Inhibition of *Escherichia coli* Heat-labile Enterotoxin B Subunit Pentamer (EtxB₅) Assembly *in Vitro* Using Monoclonal Antibodies*

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Monoclonal Antibodies*

Heat-labile enterotoxin (Etx) produced by certain strains of *Escherichia coli* is a major virulence factor related to cholera toxin. Both are hexameric proteins comprising one A-subunit and five B-subunits. The pentameric B-subunit of *E. coli* has a high affinity for GM₁-ganglioside receptors on gut epithelial cells and is directly responsible for toxin entry. The pentameric B-subunit (EtxB₅) is an exceptionally stable protein, being able to maintain its quaternary structure over a wide pH range (2.0–11.0). However, little is known about the formation of the pentameric structure (EtxB₅) from newly synthesized B-subunit monomers (EtxB₁). We previously described and characterized a mAb (LDS47) that was shown to be highly specific for an N-terminal decapeptide region of EtxB₁ (Amin, T., Larkins, A., James, R. F. L., and Hirst, T. R. (1995) *J. Biol. Chem.* 270, 20143–20150).

Here we also describe a mAb (LDS16) with exquisite specificity for pentameric EtxB. In this study, we have used these two mAbs, in combination, to probe the *in vitro* assembly of EtxB₅ from EtxB₁. EtxB pentamers disassemble in highly acidic conditions, giving rise to monomeric B-subunits that can reassemble if placed in buffers of neutral pH. Using this *in vitro* assembly model, it was found that at a molar ratio of 1:1; LDS47:EtxB₅, 50% of reassembly was inhibited, and that this inhibition increased to 90% at a ratio of 2:1. These results infer that the N-terminal decapeptide region (APQSITELC) defined by the LDS47 antibody is crucial for competent pentameric B-subunit assembly and stabilization.

Heat-labile enterotoxin (Etx)² produced by certain strains of *Escherichia coli* is related to cholera toxin (Ctx) and causes diarrheal disease in humans and animals (2, 3). Enterotoxigenic *E. coli* diarrhea occurs in all age groups and has a high mortality rate in infants, particularly in developing countries (4, 5).

The pathogenesis of enterotoxigenic *E. coli* diarrheal disease requires three processes: firstly, colonization and adherence to the mucosal epithelium by the bacterial fimbriae; secondly, binding of secreted toxin via a specific receptor (which has been identified as G₅₁-ganglioside); and lastly, uptake of the toxin into the cell, causing disruption of the electrolyte balance in gut epithelium (6, 7). Heat-labile enterotoxin and Ctx are both heterohexameric complexes comprising one A subunit (possessing ADP-ribosyl transferase activity (Mₛ = 28,000)) and five identical B subunits (Mₛ = 12,000 each), which, in vivo naturally assemble into a stable pentameric structure that has a high affinity for G₅₁-ganglioside ubiquitously found on mammalian cells (8–10).

The B-subunit of Etx from *E. coli* strains of human origin show a 96% sequence identity to one another and approximately an 80% identity to the B-subunit of cholera toxin (11). Crystallographic studies have shown that each B-subunit in the pentameric structure is folded into two three-stranded anti-parallel β-sheets (with one sheet on each side of the monomer facing an adjacent B-subunit) and a large central helix positioned at the wall of the central pole, with the five helices (one from each subunit) forming a pentagonal helical barrel (12–14). The pentasaccharide moiety exposed at the surface of G₅₁ allows binding to the five pockets formed at the interfaces of the B-subunit monomer interaction (15). It has been shown that the underside of the B₅ pentamer, where the G₅₁ ganglioside binding sites are located, has a positive surface potential. This is consistent with the fact that the terminal saccharide of G₅₁ ganglioside is negatively charged and that cell membranes themselves are also negatively charged (Fig. 1). Following binding to G₅₁ on mucosal cells and trafficking of the toxin to the endoplasmic reticulum, the A subunit enters the cytosol and displays ADP ribosyltransferase activity.

