Translocation-Specific Conformation of Adenylate Cyclase Toxin from *Bordetella pertussis* Inhibits Toxin-Mediated Hemolysis

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*Bordetella pertussis* adenylate cyclase (AC) toxin belongs to the RTX family of toxins but is the only member with a known catalytic domain. The principal pathophysiologic function of AC toxin appears to be rapid production of intracellular cyclic AMP (cAMP) by insertion of its catalytic domain into target cells (referred to as intoxication). Relative to other RTX toxins, AC toxin is weakly hemolytic via a process thought to involve oligomerization of toxin molecules. Monoclonal antibody (MAb) 3D1, which binds to an epitope (amino acids 373 to 399) at the distal end of the catalytic domain of AC toxin, does not affect the enzymatic activity of the toxin (conversion of ATP into cAMP in a cell-free system) but does prevent delivery of the catalytic domain to the cytosol of target erythrocytes. Under these conditions, however, the ability of AC toxin to cause hemolysis is increased three- to fourfold. To determine the mechanism by which the hemolytic potency of AC toxin is altered, we used a series of deletion mutants. A mutant toxin, ΔAC, missing amino acids 1 to 373 of the catalytic domain, has hemolytic activity comparable to that of wild-type toxin. However, binding of MAb 3D1 to ΔAC enhances its hemolytic activity three- to fourfold similar to the enhancement of hemolysis observed with 3D1 addition to wild-type toxin. Two additional mutants, ΔN489 (missing amino acids 6 to 489) and ΔN518 (missing amino acids 6 to 518), exhibit more rapid hemolysis with quicker onset than wild-type toxin does, while ΔN549 (missing amino acids 6 to 549) has reduced hemolytic activity compared to wild-type AC toxin. These data suggest that prevention of delivery of the catalytic domain or deletion of the catalytic domain, along with additional amino acids distal to it, elicits a conformation of the toxin molecule that is more favorable for hemolysis.

Adenylate cyclase (AC) toxin is an essential virulence factor produced by *Bordetella pertussis*, the organism responsible for whooping cough (16, 34–36). This toxin is an acylated, 177-kDa single polypeptide that delivers a portion of its catalytic domain to the interior of target cells (7, 12, 17). Once inside the cell, endogenous calmodulin binds to a site on the catalytic domain, activating enzymatic activity and leading to the production of supraphysiologic levels of intracellular CAMP from host ATP (4), a process referred to as intoxication. Insertion of AC toxin into target cell membranes also causes efflux of intracellular K+ and, in a process requiring a higher concentration of toxin and longer time, causes lysis of erythrocytes (10, 14, 28).

Removal of the N-terminal catalytic domain (amino acids [aa] 1 to 373) does not affect hemolysis or pore formation in a lipid bilayer (3, 31). However, aa 1 to 384 are required for full enzyme activity, namely, conversion of ATP into cyclic AMP (cAMP) in a cell-free system (D. Ladant, personal communication). The portion of the molecule distal to the catalytic domain is homologous to *Escherichia coli* hemolysin and other members of the RTX (repeats-in-toxin) family of bacterial toxins (8, 31, 37). However, relative to the other RTX toxins, AC toxin is weakly hemolytic (2, 8–10, 13, 28). The C-terminal portion of AC toxin is also responsible for binding and internalization of the catalytic domain into eukaryotic cells (2, 29).

Binding and insertion of AC toxin, intoxication, K+ efflux, and hemolysis all require the presence of calcium (18, 19, 30) as well as posttranslational acylation of AC toxin, which is catalyzed by an accessory protein, CyaC (1, 17, 20). Manipulation of incubation conditions can, however, dissociate the activities of AC toxin. For example, delivery of the catalytic domain to the cell interior requires temperatures above 20°C (14, 27), whereas AC toxin-elicited K+ efflux occurs with similar rates at 0 to 2 and 37°C. Hemolysis can occur at 0 to 2°C but is considerably reduced compared to that at 37°C (14). In addition, the time courses of the functional activities of AC toxin are very different. AC toxin increases intracellular cAMP and K+ efflux within seconds to minutes after toxin addition (14, 26), whereas hemolysis with wild-type AC toxin is not observed before 1 h (14, 28). We have shown previously that AC toxin monomers are sufficient for both intoxication and K+ efflux (14), but several studies indicate that hemolysis is a highly cooperative event that may require oligomerization of more than three toxin molecules (5, 14, 33).

