Receptor-Based Antidote for Diphtheria

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Although equine diphtheria antitoxin may be an effective therapy for human diphtheria, its use often induces serum sickness. We describe here a strategy for developing an alternative treatment based on the human diphtheria toxin (DT) receptor/heparin-binding epidermal growth factor-like growth factor (HB-EGF) precursor. Recombinant mature human HB-EGF acts as a soluble receptor analog, binding radioidinated DT and preventing its binding to the cellular DT receptor/HB-EGF precursor. However, the possibility existed that radioidinated DT-HB-EGF complexes associate with cells due to the binding of the heparin-binding domain of recombinant HB-EGF to cell surface heparan sulfate proteoglycans. This possibility was confirmed by performing DT binding studies in the presence of heparin. A recombinant truncated HB-EGF (residues 106 to 149), which lacks most of the heparin-binding domain, showed an essentially heparin-independent binding of radioidinated DT to cells. Furthermore, it was a more effective inhibitor of DT binding than was recombinant mature HB-EGF. Since mature HB-EGF is a known ligand for the EGF receptor and is thus highly mitogenic (tumorigenic), we then changed amino acid residues in the EGF-like domain of the recombinant truncated HB-EGF and demonstrated that this DT receptor analog (I117A/L148A) displayed a low mitogenic effect. The truncated (I117A/L148A) HB-EGF protein retained high DT binding affinity, as confirmed by using surface plasmon resonance. Our results suggest that the truncated (I117A/L148A) HB-EGF protein could be an effective, safe antidote for human diphtheria.

Although several years ago it appeared that diphtheria was no longer a major public health threat, the recent resurgence of diphtheria in the New Independent States of the former Soviet Union (8, 9), Ecuador (35), Thailand (7), Algeria, and elsewhere (21) suggests that this old menace remains a significant global health problem. Historically, diphtheria patients have been treated with equine antitoxin, which neutralizes unbound toxin; however, surviving patients often have developed serum sickness, an immune complex-type disease. Thus, a better treatment for diphtheria patients is needed. Diphtheria toxin (DT) is a Corynebacterium diphtheriae exotoxin that inhibits protein synthesis of toxin-sensitive eukaryotic cells. DT is translated as a single polypeptide of 535 amino acid residues (M, 58,342). Partial proteolysis yields a disulfide-linked polypeptide composed of two fragments: the enzymatic A fragment (M, 21,167) and the receptor-binding/translocation-mediating B fragment (M, 37,195) (13, 15, 20, 31, 36).

DT binds to cell surface DT receptors and is internalized by receptor-mediated endocytosis (18). Upon subsequent acidification of the endosome, the A fragment is translocated into the cytosol, where the toxin inhibits protein synthesis by ADP ribosylation of elongation factor 2. Although all elongation factor 2s in mammalian species are sensitive to DT (30), only those cells displaying a specific DT receptor are sensitive to the toxin (32).

One laboratory previously cloned from highly toxin-sensitive monkey Vero cells the cDNA encoding a DT receptor that conferred DT sensitivity to normally toxin-resistant mouse L-M(TK−) cells (34). The predicted amino acid sequence of the DT receptor was found to be identical to that of the cell surface-expressed heparin-binding epidermal growth factor-like growth factor (HB-EGF) precursor (proHB-EGF) (23). HB-EGF is a member of the EGF growth factor family, which includes EGF (14), transforming growth factor α (TGF-α) (17), epiregulin (44), amphiregulin (AR) (40), betacellulin (BTC) (39), and heregulins (25). Although these members were first purified and identified as secreted mature peptides, subsequent sequencing of their cDNAs revealed that all are derived from membrane-bound precursors that are proteolytically cleaved from the cellular membrane. Although proHB-EGF is cleaved and released as soluble mature HB-EGF (22), a significant amount of proHB-EGF remains on the cell surface and functions as a juxtacrine growth factor (24) and as a DT receptor (27, 34). Soluble mature HB-EGF binds to the EGF receptor and is a more potent mitogen for smooth muscle cells than is EGF (23). In addition to the monkey (Mk) proHB-EGF, the proHB-EGF cDNAs of human (Hu) (23), mouse (1), rat (1, 42), and hamster (11) have been cloned. The predicted structure of proHB-EGFs (Fig. 1A) includes a signal sequence (residues 1 to 23), a pro region (residues 24 to 62), the mature growth factor (residues 63 to 148), a juxtamembrane domain (residues 149 to 159), a transmembrane domain (residues 160 to 184), and a carboxyl-terminal cytoplasmic domain (residues 185 to 208). HB-EGF, like all members of the EGF growth factor family, contains an EGF-like domain, which has 6 conserved cysteine residues that form three disulfide bonds (linked C1—C3, C2—C4, and C5—C6 [Fig. 1B]). This EGF-like motif is important for tertiary structure stabilization, EGF receptor binding, and DT binding.

