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Conformational changes in diphtheria toxoids

Analysis with monoclonal antibodies

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Monoclonal antibodies (Mab) were raised against CRM197, a non-toxic mutant of diphtheria toxin (DT). The ability of four Mabs to bind DT and the six functional mutants CRM197, CRM176, CRM228, CRM1001, CRM45 and CRM30 was assessed by immunoblotting and by a radioimmunoassay in which the protein antigen in solution competes with labeled CRM197 for the Mab binding site. The results show that the peptides recognized by Mab11.3, Mab53 and Mab23 are accessible in the mutant molecules in solution but not when they are part of the native DT structure, which could therefore be described for this purpose as 'closed' in contrast with an 'open' conformation of CRM197, CRM176 and CRM228. In particular, the behaviour of Mab53 indicates that the single amino acid substitutions in the A fragments of CRM197 and CRM176 also affect the conformation of their B fragments.

Diphtheria toxin; Monoclonal antibody; Mutant structure

1. INTRODUCTION

Diphtheria toxin (DT) is a 58 350 Da protein [1,2] which is synthesized by *C. diphtheriae* when lysogenized by a corynephage *(tox+)* and is released extra-cellularly as a single polypeptide [3,4]. DT can be easily split by limited proteolytic digestion into two functionally independent fragments, A and B (fig.1). The amino-terminal fragment A is an enzyme responsible for the potent cytotoxic action of DT since it catalyses ADP-ribosylation, and consequent inactivation, of elongation factor-2, present in all eukaryotic ribosomes. However, fragment A is toxic only in the presence of the carboxy-terminal fragment B, which is required for the binding to specific receptors on the surface of sensitive eukaryotic cells, consequent uptake into endosomes, and translocation of fragment A into the cytosol [5].

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Although DT has been crystallized in a form suitable for *x*-ray diffraction studies [6,7], direct information on the tertiary folding of this protein has not been forthcoming so far, and models aiming to understand structure-function relationships in DT rely mainly on analysis of functional mutants. The most important group of such mutants was produced by nitrosoguanidine mutagenesis of the *tox* gene of corynephage-β *(tox+)* and infection of a virus-free *C. diphtheriae* strain. Several proteins were obtained which were either non-toxic or had a markedly reduced toxicity and were serologically related to DT [8-10]. Several of these, generally called cross-reacting materials (CRMs), are well characterized [4] and, recently, the alteration in their primary structure was analyzed by sequencing the corresponding genes ([11-14] and our unpublished data); see fig.1. In this work we used monoclonal antibodies raised against CRM197, as structural probes for the tertiary structure by testing their interaction with DT and six functional mutants.

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2. MATERIALS AND METHODS

2.1. *Cell culture media*

10% or 20% DMEM: Dulbecco's modified Eagle's medium plus 10% or 20% fetal calf serum (Flow Laboratories). All sera were inactivated (60°C, 30 min) before use.

HAT medium: 20% DMEM supplemented with hypoxantine (13.6 μg/ml), thymidine (3.87 μg/ml) and aminopterin (0.176 μg/ml).

HT medium: HAT medium without aminopterin.

2.2. *Dipheria toxin and related mutants*

Diphtheria toxin and related mutants (CRMs) were produced and purified as described [15–17]. The concentration of DT and CRMs were determined by the method of Bradford [18], and by rocket immunoelectrophoresis, as described [19].

2.3. *Production of monoclonal antibodies anti-CRM 197*

4-week-old Balb/c mice were immunized intraperitoneally with a total of 130 μg of purified CRM197 (4 injections at one week intervals). Hybridoma cell lines were prepared according to Galfrè and Milstein [20].

2.4. *Purification of antibodies*

All the Mabs chosen belonged to the IgG1 subclass, and were purified by affinity chromatography on Protein A Sepharose CL-4B (Pharmacia) [21].

2.5. *Immunoblot analysis*

Electrophoresis was performed using partially nicked and reduced DT or CRM samples on SDS-polyacrylamide gels according to Laemmli [22]. Western blot analysis of purified monoclonal antibodies was performed as described by Towbin et al. [23] and Robb et al. [24].

