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A Superactive Hormonotoxin Prepared with Truncated Diphtheria Toxin

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SUMMARY A chemically truncated form of diphtheria toxin, DT51, which lacks the cell-binding site but retains the membrane-translocating function, was covalently linked to luteinizing hormone (LH) and compared to similar conjugates containing diphtheria toxin (DT) or diphtheria toxin A-chain (DTA). The DT51 hormonotoxin killed cells possessing an LH receptor at concentrations similar to that of DT hormonotoxin and orders of magnitude lower than DTA hormonotoxin. The DTA hormonotoxin exhibited an LD-50 similar to that of previously reported hormonotoxins which employed DTA, ricin A-chain, or gelonin as toxic moieties.

Over the past decade the possibility of preparing a new class of antibiotics, which selects targeted cells on the basis of peculiar cell surface molecules, has been increasingly investigated [1]. These "magic bullets" are composed of a cell-binding moiety, which confers selectivity, covalently linked to a catalytic toxin. When the cell-binding moiety is a monoclonal antibody, this type of selective cytotoxic agent has been commonly called an immunotoxin. Similarly, when the cell selective moiety is a hormone they have been called hormonotoxins.

Some catalytic toxins, for example ricin, abrin, and diphtheria toxin, consist of two peptide chains [2]. One of the chains, designated the A-chain, is an enzyme that damages some essential function of the cell when it gains access to the cytoplasm. The other peptide chain, designated the B chain, possesses two functional domains which are involved in binding the toxin to the cell and facilitating the transport of the A-chain through cell membranes. The toxin binding site is usually specific for a ubiquitous cell surface chemical grouping, and therefore interferes with the target selectivity of immunotoxins and hormonotoxins. Consequently, most investigations have used A-chains, or a category of catalytic toxins which do not possess B-chains (e.g. pokeweed antiviral protein and gelonin), as the toxic moiety in immunotoxins or hormonotoxins.

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Abbreviations used in this report are: DT=diphtheria toxin, DTA=diphtheria toxin A-chain, DT51=truncated diphtheria toxin of approximately 51,000 molecular weight, LH=Bovine luteinizing hormone. MLTC-1=Mouse Leydig Tumor Cells, line 1. LD-50=Lethal Dose required to kill 50% of susceptible cells.

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A-chain immunotoxins are typically several orders of magnitude less potent than immunotoxins prepared with A-B chain toxins because they lack B-chain facilitation of A-chain entry into the cytoplasm [3]. Further, A-chain immunotoxins act considerably slower than A-B immunotoxins for the same reason. These, and perhaps other factors have made A-chain immunotoxins relatively ineffective in whole animal applications such as regressing a tumor completely. Other modifications designed to extend the circulation time of ricin A-chain immunotoxins, e.g. deglycosylation of ricin A-chain [4] and altering the method of covalent linkage of toxin and antibody [5], will undoubtedly improve *in vivo* effectiveness, perhaps to the degree that some particular *in vivo* applications will prove practical.

Alternatives exist to circumvent the lower toxicity and slower action exhibited by the A-chain toxin conjugates. Rather than discarding the membrane permeation domain along with the toxin binding site (B-chains), it is theoretically possible to delete only the binding site of catalytic toxins. Although more difficult to achieve in practice, the original potential toxicity would be retained while the selectivity of immunotoxins and hormonotoxins would be fully as great as those prepared with A-chains. Murphy and coworkers [6], and Pastan and coworkers [7] demonstrated one method of achieving this goal using genetic manipulation, while Youle and coworkers [8] employed a mutant diphtheria toxin. Recently, we used selective proteolysis to prepare truncated diphtheria toxin, which lacks the toxin binding site but retains most, if not all, of the membrane permeation function [9]. This report demonstrates that a hormonotoxin prepared with one of these truncated diphtheria toxins is orders of magnitude more potent than the A-chain hormonotoxins that have been reported previously [10-11].