The binding of growth factors to cell surfaces and extracellular matrices is often mediated by proteoglycans. Many growth factors are known to interact with heparin and heparan...
FIG. 1. (A) Comparison of Mk (upper line) (34) and human (Hu) (lower line) (23) HB-EGF precursors. Identical amino acid residues are denoted by dashes, and differing residues are denoted by their single-letter amino acid code. The signal sequence, mature growth factor domain, and the transmembrane domain are indicated by shaded boxes and are based on the designations of Abraham et al. (1). Three stretches of basic amino acids in the HB domain (93KRKKKGK, 103KKR, and 110RKYK) are indicated by the three black boxes. Ile 117 and Leu 148 are indicated by the residue number. The beginning (residue 106) and the end (residue 148) of the EGF-like domain are denoted by asterisks. (B) EGF-like domains sequence comparison of Hu HB-EGF with the other members of the Hu EGF family. EPR, epiregulin. All sequences were obtained from GenBank. The numbers at the top refer to the position of the residues in the Mk HB-EGF precursor, including the signal sequence shown in panel A (34). The shaded boxes indicate residues that are identical to those in Mk HB-EGF. The characteristic six conserved cysteine residues, which form three disulfide bonds (linked C1—C3, C2—C4, and C5—C6), are marked with arrows. Critical residues (I133, H135, and E141) for DT binding are marked with asterisks. The residues noted for Hu EGF correspond to residues 4 to 47 of its mature form, the residues noted for Hu TGF-α correspond to residues 6 to 48 of its mature form, the residues noted for Hu AR correspond to residues 44 to 84 of its mature form (41).
sulfate proteoglycans (HSPGs); these growth factors include HB-EGF, AR, BTC, platelet-derived growth factor, acidic fibroblast growth factor, basic fibroblast growth factor, vascular endothelial growth factor, and granulocyte-macrophage colony-stimulating factor (16). Using a combination of site-directed mutagenesis and synthetic peptide studies, the sequences within HB-EGF that mediate its interaction with heparin were located in the middle of the protein (residues 93 to 113 in the precursor sequence [Fig. 1A]) (3, 43). Three stretches of basic amino acids within this region (KRKKKGK, 108KRR, and 110RKKY [Fig. 1A]) have been shown to play a critical role in HB; interestingly, the third region is within the EGF-like domain of HB-EGF.

The discovery that the DT receptor is a growth factor precursor raises the possibility that a soluble receptor analog might be used therapeutically to block the binding of toxin to the membrane DT receptor. A laboratory earlier reported that recombinant mature Hu HB-EGF (residues 63 to 148), which is identical to the mature Mk HB-EGF (except that Asn87 of human is replaced with Ser77 in the monkey [Fig. 1A]), strongly inhibits the binding of radiolabeled DT to toxin receptor-bearing cells (26). This result suggested that it would be theoretically possible to treat diphtheria patients with mature HB-EGF, which is a natural growth factor. This approach would have significant advantages over the presently used equine antitoxin, which often causes serum sickness, an immune complex-type disease. However, mature HB-EGF might produce side effects due to its growth factor activity.

We present here a rationale for the design of such a modern antidote for diphtherial disease. The newly designed recombinant protein is a more effective inhibitor of DT binding and is significantly less mitogenic (tumorigenic) than recombinant mature HB-EGF.