2.6. *Radioimmunoassays*

The affinity constants of the Mabs were determined by non-linear regression analysis after competitive solid-phase radioimmunoassay [25] with CRM197 labeled with ^{125}I using NaI and lactoperoxidase as described by Marchalonis [26]; the specific activity was 60 μCi/μg. The test was performed by adsorbing the antibody on the wells of microtiter plates and measuring the amount of ^{125}I-labeled CRM197 which was bound to the solid phase in the presence of increasing amounts of the antigen, i.e. DT or CRM, under examination. The epitope specificity of the Mabs was determined by a competitive solid-phase antibody binding assay, according to Ivanyi [27] and Stähli et al. [28].

2.7. *Cytotoxicity assays*

Cytotoxicity neutralization tests were performed as described [29,30]. The antibody solutions were adjusted to 100 μg/ml and 10-fold serial dilutions were made in 10% DMEM. These samples were pre-incubated with DT at different concentrations, for 1 h at 37°C, and transferred onto the CHO cell cultures. After 3 days, live cells were stained with gentian violet and the amount of dye estimated with the help of a Titertek plate reader (Flow Laboratories) at 560 nm.

3. RESULTS

14 monoclonal antibodies against CRM197 were obtained and divided into 4 groups on the basis of

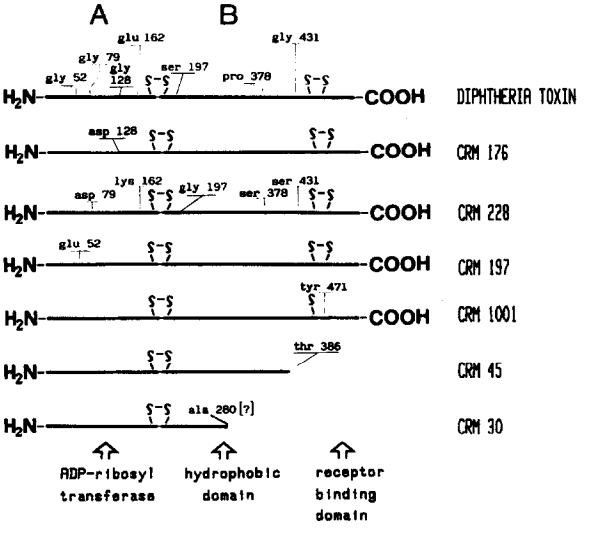

Fig.1. Schematic representation of diphtheria toxin and related functional mutants (CRMs). Fragment A (21 kDa) and fragment B (37 kDa) and the amino acid residues involved in the mutations are indicated. The data are deduced from [1 and 2] for DT, [10] for CRM228, [11] for CRM197, CRM45 and CRM30, [12] for CRM176, and our unpublished DNA sequencing data for CRM176 and CRM1001.

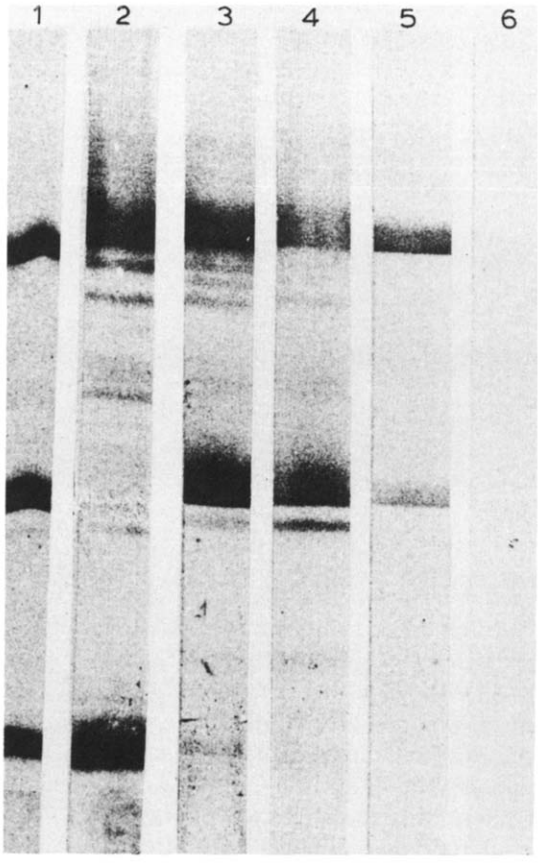

Fig.2. Antibody binding to diphtheria toxin on Western blots. A purified DT preparation in which ~50% molecules were split into fragments A and B, was used to obtain a blot which was cut into stripes and probed with different antibodies. Lane 1: blot of DT stained with amido-black; the major bands are from top to bottom: intact DT, fragment B, fragment A. Lanes 2 to 6: binding to different antibodies; Mab11.3, Mab1, Mab53, Mab23 and a control with a monoclonal IgG1 to the human chorionic somatotropin, respectively.

