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Research Note

PROTECTIVE MONOCLONAL ANTIBODIES TO DIPHTHERA TOXIN

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Diphtheria toxin (DT) is secreted by strains of *Corynebacterium diphtheriae* as single 58,350 Da polypeptide chain composed by two functionally distinct regions: fragments A (21,150 Da) and B (37,200 Da). Both fragments are linked by a disulphide bridge and they can be dissociated by the action of reduction agents (M. D. Gill & L. L. Dinus, 1971, *J. Biol. Chem., 246:* 1485-1491). Fragment A (FA) is a potent enzyme which catalyses the transfer of the ADP-ribose moiety from NAD^+ to elongation factor 2 and, as a result, this factor is inativated (D. W. Chung & R. J. Collier, 1977, *Biochem. Biophys. Acta, 483:* 248-257). Fragment B (FB) has no enzymatic activity but is necessary for the binding to cellular receptors so that FA reaches the cytoplasm of the target cell to inhibit the protein synthesis (P. Boquet & A. M. Pappenheimer, Jr, 1976, *J. Biol. Chem., 251:* 5770-5778). Monoclonal antibodies (MAbs) against DT have been useful in studies on the molecular structure and function of the toxin (D. R. Zucker & J. R. Murphy, 1984, *Mol. Immunol., 21:* 785-793; D. R. Zucker et al., 1984, *Mol. Immunol., 21:* 795-800; T. Yoshimori & T. Uchida, 1986, p. 229-246 In A. J. L. Macario & E. C. Macario, *Monoclonal antibodies against bacteria,* vol. III, Academic Press, Inc., Orlando, Florida, USA) and also for the development of rapid tests for detecting DT in cultures (T. Krech & C. Wittelsburger, 1987, *Zbl. Bakt. Hyg. A., 265:* 124-135; P. B. Nielsen et al., 1987, *J. Clin. Microbiol., 25:* 1280-1284).

The present communication describes the production and analysis of MAbs to DT which were able to neutralize its toxic action *in vivo.*

Corynebacterium diphtheriae Park-Williams 8 (PW8) was grown in C-Y medium under conditions for expression of the tox gene product (D. M. Gill et al., 1972, *Virol., 50:* 664-668); DT was obtained from culture supernatants by precipitation with 40-70% ammonium sulfate and chromatography on DE-52 and Sephadex G-100 (R. R. Holmes & R. B. Perlow, 1975, *Infect. Immun., 12:* 1392-1400). Diphtheria toxoid was obtained after treatment of DT with 0.2% formalin (M. Porro et al., 1980, *J. Infect. Dis., 142:* 716-724). For MAbs production, two lots of BALB/c mice were immunized according to slightly different protocols as previously described (M. G. M. Danelli et al., 1990, *Rev. Microbiol., São Paulo, 21:* 41-48). In the first protocol, mice were immunized with 100 μg of diphtheria toxoid, administered intraperitoneally, on days 1, 14 and 28. The toxoid was injected after being emulsified with complete Freund adjuvant (1:1) on day 1, in incomplete Freund adjuvant (1:1) on day 14 and in PBS 0.01 M, pH 7.0 on day 28. The second protocol was identical to the first one, except that 100 μg of toxin were treated with trypsin and 2-mercaptoethanol (R. Drazin et al., 1971, *J. Biol. Chem., 246:* 1504-1510), and administered as the final injection on day 28. Three days after the last injection, spleens were removed and the spleen cells were collected in RPMI. Hybrid cell clones were developed by fusing 2×10^7 spleen cells with 1×10^6 SP2/0 Ag 14 mouse myeloma cells in presence of 41% polyethylene glycol 4000 (w/v). After fusion, cells were distributed into 96 wells microtiter tissue culture plates in HAT medium (S. Hayakawa et al., 1983, *J. Biol. Chem., 258:* 4311-4317). Cell culture supernatants were screened for their reactivity by an enzyme-linked immunosorbent assay

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(ELISA) using diphtheria toxoid as antigen (M. E. Camargo et al., 1984, *J. Clin. Microbiol.*, *20:* 772-774). Positive hybrid cells were cloned by the limiting dilution method. Fifteen MAbs screened by ELISA were isolated and the clones were inoculated into BALB/c's peritoneal cavity for production of ascitic fluid. Some of those MAbs were purified from fluid by precipitation with 50% ammonium sulfate and ion exchange chromatography on DE-52 (Whatman Ltd., Maidstone Kent, England). Isotypes were determined by immunodifusion with class and subclass specific antisera (Litton Bionetics, Inc., Kensington, USA). Reactivities of the MAbs were also assayed by Western Blot (WB). For that, 100 μg of purified intact and nicked DT were electrophoresed in a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) under reducing conditions or not. The separated components were then transferred to nitrocellulose membranes and analyzed as previously described (V. C. W. Tsang et al., 1983, *Meth. Enzymol.*, *92*(E): 377-391).