Studying the pathways of B-subunit pentamerization *in vivo* is difficult because of the problems involved in investigating such processes in the complex environment of the periplasmic space (16). The use of *in vitro* conditions to study the disassembly and reassembly of B-subunit was first reported by Finkelstein et al. (17) in 1974, who showed that purified cholera toxin could be denatured in acid urea and subsequently reassembled into active toxin when neutralized. In this study, we have used *in vitro* denaturation/renaturation techniques to study a region of EtxB₁ that is recognized by an EtxB₁-specific monoclonal
antigenic intraperitoneally three times with each dose containing the equivalent of 10 μg immunogen in MF1 (1), either pentameric or monomeric, was used as an immunogen in pentamer formation (1). To facilitate this process, it was necessary to produce a monoclonal antibody (mAb) that did not recognize EtxB₁ and was specific for EtxB₅ (LDS16). Using the in vitro system of toxin reassembly and the highly specific mAbs, we show that the N-terminal region identified by LDS47 (APQSI[TELCS]) is crucial for competent pentameric B-subunit assembly.

MATERIALS AND METHODS

Preparation of Monoclonal Antibodies—Purified recombinant EtxB (1), either pentameric or monomeric, was used as an immunogen in MF1 × BALB/c F1 hybrid mice. These were immunized intraperitoneally three times with each dose containing the equivalent of 10 μg of EtxB/nitrocellulose suspension in 0.5 ml of PBS. The first two injections were 7 weeks apart, and the final immunization was 4 weeks later. A final 10-μg intraperitoneal dose was administered 3 days before the spleens were removed prior to fusion.

Production of mAbs—After the final antigen boost, spleen cells were isolated from immunized mice, mixed with NS0 myeloma cells in a ratio of 4:1, and fused using a modification of the method described by Köhler and Milstein (18) at 37 °C using 0.8 ml of 50% w/v polyethylene glycol (Mr = 1500). The cell suspension was added in 1-ml aliquots to each well in RPMI/Dulbecco’s modified Eagle’s medium (Invitrogen) containing 15% v/v fetal calf serum (Seralab) and incubated at 37 °C in 5% CO₂ (day 0). Hypoxanthine and azaserine (5 × 10⁻³ M and 2.85 × 10⁻⁵ M) in 1 ml of RPMI medium were added on day 1, and the medium in the wells was replaced every 2 days with 1 ml of RPMI/Dulbecco’s modified Eagle’s medium + 15% fetal calf serum + hypoxanthine and azaserine. Supernatants were screened against EtxB₁ and EtxB₅ by ELISA on day 11, and positive wells were cloned by limiting dilution.

Screening of Hybridoma Supernatants—Two ELISA assays were employed to discriminate between antibodies that recognized denatured EtxB monomers and assembled EtxB pentamers.

G₅₋₁ ELISA—This was performed essentially as described previously by Amin and Hirst (19). Each hybridoma supernatant was added to the wells of a microtiter plate (Immulon 1, Dynatech) that had been coated with GM₁-ganglioside and 0.2 μg/ml EtxB pentamer in PBS. Antibodies binding to EtxB were detected using a goat anti-mouse-horseradish peroxidase conjugate (Jackson ImmunoResearch Laboratories).

Non-G₅₋₁ ELISA—Since denatured EtxB subunits do not bind to the receptor G₅₋₁, denatured EtxB monomers were coated directly onto the plastic surface of the microtiter plates. Denatured EtxB monomers were prepared by boiling 5 μg/ml purified EtxB in 30 mM dithiothreitol for 5 min. Microtiter plates were coated with this preparation for 1 h at 37 °C, and the plate was washed with PBS. Nonspecific binding sites were blocked with 1% (w/v) bovine serum albumin, and the remainder of the assay was carried out in a manner identical with the GM₁-ELISA. In some assays, pentameric EtxB at 5 μg/ml in PBS was also coated directly onto microtiter plates in the absence of G₅₋₁.