a competitive solid-phase binding assay [23,25] where the ability of two antibodies to compete for the same epitope was assessed. One representative antibody for each group was selected (Mab1, Mab11.3, Mab53, Mab23) and used for further studies. Immunoblot analysis on electrophoretically separated fragments A and B (fig.2) showed that Mab11.3 recognizes an epitope on fragment A, while Mabs 1, 53 and 23 recognize different epitopes on fragment B. A better mapping within fragment B was then obtained by using the same technique with all the CRMs (not shown) and scoring the ability of each antibody to detect the mutated B fragments of CRM45, CRM30, CRM228 and CRM1001. In particular, the two C-terminally truncated CRMs 30 and 45 allowed a subdivision of fragment B into 3 portions (see fig.1): a first N-terminal portion (region B1) up to the COOH-end of CRM30; a second portion (region B2) comprising essentially the central hydrophobic region between the COOH-ends of CRM30 and CRM45; a third portion (region B3) comprising the last 149 amino acid residues and including the putative receptor binding domain [3,4]. Mab1 and Mab23, which recognized the B fragments of DT and all the CRMs tested, including CRM30, are therefore directed towards peptides of region B1. Mab53 recognized fragment B of DT, CRM197, CRM176 (all with the same primary structure) and also that one of CRM228 (with 3 amino acid substitutions); however, Mab 53 could not bind either CRM30 or CRM45 and therefore the target of this antibody must be within the region B3 (residues 386–535). Furthermore Mab53 did not react with CRM1001 which has a mutation in this region (Cys-471 to Tyr): it is therefore likely that Mab53 recognizes a peptide including, or very close to, Cys-471. These results are summarized in table 1.

The binding properties of the Mabs were also examined by a competition radioimmunoassay where the antibody is bound to the solid phase and the antigen under test, in solution, competes with ^{125}I-labeled CRM197. The same preparation of labeled CRM197 was used throughout all tests. Values below 1×10^5 M^{-1} were considered as background or non-specific binding, since they are sometimes obtainable even with heterologous antibodies. The results are shown in table 1. The Mabs were also tested for their ability to neutralize the cytotoxic action of DT on CHO cells in culture: only Mab1 showed any toxin neutralizing activity.

4. DISCUSSION

The properties of four monoclonal antibodies raised against the diphtheria toxoid CRM197 were studied. Immunoblot analysis using a panel of molecularly characterized mutants (see fig.1) allowed a rough mapping along the amino acid sequence. 4 regions were considered: fragment A

Table 1

Summary of interactions of antibodies with DT and CRMs

Mab	Epitope region[a]	CHO protection		Interactions with DT and CRMs						
				DT	197	176	228	1001	30	45
11.3	A	no	K_a[d]:	0	2.6	0.5	0.3	0	<0.1	0
			blot:	+[c]	+	+	+	+	+	+
53	B3	no	K_a:	0	8.7	14.6	1.1	0.1	0	0
			blot:	+	+	+	+	−	−	−
23	B1	no	K_a:	0	0.4	6.5	6.6	4.1	5.4	34.7
			blot:	+	+	+	+	+	+	+
1	B1	yes[b]	K_a:	192	120	105	540	32.5	1.5	463
			blot:	+	+	+	+	+	+	+

[a]A, fragment A; B1,B2,B3, regions of fragment B as described in the text
[b]Molar ratio Mab:toxin = 92
[c]+/− refers to the antibody's ability to detect the various proteins on immunoblots
[d] K_a, affinity constants; figures are $K_a \times 10^{-6}$ M^{-1}, values <0.1 vary from 10^{-5} to 10^{-4}

plus three regions of fragment B separated by the COOH-ends of CRM30 and CRM45 and called here B1, B2 and B3. Mab11.3 is directed towards fragment A; Mab1 and Mab23 are directed towards different epitopes in region B1; Mab53 is directed towards the C-terminal of region B3 and probably binds to a peptide including, or very close to, Cys-471.