A panel of monoclonal antibodies (MAbs) directed against AC toxin was produced in our laboratory (21). Among these is MAb 3D1, which is directed against an epitope (aa 373 to 399) at the distal end of the catalytic domain. Binding of this MAb to AC toxin prevents delivery of the catalytic domain to the target cell interior but markedly enhances the hemolytic activity of AC toxin. To determine the mechanism by which hemo-
lysis is enhanced, deletion mutants ΔN489 (missing aa 6 to 489), ΔN518 (missing 6 to 518), and ΔN549 (missing aa 6–549) were constructed. Deletion mutants ΔN489 and ΔN518 demonstrate enhanced hemolytic activity, while that of ΔN549 is reduced compared to wild-type toxin. The accumulated data support the concept that prevention of delivery of the catalytic domain or deletion of the catalytic domain, along with additional aa distal to it, elicits a conformation of the toxin molecule that is more favorable for hemolysis.

MATERIALS AND METHODS

Plasmids and recombinant DNA techniques. The ΔAC mutant was constructed by Sakamoto et al. (31). Unless otherwise noted, all PCR amplifications were performed using pfu polymerase ( Gibco) and the template pT7CACT1, which contains the coding sequence for wild-type AC toxin (5). An N-terminal deletion mutant lacking residues 6 through 489 of wild-type AC toxin (pN518) was constructed. Two DNA fragments were generated by PCR using the oligonucleotide primers 5′-TCAAGATCTTACGCTGTCGTTGCGCCG-3′ and 5′-TCCCAAGCTTGGTCTGATGCTATGCTTGTG-3′ (containing a HindIII linker) for fragment 1, which includes the DNA sequence upstream of and encoding as 5′ of AC toxin, and 5′-TCCCAAGGCTTGGTACGTTGCTTGGCCG-3′ (containing a HindIII linker) and 5′-TGGCCGGCCCAGGTGC-3′ for fragment 2, which encodes as 519 through 1008 of AC toxin. Fragments 1 and 2 were digested with HindIII and ligated with T4 DNA ligase (New England Biolabs, Beverly, Mass.). The resulting 1,708-bp DNA fragment served as the template for PCR amplification using the primers 5′-TTGCCGGCCCCGCCAGGTGC-3′ and 5′-TGGCCGGCCCAGGTGC-3′. The PCR product was digested with BamHI and XhoI and ligated into pT7CACT1 which had been digested with the same restriction enzymes. This plasmid was named pN518.

To construct an N-terminal deletion mutant lacking residues 6 through 489 of AC toxin (ΔN689), a PCR product was generated using oligonucleotides 5′-CCCAAGCTTGGTCTGATGCTATGCTTGTG-3′ (containing a HindIII linker) and 5′-TGGCCGGCCCAGGTGC-3′, which were designed to amplify the DNA sequence encoding as 490 through 1008 of AC toxin. The resulting PCR product was digested with HindIII and XhoI and subcloned into pN518 digested with the same restriction enzymes. Deletion mutant ΔN549, lacking residues 6 through 549 of AC toxin, was constructed by PCR amplification using oligonucleotides 5′-TCCCAAGGCTTGGTACGTTGCTTGGCCG-3′ (containing a HindIII linker) and 5′-TGGCCGGCCCAGGTGC-3′, which were designed to amplify the DNA sequence encoding as 550 through 1008. The resulting PCR product was digested with HindIII and XhoI and ligated into pN518 digested with the same restriction enzymes.