SDS-Polyacrylamide Gel Electrophoresis and Western Blot—EtxB₁ (8 μg) and boiled EtxB₅ (EtxB₁, 8 μg) were loaded into SDS-polyacrylamide 14% (w/v) gels for electrophoresis on a Bio-Rad Protean II system. After SDS-PAGE, gels were transferred to nitrocellulose paper with transfer buffer in a Bio-Rad Trans-Blot apparatus for 45 min to permit efficient transfer. Nonspecific binding sites were blocked overnight in PBS containing 5% (w/v) Marvel. Blots were washed in PBS containing 0.05% v/v Tween 20 for 3 × 10 min with agitation. LDS16 supernatant (~2 μg/ml) was used neat for 1 h at room temper-
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Antibody Purification—mAbs LDS16, LDS47 (1), LDS56 (20), and LDS102 (EtxB C-terminal-specific)3 were purified from ascites fluid on a protein G-agarose column (mAb trap kit, Amersham Biosciences). Ascites fluid was filtered through a 0.5-μm filter and diluted 1:5 with 20 mM phosphate buffer, pH 7.0. The absorbed IgG was eluted from the column using 0.1 M glycine-HCl buffer, pH 2.7, with 1.0-ml fractions collected into tubes containing 0.06 ml of 1 M Tris-HCl, pH 9.0. The presence of IgG in eluted fractions was monitored by measuring absorbance at 280 nm. ELISA was used to confirm its activity.

Inhibition of Reassembly of EtxB5 (Monomers) to EtxB5 (Pentamers) in the Presence of LDS47—The concentration of purified EtxB5 was adjusted with PBS to 34 μM, and then this was diluted 1:4 in 0.1 M KCl/HCl, pH 1.0. After 20 s in these acidic conditions, the samples were neutralized by dilution in a 10× volume of 3.4 μM in McIlvaine buffer (0.2 M disodium hydrogen phosphate, 0.1 M citric acid, pH 7, filtered through a sterile 0.22-μm filter before use) and then incubated for a further 60 min at 23 °C with or without affinity-purified mAb LDS47 at 10× molar excess ratio. Immediately after neutralization and after incubation for specified times at 23 °C, samples were removed and diluted 100-fold in PBS to prevent further assembly followed by analysis using a GmAb ELISA. The percentage of reassembled EtxB5 at each time point was calculated relative to treated and immediately neutralized (within 20 s) EtxB pentamers by ELISA using LDS16 as a readout. In addition, samples were also removed from the reaction mixture at specified time points and mixed at a ratio of 4:1 with 5 points and mixed at a ratio of 4:1 with 5

3 T. R. Hirst and R. F. L. James, unpublished data.

**RESULTS**

**LDS16 mAb Binding Specificity**—To characterize the precise specificity of LDS16 for EtxB, a series of ELISA experiments were performed (Fig. 2a). A maximal signal was detected with either EtxB5 bound directly to the plate or EtxB5, captured by GmAb, alone (lane 4, GmAb+EtxB). Observed were no signal with EtxB5 on a GmAb-coated plate (lane 2), preincubated LDS16+EtxB5, (lane 5, GmAb+EtxB), preincubated LDS16+EtxB5 on a GmAb-coated plate, and EtxB5, preincubated LDS16+EtxB5, (lane 1) (see “Discussion”). To use this system to elucidate the binding specificity of LDS16, two preincubation experiments were performed. EtxB1 and EtxB5 were preincubated with goat anti-mouse IgG conjugated to horseradish peroxidase diluted 1:2000 for 1 h. The blots were then washed and developed with an enhanced chemiluminescence system (ECL, Amersham Biosciences) for 10 min.