MATERIALS AND METHODS

Diphtheria toxin was obtained from Connaught Laboratories, Toronto, and purified by ion exchange chromatography [9]. Bovine luteinizing hormone, NIH-LH-B8, was obtained from the National Hormone and Pituitary Program, through NIH, Bethesda, Md. Mouse Leydig tumor cells, MLTC 1, were a generous gift from Dr. Victor Rebois, NIH, Bethesda, Md. Diphtheria toxin A-chain and the truncated diphtheria toxin DT51 [9] were prepared as described previously. All other materials were obtained from Sigma, St. Louis, Mo.

LH was linked to the various toxins as described previously [9], using N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP). Protein concentrations were 1 mg/ml. The molar ratio of SPDP to protein used in the reactions was 4 for LH, 3 for DT and DT51. The ratio of pyridyldithio propionate moieties per mole of protein introduced was 0.89 for LH, 0.82 for DT, and 2.5 for DT51. The pyridyldithio propionate-derivitized LH was reduced with 10mM dithiothreitol for 10 min. at room temperature, and the small molecules removed by Sephadex G-25 chromatography. The reduced, derivitized, LH was then reacted with derivitized DT and derivitized DT51 in a 1:1 molar ratio, for 6 h at 23° C followed by 12 h at 4° C. DTA was reacted directly with derivitized LH. The reactions were terminated by dialysis against phosphate buffered saline overnight at 4° C. The mean ratio of LH conjugated to each toxin was 0.78 for DT, 2.1 for DT51, and 0.97 for DTA.

On the day prior to the determination of cytotoxicity, cells were placed in the wells of 96-well microtiter plates at 5000 cells per well in 200 µl media. To assay cytotoxicity, various quantities of conjugates, LH, DT, DT51, and DTA, were added to the washed cells in 200 µl media in replicates of 8. After 24 hrs incubation, the cell number was determined using the acid phosphatase assay [12].

RESULTS AND DISCUSSION

The MLTC-1 cell line, like other murine cell lines, is relatively insensitive to the effects of DT [9]. MLTC-1 cells, also like other murine cells, are fully susceptible to the

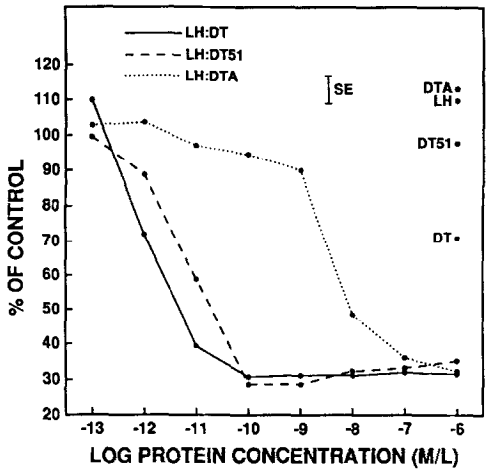

Figure 1. Cytotoxicity of LH conjugates with MLTC-1. Each point represents an average of 8 observations, and pooled standard error, SE, is given. For unlinked LH or toxin, cytotoxicity at the highest concentration is given with a single point.

effects of DTA if a means of gaining cell entry is provided. These circumstances allow a direct comparison of DT conjugates with conjugates of DTA and DT51, using the LH receptor as the means of effecting cell entry. LH, DTA, and DT51 showed no toxicity for MLTC-1 cells at concentrations up to 1 μM. DT was slightly toxic at 1 μM, killing about one-quarter of the cells, but was not toxic in the concentration range over which the LH-DT conjugate exhibited cell killing, 1 pM to 0.1 nM. Consequently, the toxicity exhibited by the LH conjugates was a result of the toxic moieties entering the cell via binding to the LH receptor.

As could be predicted from previous reports, the LH-DT conjugate exhibited an LD50 orders of magnitude less than a similar conjugate prepared with DTA, 2 pM and 5 nM respectively (Fig 1). The much greater toxicity of the LH-DT conjugate is undoubtedly the result of increased DTA membrane permeation that results from the appropriate domain of the DT B-chain being present in the LH-DT conjugate and absent in the LH-DTA conjugate. The LD50 of the LH-DT51 conjugate, 7 pM, is in the same range as that of the LH-DT conjugate. This indicates that the DT B-chain domain responsible for greater transport of the A-chain across intracellular membranes is also present in this truncated DT that has had approximately 7000 daltons of the carboxy terminus removed. That the LH-DT conjugate is a bit more toxic than the LH-DT51 conjugate is probably due to the fact that the DT51 used to make the conjugate was more derivitized than DT and has more LH molecules per toxin than does the DT conjugate. Previous observations comparing DT51 and DT conjugates with Concanavalin A as cell binding moiety indicated the same degree of toxicity [9].