To ensure that additional mutations were not introduced by PCR into the coding sequence of AC toxin in the newly constructed plasmids, pN489, pN518, and pN549 were digested with NcoI and XhoI, thereby removing the DNA fragment encoding as 621 through 1008. This fragment was replaced with the corresponding NcoI-XhoI fragment from pT7CACT1, which contains the wild-type AC toxin sequence. DNA sequencing confirmed that no additional mutations were generated in the portions of pN489, pN518, and pN549 that were generated by PCR.

Production and purification of AC toxin and deletion mutants. E. coli XL-1 Blue cells (Stratagene, La Jolla, Calif.) containing the plasmid encoding wild-type AC toxin or the N-terminal deletion mutants were grown as described previously (21, 31). Cultured bacteria were centrifuged, and the resulting pellet was resuspended in 50 mM Tris (pH 7.5), sonicated, and extracted with 8 M urea. Urea-extracted AC toxin was purified on a DEAE ion-exchange column (for wild type) as described previously (15). Deletion mutants N518 and N549 were produced by Covance Research Projects, Inc. (Denver, Pa.). These polyvalent mouse ascites against AC toxin was produced and characterized as described previously (15). Rabbit polyclonal antibody against AC toxin was produced by the ImmunePure Fab preparation kit (Pierce) as specified by the manufacturer. The purity of these fragments was assessed by SDS-polyacrylamide gel electrophoresis under nonreducing conditions. Fab fragments appeared as a single band at 50 kDa with no detectable intact antibody present (data not shown). Production of MAb against AC toxin used in this study was described elsewhere (21). Two MBAs, 3D1 and 5D1, recognized the same 26 aa of AC toxin (aa 373 to 399) and have the same isotype (immunoglobulin G1 [IgG1]). They correspondingly appeared similar in all other aspects tested. For this reason, we have chosen to present the data on 3D1 only. MBA 9D4 recognizes the repeat region of AC toxin and has an isotype of IgG2. Mouse IgG was obtained from Sigma (St. Louis, Mo.). None of the antibodies used in this study had an effect on basal CAMP levels or hemolysis when used alone.

Fragmentation of MAB 3D1 into Fab fragments was carried out using the ImmunePure Fab preparation kit (Pierce) as specified by the manufacturer. The purity of these fragments was assessed by SDS-polyacrylamide gel electrophoresis under nonreducing conditions. Fab fragments appeared as a single band at 50 kDa with no detectable intact antibody present (data not shown).

Flow cytometry. Sheep erythrocytes were washed and resuspended at 108/ml in HBSS with 75 mM sucrose to prevent hemolysis. After addition of AC toxin, the cells were washed twice. The cell pellets were resuspended in HBSS with 75 mM sucrose plus 100 μl of each mouse polyclonal antibody against AC toxin at a 1:100 dilution, MBA 3D1 at 22 μg/ml, or rabbit polyclonal antibody at a 1:300 dilution for 1 h at 0°C. Washed cells were incubated for 4 h at 0°C in HBSS containing 75 mM sucrose. Samples were washed three times, and pellets were resuspended in 100 μl of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma) at a 1:25 dilution or FITC-conjugated goat anti-rabbit IgG (Sigma) at a 1:50 dilution for 1 h. Erythrocytes were washed
three times, and 10,000 cells were assayed using a FACScan flow cytometer (Becton Dickson Immunocytometry Systems, San Jose, Calif). Control samples consisted of cells incubated without toxin but with primary antibody against AC toxin and FITC-conjugated secondary antibody.

RESULTS AND DISCUSSION

Our laboratory developed and characterized a panel of MAbs, two of which (3D1 and 5D1) recognize aa 373 to 399, an epitope at the distal end of the catalytic domain of AC toxin. On initial screening, these MAbs inhibited AC toxin-induced cAMP accumulation in Jurkat cells but had no effect on enzyme activity (conversion of ATP into cAMP in a cell-free system), suggesting that they might affect the delivery of the catalytic domain to the cell interior (21). Because 3D1 and 5D1 recognize the same epitope, are the same isotype, and are similar in all other aspects tested, we present only the data on 3D1.