MATERIALS AND METHODS

Materials. The plasmid vector pcDNA3 and the Escherichia coli host strains TOP10F and DH5α were purchased from Invitrogen. The protein expression vector pET21a, the protein expression E. coli host strain BL21(DE3), and the T7 tag purification kit were obtained from Novagen. The recombinant mature Hu HB-EGF and AR were from R&D Systems. Waymouth’s medium powder was from Life Technologies, Inc. Insulin-transferrin-selenium-X culture supplement medium powder was obtained from Novagen. The recombinant mature Hu HB-EGF (residues 63 to 149 for those proteins with a deletion of the HB domain) were amplified by PCR with pMkHB-EGF, pMkHB-EGF/I117A/L148A, or pMkHB-EGF/Y138A/L148A templates and were cloned into pET21a using the BamHI and HindIII sites, resulting in pET21a/WT, pET21a/HBD, pET21a/HBD/I117A/L148A, and pET21a/HBD/Y138A/L148A plasmids. The mutated sequences of HB-EGF were confirmed by automated sequencing as described above.

Transformation. Electroporation was used to transform plasmid DNA into LDC9 cells using the BTX ECM 600 System as described previously (10) with the following modifications. The LDC9 cell line is DT resistant and hygromycin resistant; the construction and biochemical characterization of this cell line are described in detail elsewhere (5). LDC9 cells were transfected with the wild-type DT receptor/Mk proHB-EGF plasmid pMkHB-EGF (10) and with the seven DT receptor mutant plasmids (pMkHB-EGF/F115A, pMkHB-EGF/I117A, pMkHB-EGF/Y138A, pMkHB-EGF/I117A/L148A, pMkHB-EGF/F115A/L148A, pMkHB-EGF/I117A/L148A, and pMkHB-EGF/Y138A/L148A), and cloned cell lines stably expressing the wild-type or mutant DT receptor were obtained. The cell lines were screened for the presence of cell surface proHB-EGF by the abbreviated cytotoxicity assay.

Abbreviated cytotoxicity assay. We used the previously described abbreviated cytotoxicity assay (2, 10) to observe the inhibition of protein synthesis by DT. Cells were tested with one concentration of DT (1 μg/ml) and no DT as a negative control. Protein synthesis inhibition was measured by [3H]leucine incorporation into acid-precipitable radioactivity as described previously (19, 38). Each assay included the LDC9/Mk HB-EGF cell line described previously (10) as a positive control and the LDC9 cell line as a negative control. Cell lines in which protein synthesis was inhibited by 50% or more at 1 μg of DT/ml were considered to be DT sensitive. All individual DT-sensitive cell lines were subcloned to obtain pure stable cell populations as described previously (10); the presence of cell surface Mk CD9 was verified by a quantitative whole-cell enzyme-linked immunosorbent assay as described previously (10) and was further analyzed by a radiolabeled DT binding assay described below.

Radiolabeled DT binding assay. The ability of wild-type and mutant DT receptor-expressing cell lines to bind radiolabeled DT was examined as previously described (5, 34) with modifications. For this assay, 125I-labeled DT (10 to 400 nM) was added to the cell monolayers with or without a 100-fold-excess unlabeled DT. Cell-associated radioactivity was determined employing a 1 μCi 125I Analytical Gamma Trac 1290 g counting system. Specific binding was determined as the difference between the total cell-associated radioactivity without excess unlabeled DT and the cell-associated radioactivity obtained when a 100-fold excess of unlabeled toxin was included. An acceptable level of nonspecific binding was 20% or less. Specific binding data were subjected to Scatchard analyses. All radiolabeled DT binding assays were performed in duplicate, and at least two assays were performed for each cell line. Each assay included the same positive and negative control cell lines described above for the abbreviated cytotoxicity assay.

Expression and purification of recombinant HB-EGF proteins. The recombinant HB-EGF proteins [mature Mk HB-EGF, HBΔ-EGF, HBΔ-EGF/I117A/L148A, and HBΔ-EGF/Y138A/L148A] were expressed from pET21a/WT, pET21a/HBD, pET21a/HBD/I117A/L148A, and pET21a/HBD/Y138A/L148A, respectively, in E. coli BL21(DE3). All recombinant HB-EGF proteins contained a T7 tag at their amino terminus to facilitate the purification step. To prevent the expression of the histidine tag at the carboxyl terminus of the proteins, which might interfere with DT binding, a termination codon was introduced into the plasmids before the histidine tagging domain. The overexpressed recombinant HB-EGF proteins formed inclusion bodies, which were isolated and dissolved in 8 M urea. The recombinant HB-EGF proteins were refolded by sequential dialytic removal of urea in 25 mM Tris buffer with 4, 2, 1, and 0 M urea steps, in the presence of reduced/oxidized glutathione (2/0.2 mM) (29). The refolded recombinant HB-EGF proteins were concentrated and purified by T7 affinity column as described in the manufacturer’s instructions.

