaemolyticus* and related to the thermostable direct hemolysin. Infect. Immun. **56:**961–965.
9. **Honda, T., Y. Ni, T. Miwatani, T. Adachi, and J. Kim.** 1992. The thermostable direct hemolysin of *Vibrio parahaemolyticus* is a pore-forming toxin. Can. J. Microbiol. **38:**1175–1180.
10. **Honda, T., Y. Ni, M. Yoh, and T. Miwatani.** 1989. Production of monoclonal antibodies against thermostable direct hemolysin of *Vibrio parahaemolyticus* and application of the antibodies for enzyme-linked immunosorbent assay. Med. Microbiol. Immunol. **178:**245–253.
11. **Huntley, J. S., A. C. Hall, V. Sathyamoorthy, and R. H. Hall.** 1993. Cation flux studies of the lesion induced in human erythrocyte membranes by the thermostable direct hemolysin of *Vibrio parahaemolyticus*. Infect. Immun. **61:**4326–4332.
12. **Iida, T., G. Tang, S. Suttikulpitug, K. Yamamoto, T. Miwatani, and T. Honda.** Submitted for publication.
13. **Iida, T., and K. Yamamoto.** 1990. Cloning and expression of two genes encoding highly homologous hemolysins from a Kanagawa-phenomenon-positive *Vibrio parahaemolyticus* T4750 strain. Gene **93:**9–15.
14. **Joseph, S. W., R. R. Colwell, and J. B. Kaper.** 1982. *Vibrio parahaemolyticus* and related halophilic vibrios. Crit. Rev. Microbiol. **10:**77–124.
15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
16. **Li, S. C., and C. M. Deber.** 1992. Glycine and beta-branched residues support and modulate peptide helicity in membrane environments. FEBS Lett. **311:**217–220.
17. **Lowry, O. H., N. I. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
18. **Ouchterlony, O.** 1969. Antigen-antibody reactions in gels. Acta Pathol. Microbiol. Scand. **26:**507–515.
19. **Rojanasakul, Y., L. Wang, C. J. Malanga, J. Y. Ma, D. E. Banks, and J. K. Ma.** 1993. Altered calcium homeostasis and cell injury in silica-exposed alveolar macrophages. J. Cell. Physiol. **154:**310–316.
20. **Sakurai, J., M. A. Bahavar, Y. Jinguji, and T. Miwatani.** 1975. Interaction of thermostable direct hemolysin of *Vibrio parahaemolyticus* with human erythrocytes. Biken J. **18:**187–192.
21. **Takeda, Y., T. Takeda, T. Honda, and T. Miwatani.** 1976. Inactivation of the biological activities of the thermostable direct hemolysin of *Vibrio parahaemolyticus* by ganglioside G_{T1}. Infect. Immun. **14:**1–5.
22. **Takeda, Y., T. Takeda, T. Honda, J. Sakurai, N. Ohtomo, and T. Miwatani.** 1975. Inhibition of hemolytic activity of the thermostable direct hemolysin of *Vibrio parahaemolyticus* by ganglioside. Infect. Immun. **12:**931–933.
23. **Tang, G., T. Iida, K. Yamamoto, and T. Honda.** Unpublished data.
24. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
25. **Yoh, M., T. Honda, T. Miwatani, and M. Nishibuchi.** 1991. Characterization of thermostable direct hemolysins encoded by four representative *tdh* genes of *Vibrio parahaemolyticus*. Microb. Pathog. **10:**165–172.