COMPETITIVE ANTAGONISTS OF THE ACTION OF DIPHTHERIA TOXIN IN HeLa CELLS

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1. Introduction

Native diphtheria toxin molecule (mol. wt. 62 000) consists of two distinct functional regions, fragments A and B of 24 000 and 38 000 daltons respectively that are originally connected to each other by a disulfide bridge and a peptide bond. The latter may be hydrolyzed very easily either spontaneously by the proteases of the culture medium or by trypsin. This nicked toxin is the enzymatically active form of the molecule [1,2].

Fragment A catalyzes the transfer of the ADP-ribose moiety from NAD to elongation factor 2 (EF2) which is inactivated by this modification [3]. The decline in EF2 activity is the primary result of the action of diphtheria toxin and is responsible for the decrease in protein synthesis of the intoxicated cells or organisms [4].

Although devoid of any enzymatic activity, Fragment B is nevertheless needed for the penetration of A into the sensitive cells. Its function seems to be the recognition of some cell surface receptors and to help, in this way, the penetration of A [1,2].

No direct measurement of the binding of fragment B to cells has been described until now because of its insolubility in usual buffers. The only quantitative determination of binding was performed by Ittelson and Gill [5] with crm 197, a protein isolated by Uchida [6] and produced by a mutant toxin gene. This protein which apparently has a normal B moiety but an inactive fragment A competes with the cell surface receptors of toxin.

The present paper describes the binding of two competitive proteins for HeLa cells receptors:

(a) a nitrated toxin which has lost enzymatic activity (and thus toxicity).

(b) the fragment B itself which has been obtained in a very soluble form.

We also tested for competition diphtheria toxoid, the formaldehyde detoxified currently used for immunization of men and animals.

2. Materials and methods

2.1. *Proteins*

Diphtheria toxin: crystallized toxin obtained from the Institut Pasteur de Paris in a completely nicked form.

Nitrated toxin: tetranitromethane (100 × molar excess) was added to a solution of diphtheria toxin at 1 mg/ml in 0.05 M Tris-HCl 0.5 M NaCl, pH 8.0. The solution was incubated 2 h at 30°C and dialyzed to eliminate reagent. Nine tyrosines per toxin molecule (out of 20) were transformed into 3-nitro-tyrosines (determined by amino acid analysis).

Enzymatic activity was 0.1% of native toxin and toxicity less than 0.05% (determined as minimal lethal doses in guinea pigs).

Fragment B: after carboxymethylation of the disulfide bridge between A and B, the fragment B was purified by molecular filtration on Sephadex G-200, in 0.05 M Tris-HCl, 6 M Urea pH 8.0 and then dialyzed against 0.02 M sodium borate buffer 0.02 M KCl, pH 8.0, in which it remains soluble.

Toxoid was prepared as described by Pappenheimer [7].

2.2. *HeLa cell protein synthesis determinations*

Suspensions at $4 \cdot 10^5$ cells/ml of HeLa cells S-3 (6 ml) in Eagle's medium with 5% calf serum are distributed into 25 ml erlenmeyer flasks and incubated

at 37°C in a water bath shaker for 150 min in the presence of different amounts of toxin and competitive protein. [^{14}C]Leucine (0.1 μCi) was then added to each flask and the suspension incubated one hour more. 2 ml aliquots were cooled, filtrated on millipores, washed with serum-free medium and then with 5% trichloroacetic acid. Filters are dried and counted by liquid scintillation.

3. Results

Fig.1 shows the dose–response curves of the action of diphtheria toxin on protein synthesis of HeLa cells in the presence of increasing amounts of nitrated toxin (fig.1A) fragment B (fig.1B) or toxoid (fig.1C).

Nitrated toxin and fragment B show competitive properties: the dose–response curves are shifted progressively to the right as expected in the presence of increasing concentration of a competitive antagonist. Toxoid, on the other hand, has no effect on the intoxication curve of diphtheria toxin (fig.1C). This formaldehyde detoxified protein has thus a non-functional fragment B.

In order to determine the apparent dissociation constant of the two competitive blocking proteins, we measured from fig.1A and B, the toxin concentration required to give 50% protein synthesis inhibition in the presence (T′) and in the absence (T) of the competitive protein.

The dissociation constant K_d was then calculated from the relation $T'/T - 1) = I/K_d$ where I is the competitive protein concentration [5]. Fig.2 represents the logarithmic plot of this relation (slope = 1). K_d is obtained by determination of the competitive protein concentration I for $(T'/T - 1) = 1$.

K_d values were found to be $1.3 \cdot 10^{-8}$ M for nitrated toxin and $0.8 \cdot 10^{-8}$ M for fragment B (fig.2A and B).