Assay for 125I-DT binding inhibition. A LDC9/Mk HB-EGF cell line (n = 15,000) was used for this assay. LDC9/Mk HB-EGF cells are mouse LDC9 cells that were obtained by transfection with pMkHB-EGF and stably express cell surface DT receptor/Mk proHB-EGF (12). Forty-eight-well plates were seeded at 4 x 103 LDC9/HB-EGF cells per well. After 3 days of incubation at 37°C in 5% CO2, the monolayers were washed twice with ice-cold phosphate-buffered saline (PBS) (8.8 mM Na2HPO4, 1.2 mM KH2PO4, 140 mM NaCl, 10 mM KCl, 0.5 mM MgCl2, and 1.0 mM CaCl2 [pH 7.4]), and then ice-cold binding medium (medium 199) (50 μg/ml) bovine serum albumin, 100 μg/ml gelatin, and 20 mM HEPES, pH 7.4) was added to the monolayers and preincubated for 30 min at 4°C. The preincubation medium

The pET21a plasmid was used for protein expression in E. coli. The coding region residues 63 to 149 (residues 63 to 149 for those proteins with a deletion of the HB domain) were amplified by PCR with pMkHB-EGF, pMkHB-EGF/I117A/L148A, or pMkHB-EGF/Y138A/L148A templates and were cloned into pET21a using the BamHI and HindIII sites, resulting in pET21a/WT, pET21a/HBD, pET21a/HBD/I117A/L148A, and pET21a/HBD/Y138A/L148A plasmids. The mutated sequences of HB-EGF were confirmed by automated sequencing as described above.
was removed, and the monolayers were again washed twice with ice-cold PBS. The monolayers were incubated with the recombinant HB-EGF proteins in the absence or in the presence of heparin (10 U/ml) for 5 h at 4°C. Binding medium (in the absence or in the presence of heparin) containing 

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\text{DT was allowed to pass over immobilized AR as a negative control (data not shown). The commercially available mature Hu HB-EGF (as a positive control), the commercially available mature Hu HB-EGF which lacks the amino terminus, and the recombinant HB-EGF proteins in the absence or the presence of heparin, was a more effective inhibitor of DT binding than was recombinant mature Mk HB-EGF proteins in the absence or the presence of heparin.}
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\text{RESULTS AND DISCUSSION}
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Recombinant mature Hu HB-EGF (residues 63 to 148 [Fig. 1A]) binds radiolabeled DT and therefore inhibits the binding of radiolabeled DT to toxin receptor-bearing cells (26), suggesting that mature HB-EGF could be used as an antidote for diphtherial disease.

Inhibition of DT binding by mature HB-EGF in the absence or presence of heparin. Preliminary experiments showed that wild-type mature Mk HB-EGF and the commercially available mature Hu HB-EGF inhibited the binding of 

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\text{125I-DT to wild-type receptor-bearing cells (LCD9/Mk HB-EGF); a detailed analysis of the inhibition is shown in Fig. 2. Both proteins showed the same pattern of DT binding inhibition, suggesting that our purified recombinant mature Hu HB-EGF (residues 106 to 149, HB1-ΔEGF) which lacks the amino terminus, including most of the HB domain to cell surface HSPGs. In the presence of heparin, there was a dramatic decrease in the heparin-dependent binding of radioiodinated DT to cells (Fig. 2). Therefore, to eliminate the HB effect, we prepared a recombinant truncated HB-EGF protein (residues 106 to 149, HBD-ΔEGF) which lacks the amino terminus, including most of the HB domain (i.e., lacks KKKKKGK and but not 110KKYK [Fig. 1A]).}
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Inhibition of DT binding by truncated HBD-ΔEGF. Experiments on the inhibition of radiolabeled DT binding were then performed with the purified recombinant HBD-ΔEGF and mature Mk HB-EGF proteins in the absence or the presence of heparin (Fig. 3). The recombinant HBD-ΔEGF protein at all concentrations, in the absence of heparin, was a more effective inhibitor of DT binding than was recombinant mature Mk HB-EGF, and addition of heparin showed only a small effect on toxin binding. Thus, HBD-ΔEGF appears to be a better candidate for neutralizing DT than wild-type mature HB-EGF.