**Antibody Purification**—mAbs LDS16, LDS47, LDS56, and LDS102 (EtxB C-terminal-specific)3 (all IgG, isotype) were purified from ascites fluid on a protein G-agarose column (mAb trap kit, Amersham Biosciences). Ascites fluid was filtered through a 0.5-μm filter and diluted 1:5 with 20 mM phosphate buffer, pH 7.0. The absorbed IgG was eluted from the column using 0.1 M glycine-HCl buffer, pH 2.7, with 1.0-ml fractions collected into tubes containing 0.06 ml of 1 M Tris-HCl, pH 9.0. The presence of IgG in eluted fractions was monitored by measuring absorbance at 280 nm. ELISA was used to confirm its activity.

**Inhibition of EtxB Reassembly**—Samples of the reassembly mixture were taken after 1 h, diluted 100-fold to a concentration of 34 nM, and then added to ELISA plates that had previously been coated with 200 ng of GmAb and subsequently blocked with 1% Marvel in PBS. Samples were serially diluted 2-fold in PBS, and bound EtxB5 was detected using LDS16 (1.66 mg/ml) at a 1:200 dilution. For quantification of the amount of EtxB5 bound to the ELISA plates for each test sample, optical density readings corresponding to dilutions located on the linear part of the curve were compared with the dilution of an EtxB5 standard (1 μg/ml, diluted 2-fold), giving the same optical density reading.

**Graphical representations of the three-dimensional molecular features were generated using a previously published homologous structure of CtxB5 (2CHB.pdb) (21) and the SWISS-model software package (22).**

**ELISA Experiments**—These were repeated at least three times with each data point done in triplicate. The results are plotted as mean ± S.D.

**RESULTS**

**LDS16 mAb Binding Specificity**—To characterize the precise specificity of LDS16 for EtxB, a series of ELISA experiments were performed (Fig. 2a). A maximal signal was detected with either EtxB5 bound directly to the plate or EtxB5, captured by GmAb, alone (lane 4, GmAb+EtxB). No signal was detected with EtxB5 on a GmAb-coated plate (lane 7), preincubated LDS16+EtxB5, on a GmAb-coated plate, and EtxB5, preincubated LDS16+EtxB5, (lane 1) (see “Discussion”). To use this system to elucidate the binding specificity of LDS16, two preincubation experiments were performed. EtxB1 and EtxB5 were preincu-
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bated with LDS16 at equimolar concentrations prior to adding to a G\textsubscript{M\textsubscript{1}}-coated plate (lanes 6 and 7). In neither case was a signal detected.

Having shown by ELISA (Fig. 2a) that LDS16 was highly specific for EtXb\textsubscript{5}, we went on to examine its specificity using Western blotting (Fig. 2b). In non-reducing conditions, LDS16 gave, as expected, a single band with EtXb\textsubscript{5} running in its oligomeric configuration at 43 kDa (Fig. 2b, lane 2). After boiling in non-reducing conditions, LDS16 only detected a faint band in the pentameric form (incomplete dissociation, Fig. 2b, lane 1).

Concentration-dependent Inhibition of LDS16-EtXb\textsubscript{5} Binding—As seen in the ELISA experiments described in the legend for Fig. 2, LDS16 binds strongly to both EtXb\textsubscript{5} either bound directly to the plate or bound via G\textsubscript{M\textsubscript{1}}. We show in this experiment that free EtXb\textsubscript{5} in solution will strongly inhibit LDS16 binding to EtXb\textsubscript{5} on the plate but only to a plateau level in which ~70% of LDS16 binding is inhibited (Fig. 3a, panel i). Interestingly, the level of inhibition achieved by free EtXb\textsubscript{5} when the plates are coated with G\textsubscript{M\textsubscript{1}}-EtXb\textsubscript{5} is far less (~30%) (Fig. 3a, panel i), with a plateau level reached at ~2 \mu g/ml free EtXb\textsubscript{5}. As shown in Fig. 3b, LDS47 does show a faint band in Western blotting with boiled EtXb\textsubscript{5}, possibly indicating the detection of the intermediate form of EtXb\textsubscript{5} (see “Discussion”). In the ELISA experiment (Fig. 3a, panels i and ii), LDS47 does give a consistent signal with EtXb\textsubscript{5} bound directly to plates, consistent with the Western blot (Fig. 3b). However, this binding is not inhibited by free EtXb\textsubscript{5} (Fig. 3a, panels i and ii) in solution, consistent with the Western blot, which shows that LDS47 does not detect the pentameric form of EtXb\textsubscript{5} (Fig. 3b). Consistent with Fig. 2a, LDS16 binds strongly to both directly coated EtXb\textsubscript{5} and EtXb\textsubscript{5} bound to G\textsubscript{M\textsubscript{1}} (Fig. 3a, panel ii), but neither of these reactivities were inhibited by EtXb\textsubscript{1}, confirming the strong specificity shown by LDS16 to EtXb\textsubscript{5} when compared with EtXb\textsubscript{1}.