Approximately one-third of the MLTC-1 cells were not susceptible to the LH conjugates, a fact that is most probably due to a loss of the LH receptor in that proportion of cells as a result of continuous culture of this particular cell line. Evidence suggesting this interpretation is provided by previous results with this MLTC-1 line using Concanavalin A-DT conjugates [9] which killed all the cells. Also, for another mouse Leydig tumor cell line, M5480P, altered expression of the LH receptor in cultured cells compared to Leydig cells *in vivo* has been reported [13]. The lack of susceptibility of a proportion of the MLTC-1 cells provides an internally controlled demonstration of the target specificity that can be achieved with hormonotoxins. It also

suggests that hormonotoxins might be useful in studying the heterogeneity of seemingly homogeneous populations of cells.

The LD50 of the LH-DTA conjugate reported here, 5 nM, corresponds well with those for hormonotoxins reported previously. LH-gelonin conjugates have been reported to have an LD50 of 2.2 nM [11] with another mouse Leydig tumor cell line, MA-10. Also, human chorionic gonadotropin (hCG) conjugates [10] of gelonin, ricin A-chain, and DTA have been reported to have LD50 values of 10 nM, 1 nM, and 50 nM, respectively, using still another mouse Leydig tumor cell line, M5480P. Consequently, the picomolar LD50 exhibited by the LH-DT51 conjugate represents orders of magnitude increase in cytotoxicity over A-chain hormonotoxins. Since DT51 does not possess the DT cell-binding site, the increased cytotoxicity of DT51 conjugates should not be accompanied by a decrease in target selectivity, as compared with A-chain conjugates. Evidence for this prediction has been demonstrated previously [9], using a human breast tumor cell line, MCF-7, and is supported by these results in that the MLTC-1 cells remain untouched by DT51 at a concentration at which DT kills some of the cells. In conclusion, then, it appears that more effective hormonotoxins can be constructed with DT51 than could be synthesized with A-chains.

REFERENCES

1. Houston, L.L. (1988) in "Immunotoxins", A. F. Frankel, ed., Kluwer Acad. Pub., Norwell, MA, pp 1-7.
2. Olsnes, S. and Pihl, A. (1982) in "Molecular Action of Toxins and Viruses", P. Cohen and S. van Heyningen, eds., Elsevier, Amsterdam, pp. 51-105.
3. Olsnes, S. and Sanvig, K. (1985) in "Endocytosis", I. Pastan and M. Willingham, eds., Plenum, New York, pp, 195-234.
4. Thorpe, P.E. et al (1985) Eur. J. Biochem., 147, 197-206.
5. Thorpe, P.E. et al (1987) Cancer Res., 47, 5924-5931.
6. Murphy, J.R. et al (1986) Proc. Natl. Acad. Sci., USA, 83, 8258-8262.
7. Chaudhary, V.K., FitzGerald, D.J., Adhya, S., and Pastan, I. (1987) Proc. Natl. Acad. Sci., USA, 84, 4538-4542.
8. Greenfield, L., Johnson, V.V., and Youle, R.J. (1987) Science, 233, 536-539.
9. Myers, D.A. and Villemez, C.L. (1988) J. Biol. Chem., 263, 17122-17127.
10. Oeltmann, T.N. (1985) Biochem. Biophys. Res. Com., 133, 430-435.
11. Singh, V., Sairam, M.R., Bhargavi, G.N., and Akhras, R.G. (1989) J. Biol. Chem., 264, 3089-3095.
12. Connolly, D.T., Knight, M.B., Harakas, H.K., Wittwer, A.J., and Feder, J. (1986) Anal. Biochem., 152, 136-140.
13. Ascoli, M. and Puett, D. (1978) Proc. Natl. Acad. Sci., USA, 75, 99-102.