We then measured the affinity constants of the Mabs for their epitopes when these are in their native tertiary structure (table 1).

Mab1 (which gives a fairly strong interaction with all the molecules tested and has the value of a positive control) is the only antibody capable of neutralizing DT's toxicity for CHO cells. In this respect it is interesting to note that cytotoxicity neutralization does not necessarily correlate (in an assay similar to the one we used) with a high affinity but with inhibition of toxin binding to its receptor [31]. The location of the site recognized by Mab1 within the region B1 supports the suggestion that an N-terminal portion of fragment B is also involved in this process besides the primary receptor-binding domain located within region B3.

Mab 11.3 discriminates between the A fragments of CRM197, CRM176 and CRM228 (all mutated and fully or partially inactivated) and the same fragment in its wild-type configuration (as in DT, CRM45, CRM30 and CRM1001).

Mab53 has no affinity for CRM1001, CRM30 and CRM45 in agreement with the immunoblot analysis. However, its behaviour with the wild-type B fragments present in DT, CRM197 and 176 is unexpected since it fails to bind DT while reacting well with both CRM197 and CRM176.

Mab23 binds all the CRMs of the panel but, as Mab53, does not bind DT. The variation in affinity values of Mab23 for the CRMs could be related to different structures of their B fragments. The highest affinity was obtained for CRM45, possibly because the epitope involved is better exposed in this C-terminally truncated molecule.

The results reported here support the view that DT's structure can assume 'open' and 'closed' configurations. Mab1 would bind to a major surface determinant exposed in both configurations (higher affinities, toxicity neutralization), whereas Mabs 53, 23 and 11.3 would bind to epitopes accessible only in the 'open' structures adopted by the functional mutants here examined. Zucker and Murphy [31] reported in a recent study the analysis of 43 monoclonal antibodies raised using a detoxified DT preparation and found that none of them was directed against the amino-terminal B1 region of fragment B; also, when they tested the reactivity of the peptide between Cys-461 and Cys-471 they found that none of their monoclonal antibodies or a polyclonal antitoxin serum reacted with this peptide. Furthermore, all the 16 monoclonal antibodies directed against fragment A mapped within a region comprising only 20% of the fragment. All this is consistent with the view that Mabs

11.3, 23 and 53 interact in CRM197 and CRM176 with regions which are not exposed in native DT in solution at a neutral pH.

The data obtained with Mab53 indicate that the structure of fragment B in CRM197 (which has a primary structure identical to that of DT and can complement in vitro a native fragment A to reconstitute a fully toxic molecule, [9]) differs from the same polypeptide in the wild-type configuration as a consequence of a single amino acid substitution in fragment A, which introduces a charged residue in the place of a glycine. These findings point to a tight interaction between the two fragments, and are in agreement with a number of previous observations: namely, (i) the CD spectra of DT and CRM197 are different [32,33]; (ii) CRM197 is more susceptible than DT to the action of proteases ([7] and unpublished observations); (iii) the interaction of fragment B of CRM197 with lipid membranes is stronger than that of the same fragment in DT [34]. This last property described in the recent work of Papini et al. [34] could explain why the affinity of CRM197 for the cell receptors is apparently higher than that of DT [32].

Similar considerations applied to the structure of CRM176 would explain why its toxicity is 200-times lower than that of DT [23], although the enzymatic activity in vitro of its fragment A is only 10-times lower than that of wild-type fragment A [8–10] and its fragment B has the same primary structure as DT. It seems likely that such a tight interaction between A and B fragments as in CRM176 and CRM197 can exist in the native toxin as well.