Time-dependent Reassembly of EtXb\textsubscript{5}—To investigate the assembly of EtXb monomers to EtXb pentamers, we utilized the method described by Sixma et al. (12). Essentially, EtXb pentamers were exposed briefly to strong acidic conditions (pH 1.0–2.0), neutralized, and allowed to reassemble with or without the presence of inhibiting mAbs. Time-dependent reassembly was assessed using G\textsubscript{M\textsubscript{1}}-coated plates probed with LDS16. By plotting the level of EtXb\textsubscript{5} obtained against time (Fig. 4a), it was possible to carry out one phase exponential association analysis. The half-rate of assembly either with or without the presence of LDS47 was similar (EtXb alone $t_{1/2} = 9.416 \text{ min}$ versus EtXb + LDS47 $t_{1/2} = 10.82 \text{ min}$), and the rate of assembly was also comparable (EtXb alone $k = 0.073 \pm 0.008 \text{ min}^{-1}$ versus EtXb + LDS47 $k = 0.064 \pm 0.025 \text{ min}^{-1}$). The clear difference observed in the presence of LDS47 was in the maximum reassembly. In the absence of LDS47, 91.09% reassembly occurred by 60 min, whereas in the presence of LDS47, maximal reassembly reached only 38.12% (a 59% reduction). Importantly, this inhibition appeared irreversible (Fig. 4a). The inhibition of reassembly was also confirmed by Western blot analysis using LDS16 as a probe (Fig. 4b).

Inhibition of EtXb Reassembly—Using the same in vitro system of acid dissociation followed by neutralization, we studied the effects of the presence of various mAbs in the system. As can be seen from Fig. 5, reassembly following denaturation/neutralization gave almost 100% recovery of EtXb\textsubscript{5} (lanes 1 and 2). In the presence of LDS47, renaturation was almost completely inhibited in the presence of either 1:1 or 2:1 molar ratios of EtXb to LDS47 (lanes 3 and 4). Other mAbs studied were LDS102, which has been shown to react specifically for EtXb\textsubscript{5}, and binding was inhibited by increasing concentrations of free EtXb\textsubscript{5} (Fig. 2a, panel ii), plates were coated either directly with EtXb\textsubscript{5} (B\textsubscript{5c}, lane 4) or with G\textsubscript{M\textsubscript{1}}/EtXb\textsubscript{5} (G\textsubscript{M\textsubscript{1}}/B\textsubscript{5c}, lane 6). Consistent with Fig. 2a, LDS16 binds strongly to both EtxB5 either bound directly to plates, consistent with the Western blot (Fig. 3b). However, this binding is not inhibited by free EtxB5 (Fig. 3a, panels i and ii) in solution, consistent with the Western blot, which shows that LDS47 does not detect the pentameric form of EtxB (Fig. 3b). Consistent with Fig. 2a, LDS16 binds strongly to both directly coated EtxB5 and EtxB5 bound to G\textsubscript{M\textsubscript{1}} (Fig. 3a, panel ii), but neither of these reactivities were inhibited by EtxB1, confirming the strong specificity shown by LDS16 to EtxB5 when compared with EtxB1.