DT binding ability of cells expressing mutant DT receptors. Since mature HB-EGF is a known ligand of the EGF receptor and is thus highly mitogenic, the mitogenic (tumorigenic) effect would need to be reduced to make the mature HB-EGF safe for in vivo use. Accordingly, we first employed site-directed
as well as Arg142, are in close contact with the toxin (29). Based on this rationale, we used site-directed mutagenesis to convert mutations change the receptor DT binding ability. Table 1 summaries the data for toxin receptor affinity and the number of DT binding sites per cell for wild-type and seven different mutant DT receptors. The DT receptor affinity was decreased ~3-fold for the LCD9/HB-EGF(F115A) cell line ($K_d = 20.0$ nM) and ~2-fold for the LCD9/HB-EGF(F115A/L148A) cell line ($K_d = 15.9$ nM), compared to the cell line expressing the wild-type DT receptor ($K_d = 7.0$ nM). This result suggests that Phe115 may also be involved in DT binding.

The DT binding affinity was increased ~2-fold for both the LCD9/HB-EGF(I117A) cell line ($K_d = 4.0$ nM) and the LCD9/HB-EGF(I117A/I148A) cell line ($K_d = 3.0$ nM). The DT binding affinity was also increased ~2-fold for the LCD9/HB-EGF(L148A) cell line ($K_d = 3.6$ nM). It is interesting that these mutant DT receptors display a higher affinity for DT than does the wild-type DT receptor. This increased affinity is probably a reflection of the decreased level of expression of cell surface DT receptor/Mk proHB-EGF (Table 1) at a constant level of expression of CD9, which we previously showed resulted in a higher Mk CD9/Mk proHB-EGF ratio and in an increased receptor affinity (12). The DT receptor affinities of the LCD9/HB-EGF(Y138A) cell line ($K_d = 6.6$ nM) and of the LCD9/HB-EGF(Y138A/L148A) cell line ($K_d = 6.8$ nM) are similar to that of the wild-type DT receptor. In summary, these results suggest that residues Ile117, Tyr138, and Leu148 are not involved in DT binding but that residue Phe115 is. Therefore, residues Ile117, Tyr138, and Leu148 are good candidates to mutate in order to attempt to reduce the tumorigenic effect without affecting the toxin binding ability. Furthermore, the double mutants I117A/L148A and Y138A/L148A, which have a mutation in the most critical residue (Leu148), might be even better candidates. Thus, the double mutants I117A/L148A and Y138A/L148A were created in the truncated HBΔ-EGF background, resulting in HBΔ-EGF(I117A/L148A) and HBΔ-EGF(Y138A/L148A) proteins. These proteins were then tested for their mitogenic activity and their ability to inhibit binding of radiolabeled DT to cells.

**HBΔ-EGF(I117A/L148A) displays a lower mitogenic activity.** The mitogenic activity of the commercial Hu HB-EGF, the purified Mk HB-EGF, HBΔ-EGF, HBA-EGF(I117A/L148A), and HBΔ-EGF(Y138A/L148A) proteins was examined using serum-starved BALB/c3T3 cells (Fig. 4). As expected, the recombinant mature Hu HB-EGF and the purified recombinant Mk

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**TABLE 1. Summary of DT binding affinity and the number of DT binding sites per cell**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>$K_d$ (nM)</th>
<th>$n$ ($10^3$)</th>
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<tbody>
<tr>
<td>LCD9/Mk HB-EGF</td>
<td>7.0</td>
<td>8.7</td>
</tr>
<tr>
<td>LCD9/F115A</td>
<td>20.0</td>
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</tr>
<tr>
<td>LCD9/I117A</td>
<td>4.0</td>
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</tr>
<tr>
<td>LCD9/Y138A</td>
<td>6.6</td>
<td>6.5</td>
</tr>
<tr>
<td>LCD9/L148A</td>
<td>3.6</td>
<td>2.4</td>
</tr>
<tr>
<td>LCD9/F115A/L148A</td>
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</tr>
<tr>
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<td>3</td>
<td>4.6</td>
</tr>
<tr>
<td>LCD9/Y138A/L148A</td>
<td>6.8</td>
<td>11.8</td>
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</table>

* These cell lines are LCD9 cells expressing wild-type or mutant Mk proHB-EGF/DT receptors.