The concentration dependence for inhibition of intoxication by MAb 3D1 is strikingly steep. As shown in Fig. 1, AC toxin activity in Jurkat cells is reduced by more than 96% when 3D1 is added at an approximately equimolar concentration to that of toxin. These data support the concept that 3D1 prevents degradation of the toxin molecule. It is likely that this reduction in MAb 3D1 binding is not due to loss, processing, or degradation of the toxin molecule. Antibody bound to AC toxin was detected using FITC-conjugated goat anti-mouse IgG, but without AC toxin (solid line). Data presented here represent the flow cytometric histogram of 10,000 cells for each condition and are representative of three separate flow cytometry experiments. The x axis measures arbitrary fluorescence intensity units on a log scale.

To understand the structural requirements necessary for the fluorescence intensity associated with 3D1 binding is markedly reduced after incubation at 37°C (a condition in which delivery of the catalytic domain does occur) compared to that at 0°C (a condition in which delivery of the catalytic domain does not occur). A decrease in intensity is not seen when polyclonal antibody against AC toxin is used (Fig. 2), indicating that the reduction in MAb 3D1 binding is not due to loss, processing, or degradation of the toxin molecule. It is likely that this reduction in fluorescence intensity reflects the disappearance of the 3D1 epitope from a location to which the antibody has access, namely, by translocation of this epitope as well as the catalytic domain from the external surface to the target cell interior. Alternatively, the peptide domain could still be present at the cell surface but could have undergone a conformational change in the course of delivery of the catalytic domain such that the epitope no longer exists. Nevertheless, these experiments show that under conditions in which intoxication occurs there is a significant change in or translocation of a portion of the epitope, including aa 373 to 399, resulting in the disappearance of the 3D1 epitope.

To understand the structural requirements necessary for the
different functional activities of AC toxin, it was important to
determine if $\text{K}^+\text{EFX}$ flux is affected by MAb 3D1. As shown in
Fig. 3A, $\text{K}^+\text{EFX}$ of intracellular $\text{K}^+\text{H11001}$ from sheep erythrocytes is
unaffected by the addition of MAb 3D1 to AC toxin prior to
incubation with erythrocytes, even when intoxication is inhib-
ited by more than 99% (Fig. 3A). This observation suggests
that 3D1 prevents the delivery of the catalytic domain without
affecting the amount of toxin inserted into sheep erythrocytes.

To address this issue directly, we performed
flow cytometry using a rabbit polyclonal antibody against AC toxin. The
amount of AC toxin detectable on sheep erythrocytes is unaf-
fected by incubation of toxin with MAb 3D1 before its addition
to cells (data not shown). These data indicate that binding of
AC toxin and insertion into the host cell membrane, which
results in $\text{K}^+\text{EFX}$, are unaffected by 3D1 and strongly support
the previously proposed concept that intoxication and $\text{K}^+\text{EFX}$
are distinct activities (14).

$\text{K}^+\text{EFX}$ is thought to occur by insertion of monomeric
AC toxin, while hemolysis appears to require subsequent oli-
gomerization of toxin molecules (5, 14, 33). MAb 3D1, when
incubated with AC toxin prior to addition to sheep erythro-
cytes, augments hemolysis 3.6-fold while at the same time
inhibiting intoxication in these cells by more than 90% (Fig.
3B). To determine if 3D1 could enhance the hemolytic activity
of AC toxin after the toxin was inserted into target
cell membranes, we incubated sheep erythrocytes with AC toxin at 0
°C (a condition in which $\text{K}^+\text{EFX}$ occurs but translocation of the catalytic
domain does not). After the target cells were washed to re-
move unbound toxin, 3D1 was added and cells were incubated
at 37°C, a temperature permissive to translocation. The results
shown in Fig. 4 illustrate that 3D1 enhances the hemolytic
ability of AC toxin 2.5-fold even after its insertion into target
cell membranes. Hemolysis in the presence of a MAb against
the repeat region, 9D4, or mouse IgG was comparable to that
with AC toxin alone.