Concentration-dependent Inhibition of LDS16-EtxB\textsubscript{5} Binding—Using the same method described by Sixma et al. (12). Essentially, EtXb pentamers were exposed briefly to strong acidic conditions (pH 1.0–2.0), neutralized, and allowed to reassemble with or without the presence of inhibiting mAbs. Time-dependent reassembly was assessed using G\textsubscript{M\textsubscript{1}}-coated plates probed with LDS16. By plotting the level of EtXb\textsubscript{5} obtained against time (Fig. 4a), it was possible to carry out one phase exponential association analysis. The half-rate of assembly either with or without the presence of LDS47 was similar (EtXb alone $t_{1/2} = 9.416 \text{ min}$ versus EtXb + LDS47 $t_{1/2} = 10.82 \text{ min}$), and the rate of assembly was also comparable (EtXb alone $k = 0.073 \pm 0.008 \text{ min}^{-1}$ versus EtXb + LDS47 $k = 0.064 \pm 0.025 \text{ min}^{-1}$). The clear difference observed in the presence of LDS47 was in the maximum reassembly. In the absence of LDS47, 91.09% reassembly occurred by 60 min, whereas in the presence of LDS47, maximal reassembly reached only 38.12% (a 59% reduction). Importantly, this inhibition appeared irreversible (Fig. 4a). The inhibition of reassembly was also confirmed by Western blot analysis using LDS16 as a probe (Fig. 4b).

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Concentration-dependent Inhibition of EtxB Reassembly

The in vitro reassembly model was also used to determine the dose-response relationship between the concentration of LDS47 and the percentage reassembly of EtxB5. As can be seen from the sigmoid dose-response curve (Fig. 6) at a molar ratio of 1:1 (IC50 equals 0.345 μM ± 0.005 (R² = 0.999)), 50% of reassembly was inhibited, and 90% inhibition was obtained at a molar ratio of 2:1 (Fig. 6).

DISCUSSION

At the present time, it is difficult to study the details of EtxB5 assembly and transport to the periplasm in vivo and, for this reason, many studies on EtxB assembly have been carried out in vitro (23, 24). A model has been put forward by Lesieur et al. (25), which suggests that in vitro (in the case of cholera toxin), following disassembly and neutralization, dimers, trimers, and tetramers can be reassembled in a mixture and that the concentrations of these decline with time, consistent with their assembly into pentamers. We have previously described and characterized a mAb (LDS47) that recognized the monomeric form of the B-subunit of E. coli heat-labile enterotoxin, which failed to recognize or precipitate the native B-subunit pentamer. In this study, we have gone on to use LDS47 with a newly described mAb LDS16 that has the converse reactivity (in that it has high specificity for pentameric EtxB but fails to bind monomeric EtxB) to directly study pentameric EtxB assembly in vitro. Previously, a lack of suitable reagents for studying toxin biogenesis has made this approach difficult. For instance, polyclonal antitoxin antisera generally have mixed specificities, and other anti-EtxB-specific mAbs recognize both pentamers and denatured subunits (26).

To fully characterize the specificities of LDS16 and LDS47, we carried out a series of ELISAs and Western blots. It can be clearly seen in the ELISA experiment shown in Fig. 2a that LDS16 will bind to EtxB5 either in its native form or when bound to GM1. Interestingly, preincubation of EtxB5 with LDS16 before binding to GM1-coated plates completely blocked EtxB5 binding (Fig. 2a, lane 6). This could be due to the mAb interfering with the GM1 binding site but is more likely to be the result of steric interference by the relatively large antibody molecules in an antigen/antibody complex.