1 All binding assays were done in duplicate and were performed at least twice for each cell line with the average calculated $K_d$ and number of binding sites per cell ($n$) being reported.
HB-EGF showed similar, high levels of mitogenicity. Interestingly, HBΔ-EGF showed a similar mitogenic effect to that of the wild-type proteins, a result consistent with the fact that the EGF-like domain is the one that interacts with the EGF receptor.

Surprisingly, the mitogenic effect of HBΔ-EGF(Y138A/L148A) in HBΔ-EGF showed a similar mitogenic effect to that of the commercial Hu HB-EGF, Mk HB-EGF, and HBΔ-EGF, although the two mutations in this truncated protein are in highly conserved residues in the EGF family which have been shown, in EGF and TGF-α, to be involved in binding to the EGF receptor (Fig. 1B).

More important was the mitogenic effect observed with HBΔ-EGF(Y138A/L148A); it was decreased at least 100-fold when the 50% effective dose (ED50) of HBΔ-EGF(1117A/L148A) (ED50 ≈ 10 nM) was compared with that of mature Hu HB-EGF, Mk HB-EGF, and HBΔ-EGF (Y138A/L148A) (ED50 ≈ 0.06 to 0.1 nM). The extremely low mitogenic activity suggested that HBΔ-EGF(1117A/L148A) would be the best candidate for an antidote against diphtherial disease.

HBΔ-EGF(1117A/L148A) inhibits binding of DT as well as HBΔ-EGF. The experiment for inhibition of radiolabeled DT binding was performed with HBΔ-EGF(1117A/L148A) and with HBΔ-EGF proteins; they both showed a similar pattern of inhibition of DT binding (Fig. 5).

High DT binding affinity of HBΔ-EGF(1117A/L148A). We examined the binding of the recombinant HB-EGF, HBΔ-EGF, and HBΔ-EGF(1117A/L148A) to DT, employing surface plasmon resonance. Table 2 summarizes the data for the association rate constant (Ka), the dissociation rate constant (Kd), and the apparent dissociation constant (KdA) of the recombinant HB-EGF proteins towards DT. Our purified recombinant wild-type Mk HB-EGF (KdA = 3.4 × 10^-8 M) showed an apparent KdA very similar to that of the commercial recombinant Hu HB-EGF (KdA = 3.8 × 10^-8 M); these apparent KdA values obtained here are in agreement with those previously reported (4). Interestingly, the apparent KdA for HBΔ-EGF (KdA = 5.2 × 10^-8 M) was similar to that of the recombinant Hu HB-EGF and of the purified recombinant Mk HB-EGF, in agreement with the DT binding inhibition results described above. High-affinity binding of HBΔ-EGF(1117A/L148A) for DT was also observed (KdA = 10 × 10^-8 M); however, it was 2-fold lower than that obtained with HBΔ-EGF.

In this study we demonstrated that the truncated mutant protein HBΔ-EGF(1117A/L148A) resulted in a DT receptor analog that both strongly inhibited binding of radioiodinated DT to cells and displayed a 100-fold-lower mitogenic effect. Therefore, we conclude that the HBΔ-EGF(1117A/L148A) protein may be a suitable candidate for a diphtheria antidote. This protein will now be tested in vivo employing a toxin-sensitive animal model. We propose to start by testing that concentration of HBΔ-EGF(1117A/L148A) that inhibits binding of radioiodinated DT by 50% (Fig. 5), a concent-

<table>
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<th>Reombinant HB-EGF protein used</th>
<th>Avg Ka (10^4) (1/Ms)</th>
<th>Avg Kd (10^-8) (1/S)</th>
<th>Avg KdA (10^-8) (M)</th>
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<td>7.1</td>
<td>10</td>
</tr>
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</table>

*The BIAcore 2000 system with CM5 sensor chips was employed. The recombinant HB-EGF proteins were immobilized on CM5 sensor chips by standard amine coupling, and the kinetic experiments were performed as described in Materials and Methods. This table shows values for a representative experiment of two separate experiments.
tation which is expected to result in about a 50%-lower mitogenic effect than that obtained with the truncated and mature HB-EGF molecules (Fig. 4).

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