These data suggest that 3D1 does not affect the insertion of
AC toxin responsible for $\text{K}^+\text{EFX}$ but does prevent translo-
cation of the catalytic domain and, in doing so, induces a
conformation of the toxin molecule that favors interaction with
other toxin molecules to form the hemolytic oligomer. How-
ever, there are two additional potential explanations for the
increase in hemolysis elicited by 3D1: (i) MAb 3D1, with or
without AC toxin bound, could bind to the Fc receptor on the
erthrocyte and, by some mechanism, promote hemolysis; or
(ii) MAb 3D1, which is a divalent IgG$_1$, could bring together

\begin{figure}
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\includegraphics[width=\textwidth]{fig3.png}
\caption{AC toxin-induced intoxication of sheep erythrocytes is
markedly reduced by MAb 3D1, while $\text{K}^+\text{EFX}$ is unaffected and its
hemolytic activity is enhanced. (A) AC toxin (5 $\mu$g/ml), alone or with
antibodies (20 $\mu$g/ml), was incubated for 15 min at room temperature.
Sheep erythrocytes were added to these mixtures and control samples,
containing buffers alone (no toxin or antibodies), and the mixtures
were incubated for 1 h at 37°C. The cells were centrifuged, and the
supernatant was used to determine the extracellular $\text{K}^+$ concentration.
cAMP was extracted from the cell pellet as described in Materials and
Methods. Data represent the means ± standard deviations of triplicate
samples from a single experiment that is representative of three sep-
arate experiments. (B) AC toxin (5 $\mu$g/ml), alone or with antibodies
(20 $\mu$g/ml), was incubated for 15 min at room temperature. Sheep
erthrocytes were added to the mixture, and the mixture was incubated
at 37°C. The incubation time for intoxication was 30 min, while that for
hemolysis was 6 h. Each bar represents the mean ± standard deviation
of triplicate determinations and is representative of four similar ex-
periments.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{MAb 3D1 enhances the hemolytic activity of AC toxin
bound to sheep erythrocytes. AC toxin (20 $\mu$g/ml) was incubated with
sheep erythrocytes for 30 min at 0°C. The cells were washed and
incubated with or without antibody (2 $\mu$g/ml) for 15 min at 0°C.
Samples were then incubated for 20 h at 37°C, 9D4, a MAb against the
repeat region of AC toxin, and mouse IgG served as controls. Results
are expressed as a percentage of total hemolysis. Each bar represents
the mean ± standard deviation of triplicates and is representative of three similar experiments.}
\end{figure}

FIG. 3. AC toxin-induced intoxication of sheep erythrocytes is
markedly reduced by MAb 3D1, while $\text{K}^+\text{EFX}$ is unaffected and its
hemolytic activity is enhanced. (A) AC toxin (5 $\mu$g/ml), alone or with
antibodies (20 $\mu$g/ml), was incubated for 15 min at room temperature.
Sheep erythrocytes were added to these mixtures and control samples,
containing buffers alone (no toxin or antibodies), and the mixtures
were incubated for 1 h at 37°C. The cells were centrifuged, and the
supernatant was used to determine the extracellular $\text{K}^+$ concentration.
cAMP was extracted from the cell pellet as described in Materials and
Methods. Data represent the means ± standard deviations of triplicate
samples from a single experiment that is representative of three sep-
arate experiments. (B) AC toxin (5 $\mu$g/ml), alone or with antibodies
(20 $\mu$g/ml), was incubated for 15 min at room temperature. Sheep
erthrocytes were added to the mixture, and the mixture was incubated
at 37°C. The incubation time for intoxication was 30 min, while that for
hemolysis was 6 h. Each bar represents the mean ± standard deviation
of triplicate determinations and is representative of four similar ex-
periments.
two toxin molecules, thereby increasing the local concentration of toxin on the target cell and making the conditions more favorable for oligomer formation and hemolysis. Monovalent Fab fragments of 3D1 were made to test both of these hypotheses. As shown in Table 1, Fab fragments of 3D1 cause both an enhancement of hemolysis and inhibition of intoxication (controls for these experiments included MAb 9D4, reactive with the repeat region of AC toxin, and mouse IgG). These findings rule out a role for the Fc receptor and for antibody-mediated cross-linking of toxin molecules as explanations for the enhanced hemolytic activity of AC toxin in the presence of MAb 3D1.