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REFERENCES

[1] Ratti, G., Rappuoli, R. and Giannini, G. (1983) Nucleic Acids Res. 11, 6581–6595.
[2] Greenfield, L., Bjoen, M.J., Horn, G., Fond, D., Buck, G.A., Collier, R.J. and Kaplan, D.A. (1983) Proc. Natl. Acad. Sci. USA 80, 6853–6857.
[3] Pappenheimer, A.M. jr (1977) Annu. Rev. Biochem. 46, 69–91.
[4] Collier, R.J. (1982) in: ADP-ribosylation Reactions (Hayaishi, D. and Ueda, K. eds) pp. 575–592, Academic Press, New York.
[5] Olsnes, S. and Sandvig, K. (1987) in: Immunotoxins (Frankel, A.E. ed.) Martinus Nijhoff Publishing, Boston, in press.
[6] McKeever, B. and Sarma, R. (1982) J. Biol. Chem. 257, 6923–6925.
[7] Collier, R.J., Westbrook, E.M., McKay, D.B. and Eisenberg, D. (1982) J. Biol. Chem. 257, 5283–5285.
[8] Uchida, T., Pappenheimer, A.M. jr and Greany, R. (1973) J. Biol. Chem. 248, 3838–3844.
[9] Uchida, T., Pappenheimer, A.M. jr and Harper, A. (1973) J. Biol. Chem. 248, 3845–3850.
[10] Uchida, T., Pappenheimer, A.M. jr and Harper, A. (1973) J. Biol. Chem. 248, 3851–3854.
[11] Kaczorek, M., Delpeyroux, F., Chenciner, N., Streeck, R.E., Murphy, J.R., Boquet, P. and Tiollais, P. (1983) Science 221, 855–858.
[12] Giannini, G., Rappuoli, R. and Ratti, G. (1984) Nucleic Acids Res. 12, 4063.
[13] Maxwell, F., Maxwell, I.H. and Glode, L.M. (1987) J. Cell Biol., in press.
[14] Rappuoli, R., Ratti, G., Giannini, G., Perugini, M. and Murphy, J.R. (1986) in: Protein Carbohydrates Interactions in Biological Systems (Lark, D.L. ed.) pp. 295–296, Academic Press, London.
[15] Rappuoli, R. (1983) Appl. Env. Microbiol. 46, 560–564.
[16] Rappuoli, R., Ratti, G., Perugini, M. and Marsili, I. (1985) Biotechnology 3, 161–163.
[17] Rappuoli, R., Perugini, M., Marsili, I. and Fabbiani, S. (1983) J. Chromatography 268, 543–548.
[18] Bradford, M. (1976) Anal. Biochem. 72, 240–250.
[19] Mueller, J.H. and Miller, P.A. (1941) J. Immunol. 40, 21–32.
[20] Galfrè, G. and Milstein, C. (1981) Methods Enzymol. 73, 1–46.
[21] Stephenson, J.R., Lee, J.M. and Wilton-Smith, P.D. (1984) Anal. Biochem. 142, 189–195.
[22] Laemmli, U. (1970) Nature 227, 680–685.
[23] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4650–4655.
[24] Robb, M., Nichols, J.C., Whoriskey, S.K. and Murphy, J.R. (1982) Infect. Immun. 38, 267–272.
[25] Antoni, G. and Mariani, M. (1985) J. Immunol. Method. 83, 61–68.
[26] Marchalonis, J.J. (1969) Biochem. J. 113, 299–305.
[27] Ivanyi, J. (1982) Mol. Immunol. 19, 1611.
[28] Stähli, C., Miggiano, V., Stocker, T., Staehelin, T., Häring, P. and Takacs, B. (1983) Methods Enzymol. 42, 242.

[29] Miyamura, K., Nishio, S., Ito, A., Murata, R. and Kono, R. (1974) J. Biol. Standardization 2, 189–201.
[30] Murphy, J.R., Bacha, P. and Teuf, M. (1978) J. Clin. Microbiol. 7, 91–96.
[31] Zucker, D.R. and Murphy, J.R. (1984) Mol. Immunol. 21, 785–793.
[32] Mekada, E. and Uchida, T. (1985) J. Biol. Chem. 260, 12148–12153.
[33] Collins, C.M. and Collier, R.J. (1985) Biochim. Biophys. Acta 828, 138–143.
[34] Papini, E., Colonna, R., Schiavo, G., Cusinato, F., Tomasi, M., Rappuoli, R. and Montecucco, C. (1987) FEBS Lett., in press.