Due to their different reactivities when assayed with diphtheria toxoid by ELISA and with DT and FA and FB by WB (Table), the MAbs B6, D8 and G6, obtained from fusions using two different immunization protocols, were selected to be analyzed by toxycity neutralization assay (TNA).

TABLE

Results of immunoenzymatic assays (ELISA and WB) and toxicity neutralization assay (TNA) with three MAbs to diphtheria toxin

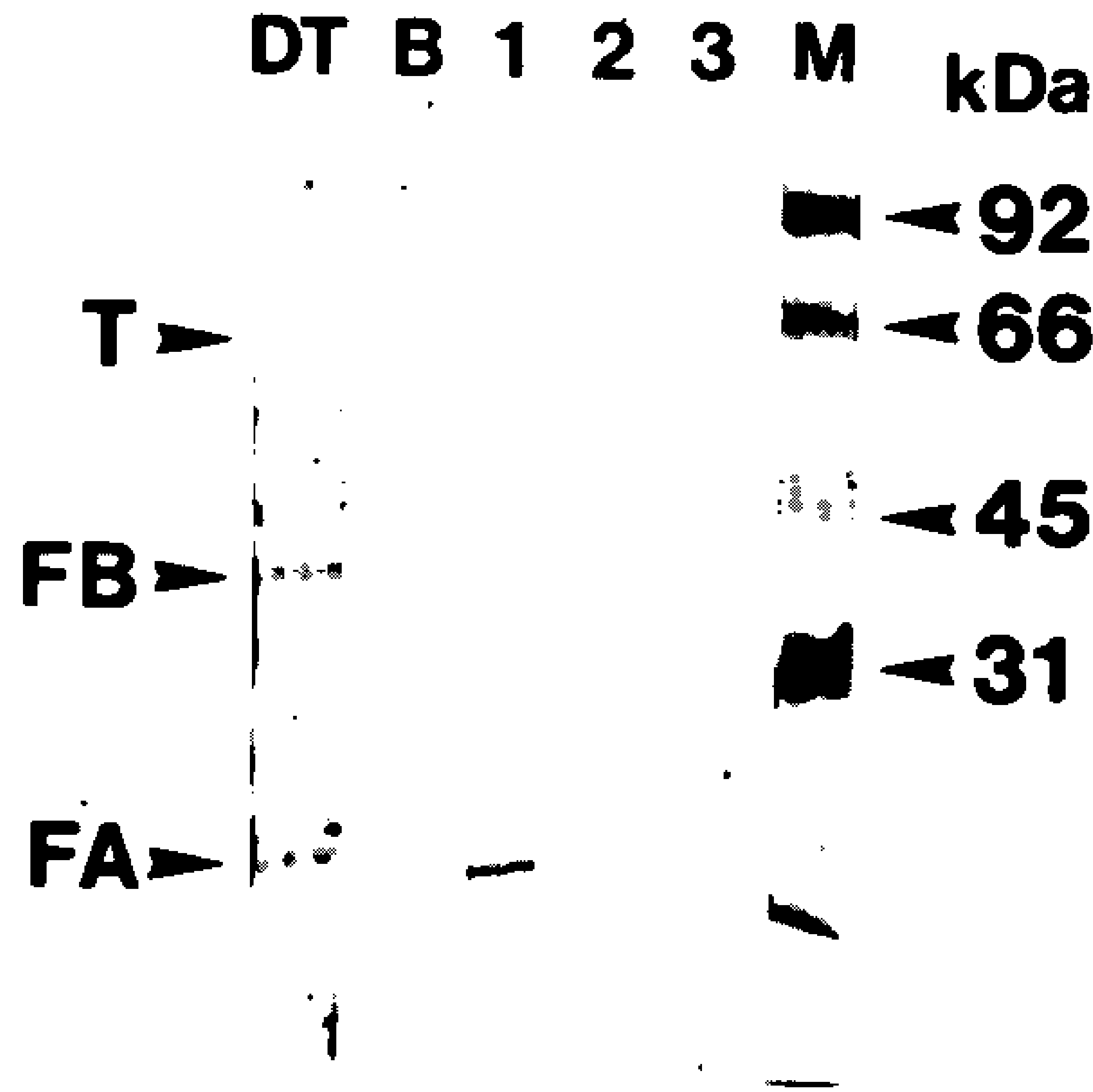
MAbs[a, b]	ELISA[c]	WB			TNA[d]
		Toxin	Fragment A	Fragment B	
B6	+++	++	–	+	+
D8	+	++	+	+	+
G6	+	++	+	–	+
Control[e]	–	–	–	–	–

a: all MAbs were shown to belong to the IgG2b k isotype.
b: the MAb B6 was obtained from mice immunized according to the first protocol and MAbs D8 and G6 were derived from another immunization schedule, as detailed in the text.
c: diphtheria toxoid was used as antigen.
d: positivity in this test was expressed by protection of guinea-pigs from death after challenge with diphtheria toxin.
e: IgG fraction from normal mouse sera was used as a control.