Exposure of AC toxin to calmodulin prior to incubation with sheep erythrocytes has also been observed to inhibit intoxication and augment AC toxin-induced hemolysis (11, 14, 28, 32). Furthermore, in a recent report by Osickova et al., substitution of lysines for glutamates 509 and 516 in the hydrophobic domain of AC toxin was shown to ablate the capacity of the mutant toxin to intoxicate target cells, as well as to significantly enhance hemolytic activity (25). These data raise the possibility that any process blocking delivery of the catalytic domain also enhances hemolysis, but this explanation may be oversimplified for the following reasons. A mutant AC toxin (ΔAC), in which aa 1 to 373 are deleted (31), exhibits hemolytic activity comparable to that seen with wild-type toxin (Table 2). These data shown that the absence of the first 373 aa, in itself, is not enough to augment hemolytic activity. Because ΔAC contains the epitope for 3D1, it was possible to test the effect of this MAb on its hemolytic activity. Addition of MAb 3D1 to ΔAC results in enhanced hemolysis, similar to that seen with MAb 3D1 and wild-type toxin. These data suggest that an additional segment of the toxin molecule, distal to aa 373, may undergo a conformational change and/or translocate when the toxin interacts with a target cell, thereby imposing an inhibitory constraint on hemolysis. In the case of ΔAC, 3D1 prevents the translocation and/or the resulting structural change involving this separate domain, resulting in the same enhancement of hemolysis that it elicits with intact toxin.

In light of this finding and the effect of the mutations generated by Osickova et al. (25), three new mutants of AC toxin, constructed by deletion of increasing amounts of the region distal to the catalytic domain, were tested for their hemolytic activity. None of these proteins contain the catalytic domain or the epitope for 3D1. As shown in Fig. 5, ΔN489 (missing aa 6 to 489) and ΔN518 (missing aa 6 to 518) exhibit more rapid hemolysis and quicker onset than do wild-type toxin and wild-type toxin plus 3D1. On the other hand, the hemolytic activity of ΔN549 (missing aa 6 to 549) is reduced relative to that of wild-type toxin, suggesting that the deletion of additional aa compromises the ability of this protein to insert and/or oligomerize in the target cell membrane.

These results identify a domain of the toxin molecule that is a major determinant of the hemolytic activity of AC toxin. This domain, defined by MAb 3D1 and our deletion mutants, consists of at least aa 373 to 489. Our data suggest that the presence and translocation of this domain, with or without the catalytic domain, impose a constraint on the toxin molecule that impairs hemolysis, perhaps by interfering with oligomerization. On the other hand, if this portion is absent (by deletion) or not delivered to the target cell interior (by incubation with MAb 3D1 or calmodulin or by point mutations at residues 509 and 516), the hemolytic activity of AC toxin is enhanced. If this hypothesis is correct, incubation of 3D1 with AC toxin prior to its addition to target cells under conditions in which translocation of the catalytic domain does not occur, should have no effect on the hemolytic activity of AC toxin. To test