The other surprising outcome of this experiment was that boiled EtxB (monomers) gave a small signal with LDS16 when added to GM1-coated plates. Given that on Western blot (Fig. 2b), LDS16 does detect a faint band with boiled EtxB in the pentameric position (43 kDa), it is likely that the high affinity of Gm1 for EtxB5 allows the capture of incompletely disassociated...
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EtxB (or reassociated EtxB₅) and subsequent detection by the EtxB₅-specific mAb.

It was possible to study the kinetics of binding of LDS16 to EtxB₅ using ELISA (Fig. 3). By using Scatchard analysis, we determined that at a level of 0.3 µg/ml (or greater) free EtxB₅, saturation binding occurred, blocking any further binding of LDS16 to EtxB₅ (Fig. 3a, panel i). Neutralization of LDS16 binding to EtxB₅ bound to G₃M₁ was 6-fold less efficient (Fig. 3a, panel ii), indicating that the binding site of LDS16 is more effectively formed when EtxB₅ is bound to G₃M₁. As can be seen in Fig. 3a, panels i and ii, free EtxB₁ does not inhibit binding of LDS16 to EtxB₅, again demonstrating the high specificity of this mAb to the pentameric structure. These experiments as a whole demonstrate the potential of the LDS16 binding site as being a key region for monitoring complete pentameric assembly.

In these experiments, we also looked at the kinetic behavior of LDS47, which we described in detail in an earlier publication (1). Although we have demonstrated the unique specificity or LDS47 toward EtxB₅, there is always a small signal obtained against EtxB₅ coated directly to plastic (Fig. 3a, panel i) but never to EtxB₅ coated via G₃M₁ (Fig. 3a, panel ii). We previously interpreted this as being due to partial unfolding of EtxB₅ on the plastic surface exposing the LDS47 binding site, but more sensitive blotting methods now available show that LDS47 can also recognize an intermediate form of EtxB₅ (Fig. 3b) (with a molecular mass of ~25 kDa). The formation of such intermediates was previously hypothesized by us when analyzing models for CtxB assembly (25). Thus the signal obtained from LDS47 reacting with directly coated EtxB₅ is more likely to be the result of recognition of an EtxB₅ intermediate form than recognition of monomers per se.

In our previous analysis of LDS47, we concluded that the epitope recognized by LDS47 needed to adopt a secondary structure and that amino acid residues, such as 6, 7, and 10, which are exposed in both the monomer and the dimer, are essential for multimer formation (1). Here we clearly show (using the in vitro model of reassembly) that indeed, the LDS47 binding site is essential for pentameterization (Fig. 4). This goes to emphasize the importance of this mAb not only in the study of possible EtxB chaperones involved in assembly but also in the processes involved in assembly itself.

To confirm that the in vitro system of reassembly was not non-specifically affected by the presence of a mAb, we used an irrelevant control (LDS56), which had no effect on reassembly (Fig. 5, lane 6). This figure also shows that another mAb we have prepared against the C-terminal region of EtxB (LDS102) will also inhibit pentamer formation but only partially (Fig. 5, lane 5). This confirms that the inhibition of reassembly seen with LDS47 is not a result of steric hindrance per se but is a direct result of interference with the crucial N-terminal APQSITELC₅S assembly sequence.

Finally, we studied the kinetics of EtxB reassembly in the presence of LDS47 (Fig. 6). Here we show that there is a classic dose-response relationship between the concentration of LDS47 and the rate of pentamer formation, again emphasizing the crucial nature of the LDS47 binding site.

In conclusion, we have extended our previous studies of the role of the EtxB binding site defined by LDS47. We now show that the epitope recognized by this mAb (amino acids 1–10) plays a crucial and essential role in EtxB pentamerization. These studies can therefore form the basis of further work involved not only in determining how such a stable pentameric structure such as EtxB is assembled but also in the design of biologics that would interfere with its production, thus having applications in therapeutics against these important diarrheal diseases.