The TNA was performed using two distinct procedures. In the first one, guinea-pigs (300 g) were injected, intraperitoneally, with MAbs (0.4 mg) or diphtheria antitoxin (SAD – 1000 UA). After 24 h, the animals were challenged, subcutaneouslly, with purified DT (250 ng) and observed during 6 days. In the second procedure, DT (250 ng) was mixed *in vitro* with SAD (50 UA/ml) or MAbs B6, D8 and G6 (125 μg/ml) and incubated at 37 °C for 30 min and then at 4 °C overnight. In the following day the animals were inoculated, subcutaneouslly, with these mixtures (0.5 ml) and observed during 6 days.

As shown in the Table, MAbs D8 and G6 had higher reactivity with DT in the WB than with diphtheria toxoid in the ELISA. On the other hand, the MAb B6 was shown to react strongly with diphtheria toxoid than with DT. This result might be related to differences in the structure of the molecules (diphtheria toxoid and DT), due to the formation of metilene bridges during the DT detoxification process (A. M. Pappenheimer Jr et al., 1972, *Immunochem.*, *9:* 891-996). It is also possible that the heating treatment of the DT and FA and FB before application in the SDS-PAGE system could alter the molecule conformation. Conformational differences have already been observed between DT and cross reacting materials (CRMs) (P. Boquet et al., 1976, *Proc. Natl. Acad. Sci. USA*, *73:* 4449-4453; M. Bigio et al., 1987, *FEBS Lett.*, *218:* 271-276; F. Pappini et al., 1987, *FEBS Lett.*, *215:* 73-78). By using a immunoblot technique for the analysis of the CRM 197 structure with MAbs M. Bigio et al. (*loc. cit.*) found that this protein had a different molecular conformation from DT. In this work, the use of FA and FB as antigens in the WB analysis led us to observe an interesting aspect. Although MAbs B6 and G6 have recognized specific epitopes in the FB and FA, respectively, the MAb D8 was shown to react with epitopes present in both fragments (Fig.). This finding could be explained by the existence of repetitive immunodominant epitopes in distinct regions of the DT. F. Triabel et al. (1986, *Eur. J. Immunol.*, *16:* 47-53) have observed that some T lymphocytes clones recognized epitopes located between FA and FB which reinforces the idea explained above.

The ability of the three MAbs analyzed in blocking the action of the DT was demonstrated by the *in vivo* assays, as long as protection of



Immunoblot analysis of MAbs reactive with fragments A (FA) and B (FB) and the intact diphtheria toxin (T). Lane DT: DT treated with tripsin and 2-mercaptoethanol after amido black staining. Lane B: control, without MAbs. Lanes 1, 2 and 3: reactivity with MAbs D_8, G_6 and B_6, respectively. Lane M: molecular weight standards after amido black staining.

guinea-pigs from lethality was obtained. Only the animals inoculated with DT were dead 72 h after the challenge with DT. The three MAbs were able to neutralize the toxin action and the animals survive until the end of the experiment. The binding of DT to target cells is known to be epitope specific (F. Mekada & T. Uchida, 1985, *J. Biol. Chem., 260:* 1248-1253; V. W. Hu & R. K. Holmes, 1987, *Biochem. Biophys. Acta, 902:* 24-30) and neutralization of the DT citotoxicity has been found to correlate with the inhibition of the DT binding to its surface receptors on target cells (D. R. Zucker & J. R. Murphy, *loc. cit.*). Therefore, we believe that the MAbs studied recognize epitopes in the native molecule that are involved either in the DT binding or entry into target cells. Policlonal antibodies against FA were not shown to be able to block the toxin action (A. M. Pappenheimer Jr et al., *loc. cit.*). However, MAb G6, specific to FA, revealed this ability *in vivo*, which further confirms previous observation on the role of FA in this process (E. Mekada & T. Uchida, *loc. cit.*).

The characteristics of the MAbs reported here encourages their use as tools to investigate molecular aspects of the binding and entrance of DT into intact cells. In addition, such MAbs would have practical utility for specific and rapid detection of DT in cultures of clinical specimens, specially in areas where diphtheria is still endemic or epidemic and upper respiratory infections are highly prevalent mainly in children. Experiments are currently being undertaken to evaluate these possibilities.

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