### Table 1. Fab fragments of MAb 3D1 enhance AC toxin-induced hemolysis but inhibits intoxication of sheep erythrocytes

<table>
<thead>
<tr>
<th>Toxin</th>
<th>% Hemolysis (4.5 h)</th>
<th>Intoxication (30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC toxin</td>
<td>15.3 ± 1.04</td>
<td>14,292 ± 713</td>
</tr>
<tr>
<td>AC toxin + MAb 3D1</td>
<td>47.4 ± 1.39</td>
<td>1,233 ± 141</td>
</tr>
<tr>
<td>AC toxin + Fab fragment of MAB 3D1</td>
<td>60.4 ± 1.36</td>
<td>1,327 ± 20.2</td>
</tr>
<tr>
<td>AC toxin + MAB 9D4</td>
<td>13.9 ± 1.79</td>
<td>13,875 ± 891</td>
</tr>
<tr>
<td>AC toxin + Mouse IgG</td>
<td>17.0 ± 0.72</td>
<td>13,392 ± 339</td>
</tr>
</tbody>
</table>

*a* AC toxin (5 μg/ml) was incubated with or without antibodies (20 μg/ml) for 10 min at 0°C before the addition of 5 x 10⁸ sheep erythrocytes per ml.

*b* Data represent the means ± standard deviations of triplicate samples from a single experiment that is representative of three comparable experiments.

### Table 2. MAb 3D1 increases AC toxin-induced hemolysis in a mutant lacking the catalytic domain

<table>
<thead>
<tr>
<th>Toxin</th>
<th>% Hemolysis (4.5 h)</th>
</tr>
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<tbody>
<tr>
<td>Wild-type toxin</td>
<td>11.3</td>
</tr>
<tr>
<td>Wild-type toxin + MAB 3D1</td>
<td>66.6</td>
</tr>
<tr>
<td>Wild-type toxin + MAB 9D4</td>
<td>11.2</td>
</tr>
<tr>
<td>Wild-type toxin + Mouse IgG</td>
<td>11.7</td>
</tr>
<tr>
<td>ΔAC</td>
<td>9.1</td>
</tr>
<tr>
<td>ΔAC + MAB 3D1</td>
<td>55.8</td>
</tr>
<tr>
<td>ΔAC + MAB 9D4</td>
<td>9.1</td>
</tr>
<tr>
<td>ΔAC + Mouse IgG</td>
<td>9.9</td>
</tr>
</tbody>
</table>

*a* Wild-type toxin and ΔAC (5 μg/ml) were incubated with or without antibodies (20 μg/ml) for 10 min at 0°C before addition of sheep erythrocytes.

*b* Data represent duplicate determinations of one experiment that is representative of two comparable experiments.
this hypothesis, sheep erythrocytes at 0°C were incubated with AC toxin with or without 3D1. Under this condition, translocation does not occur but hemolysis does, albeit at a reduced rate. As predicted, the addition of 3D1 does not enhance the hemolytic activity of AC toxin at 0°C in 31 h (percent hemolysis is as follows: AC toxin [10 μg/ml] alone, 10.4%; AC toxin plus 3D1, 4.4%; AC toxin plus 9D4, 6.2%; AC toxin plus mouse IgG, 11.0%).