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REFERENCES

1. Amin, T., Larkins, A., James, R. F. L., and Hirst, T. R. (1995) J. Biol. Chem. 270, 20143–20150
2. Hirst, T. R. (1995) Bacterial Toxins and Virulence Factors in Disease: Handbook of Natural Toxins (Moss, J., Vaughan, M., Iglewski, B., and Tu, A.T., eds) Vol. 8, pp. 123–184, Marcel Dekker Inc., New York
3. Echeverria, P., Seriwarana, J., Taylor, D. X., Yangegrate, S., and Tirapat, C. (1985) Ann. I. Trop. Med. Hyg. 34, 547–554
4. DeMol, P., Brasseur, D., and Hol, W. G. J. (1995) Curr. Opin. Struct. Biol. 5, 165–171
5. Evans, D. G., Olarte, J., Dupont, H. L., Evans, D. J., Jr., Galindo, E., Portnoy, B. L., and Conklin, R. H. (1977) J. Pediatr. 91, 65–68
6. Spangler, B. D. (1992) Microbiol. Rev. 56, 622–647
7. Merritt, E. A., and Hol, W. G. J. (1995) Curr. Opin. Struct. Biol. 5, 165–171
8. Holmgen, L., Lonnroth, I., Messing, J., and Svennerholm, L. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 2520–2524
9. Fishman, P. H., Moss, J., and Osborne, J. C., Jr. (1978) Biochemistry 17, 711–716
10. Fishman, P. H., Pauczuska, T., Hom, B., and Moss, J. (1980) J. Biol. Chem. 255, 7557–7664
11. Yamamoto, T., Gojobori, T., and Yokota, T. (1987) J. Bacteriol. 169, 1352–1357
12. Sixma, T., Kalk, K. H., van Zanten, B. A., Dauter, Z., Kingma, J., Witholt, B., and Hol, W. G. (1993) J. Mol. Biol. 230, 890–918
13. Sixma, T., Pronk, S. E., Kalk, K. H., Wartna, E. S., van Zanten, A. M., Witholt, B., and Hol, W. G. J. (1991) Nature 351, 371–377
14. Hol, W. G. J., Sixma, T. K., and Merritt, E. A. (1995) in Bacterial Toxins and Virulence Factors in Disease: Handbook of Natural Toxins (Moss, J., Vaughan, M., Iglewski, B., and Tu, A.T., eds) pp. 185–223, Marcel Dekker Inc., New York
15. Kuziemko, G. M., Stroh, M., and Stevens, R. C. (1996) Biochemistry 35, 6375–6384
16. Hardy, S. I., Holmgen, J., Johansson, S., Sanchez, J., and Hirst, T. R. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 7109–7113
17. Finkenstein, R. A., Boesman, M., Neoh, S. H., LaRue, M. K., and Delaney, R. (1974) J. Immunol. 113, 145–150
18. Kohler, G., and Milstein, C. (1975) Nature 266, 495–497
19. Amin, T., and Hirst, T. R. (1994) Protein Expression Purif. 5, 198–204
20. Stephens, J. C., Larkins, A., James, R. F., and Rathbone, B. J. (1996) J. Immuno. Methods 190, 163–169
21. Merritt, E. A., Sarfati, S., Iglewski, B., Holmes, R. K., Hirst, T. R., and Hol, W. G. (1997) Protein Sci. 6, 1516–1528
22. Guex, N., and Peitsch, M. C. (1995) Electrophoresis 18, 2714–2723
23. Ruddock, L. W., Coen, J. J. F., Cheesman, C., Freedman, R. B., and Hirst, T. R. (1996) J. Biol. Chem. 271, 19118–19123
24. Cheesman, C., Ruddock, L. W., and Freedman, R. B. (2004) Biochemistry 43, 1609–1617
25. Lesieur, C., Clift, M. J., Carter, R., James, R. F. L., Clarke, A. R., and Hirst, T. R. (2002) J. Biol. Chem. 277, 16697–16704
26. Sandkvist, M., Hirst, T. R., and Bagdasarian, M. (1990) J. Biol. Chem. 265, 15239–15244