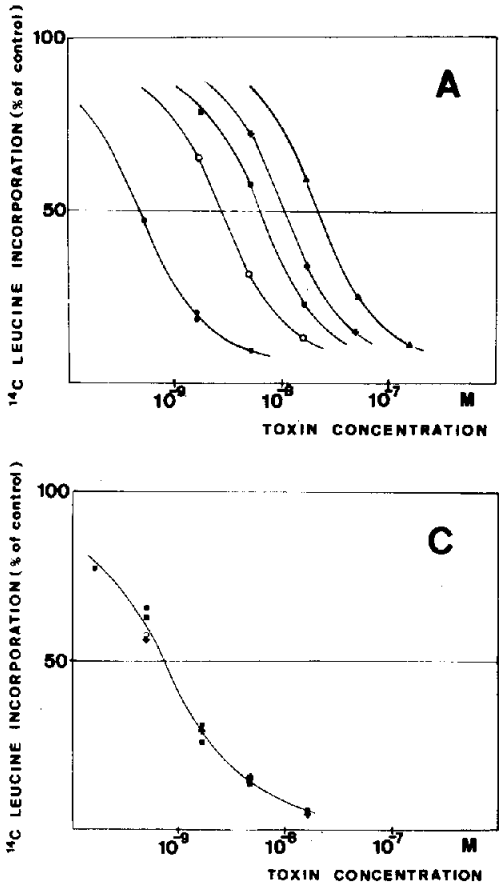

Fig.1. HeLa cells protein synthesis inhibition by diphtheria toxin. Experiments performed as described under Materials and methods. Results are expressed as % of control (without toxin). (A) In the presence of nitrated toxin. (○) 5 μg/ml, (■) 10 μg/ml, (+) 20 μg/ml, (▲) 40 μg/ml, (●) without nitrated toxin. (B) In the presence of fragment B (○) 0.25 μg/ml, (■) 2.5 μg/ml, (+) 7.5 μg/ml, (▲) 25 μg/ml, (●) without fragment B. (C) In the presence of toxoid. (○) 5 μg/ml, (■) 10 μg/ml, (+) 25 μg/ml, (●) without toxoid.

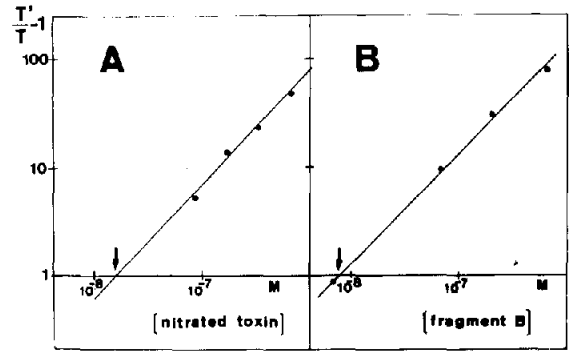

Fig.2. Logarithmic plot of the relation $(T'/T - 1) = I/K'_d$, where I is the competitive antagonist concentration: nitrated toxin (A) or fragment B (B). Dose ratios T'/T (see text) were calculated from fig.1A and B at 50% inhibition.

4. Discussion

Nitrated toxin has biological properties similar to that of the mutant protein crm 197 isolated by Uchida [6]: almost completely enzymatically inactive (0.1%) and non-toxic (< 0.05%) but with a fragment B still able to bind to cell receptors. Moreover, K_d values are very similar: Ittelson and Gill [5] reported a value of about 10^{-8} M for crm 197 in perfect agreement with the value of $1.3 \cdot 10^{-8}$ M for nitrated toxin found in the present study.

Even after 6 M urea treatment, fragment B in borate buffer seems to have recovered the conformational structure it possesses in the native toxin: antigenic properties remain unaltered [8] and its ability to recognize HeLa cells receptors and to compete with toxin is demonstrated here.

Affinity for receptors seems to be at least as good as for entire toxin analogs like crm 197 or nitrated toxin.

The value of about 10^{-8} M for the dissociation constant of the toxin itself proposed by Ittelson and Gill [5] seems thus entirely justified.

For toxoid however, detoxification by formaldehyde seems to have altered not only the enzymatic activity of fragment A but also the binding properties of fragment B to cells. This alteration may be due either to the chemical modification of some essential residue (e.g. lysine) or to the presence of methylene bridges [9] which would modify the conformational properties of the molecule.

References

[1] Gill, D. M. and Pappenheimer Jr., A. M. (1971) J. Biol. Chem. 246, 1492–1495.
[2] Collier, R. J. and Kandel, J. (1971) J. Biol. Chem. 246, 1496–1503.
[3] Honjo, T., Nishizuka, Y., Hayaishi, O. and Kato, I. (1968) J. Biol. Chem. 243, 3553–3555.
[4] Bowman, C. G. and Bonventre, P. F. (1970) Biochem. Biophys. Res. Commun. 41, 1148–1154.
[5] Ittelson, T. R. and Gill, D. M. (1973) Nature 242, 330–332.
[6] Uchida, T., Pappenheimer Jr. A. M. and Greany, R. (1973) J. Biol. Chem. 248, 3838–3844.
[7] Pappenheimer Jr. A. M., Uchida, T. and Harper, A. (1972) Immunochemistry 9, 891–906.
[8] Muyldermans, G. and Zanen, J. (1974) Arch. Intern. Physiol. Biochim. 82, 190.
[9] Blass, J., Bizzini, B. and Raynaud, M. (1967) Bull. Soc. Chem. 10, 3957–3965.