Although we and others have previously referred to aa 1 to 400 as the catalytic domain, it seems more appropriate from sequence comparison with E. coli hemolysin, HlyA (13), and from the data presented here that the catalytic domain be defined as ending between aa 373 and 384. The sequence from aa 373 to 500, the putative beginning of what has been considered the hydrophobic domain, has not previously been associated with a defined function. Osickova et al. demonstrated that the initial part of the hydrophobic domain is critical for delivery of the catalytic domain, because mutants containing single- and double-aa substitutions at residues 509 and/or 516 display reduced or absent intoxication (25). Here, we propose a domain encompassing aa 373 to 489 that undergoes a conformational change on delivery of the catalytic domain. Under conditions in which intoxication occurs, this domain appears to induce or contribute to a conformation that is less than optimal for hemolysis. Because oligomers are believed to be essential for hemolysis (5, 14, 33), it is tempting to speculate that binding of MAb 3D1 elicits or stabilizes a confirmation of AC toxin that prohibits the translocation of the catalytic domain and favors the formation of hemolytic oligomers. The observation that there is enhanced hemolysis with deletion mutants ΔN489 and ΔN518 (lacking the 3D1 epitope) but not with ΔAC (missing the first 373 aa) indicates that lack of delivery of the catalytic domain, as in ΔAC, is not sufficient to enhance hemolysis. Instead, it appears that the location and structure of the intervening region of AC toxin, defined by 3D1 and our deletion mutants, are critical to the success or failure of the oligomerization process.

Our data show that a MAb against an epitope distal to the catalytic domain of AC toxin has strikingly disparate effects on toxin activities. MAb 3D1 blocks the delivery of the catalytic domain to the target cell interior, has no effect on insertion leading to K⁺ efflux, but enhances the hemolytic activity of AC toxin. In addition, there is a conformational change or translocation of the 3D1 epitope on delivery of the catalytic domain, making it inaccessible to 3D1 present in the extracellular medium. The MAb-mediated enhancement of hemolysis occurs even when Fab fragments of 3D1 are used or when intact 3D1 is added to ΔAC. These observations, in combination with prior information about AC toxin (25, 28), strongly support a model of toxin action in which K⁺ efflux is an early event resulting from toxin insertion into the membrane. Intoxication and hemolysis, however, are antagonistic processes, as has been suggested previously (14, 25).

The data presented here clearly demonstrate that inhibition of delivery of the catalytic domain is not the sole requirement for enhanced hemolytic activity by AC toxin. Instead, our observations focus new attention on a portion of the molecule distal to the catalytic domain, including at least aa 373 to 489, that seems to be antagonistic for hemolysis. Removal of this segment or prevention of its entry into cells overcomes its inhibitory influence and causes an enhancement of hemolysis relative to wild-type toxin. Although the enhancing effect of 3D1 on toxin-mediated hemolysis was notable when it was first observed, it is now clear that the ability of 3D1 to modulate the conformational change associated with translocation is modest relative to deletion of this inhibitory segment. One explanation is that 3D1 binding to aa 373 to 399 does not completely prevent insertion of the domain that is antagonistic to hemolysis. The rapidity of hemolysis produced by ΔN489 and, to a slightly lesser extent, by ΔN518 is truly striking for AC toxin. The difference in hemolytic activity observed between ΔN489 and ΔN518 may occur because as more aa are deleted into the hydrophobic domain, the toxin inserts or oligomerizes less well. This notion is supported by the finding that ΔN549 is severely impaired for hemolysis even though it lacks the antagonistic domain.

Interestingly, Ludwig et al. demonstrated that deletion of aa 9 to 37 at the N terminus of E. coli hemolysin led to production of a protein with threefold-enhanced hemolytic activity (23). Comparison of the sequence alignments of E. coli hemolysin and B. pertussis AC toxin revealed that the corresponding aa in AC toxin are aa 330 to 363. This region is deleted in ΔAC with no increase in hemolysis compared to wild-type toxin. Relative to other RTX toxins, AC toxin is a very modest hemolysin (2, 8–10, 13, 28). In addition, AC toxin is the only member of this family that contains a catalytic domain. The observation that prevention of intoxication by MAb 3D1 or absence of the catalytic domain and the adjacent domain increases hemolysis suggests that the tertiary structure of this region may actually impair oligomerization. From our present data, it is all the more compelling to speculate that AC toxin resulted from a gene fusion between eukaryotic, calmodulin-regulated AC (24) and an ancestor of the RTX toxin family (13). The result was a molecule able to deliver this newly acquired catalytic region, but at the cost of hemolytic potency.

ACKNOWLEDGMENTS

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