Monoclonal B-cell response to diphtheria toxoid: evidence for cross-reactive epitopes

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SUMMARY

Our previous studies on the human specific B- and T-cell responses against diptheria toxoid (DT) had shown that the B- and T-cell antigenic determinants were mostly assembled topographic sites with large intra-molecular cross-reactivities. In order to characterize further the B-cell responses against DT, and to investigate these cross-reactivities, we have produced 54 murine monoclonal antibodies (moAbs) against the native DT. Determination of their antigenic specificity showed that (i) 25 moAbs recognized conformational determinants on the native DT, (ii) 20 moAbs reacted against the A fragment (DTA) of the toxin, and (iii) nine moAbs reacted with at least three distinct highly purified CNBr peptides of the B fragment (DTB); some of them simultaneously recognized two or three purified CNBr peptides of DTB. As shown by cross-inhibition studies, the epitopes cross-reacting with those moAbs were distinct. Previously described sequence data indicated that these epitopes were not induced by repetitive amino-acid sequences. Finally, (iv) a cross-reactive idiotype was shared by moAbs specific for the CB1 and the CB3 peptides of DTB. Altogether, these data indicate that anti-DT moAbs are mostly directed against conformational determinants, and may cross-react with several DTB CNBr peptides.

INTRODUCTION

Renewed interest in the prediction of antigenic determinants of proteins recognized by B and T cells arises from the recent developments of synthetic peptides as vaccines. Toxoid vaccines such as diphtheria toxoid provide good tools to analyse the B- and T-cell repertoire against native proteins. DT) a single 58,390 molecular weight (MW) polypeptide, the amino-acid sequence of which has recently been described (Kaczorek *et al.*, 1983; Greenfield *et al.*, 1983), can be divided into two functionally distinct fragments (Fig. 1): (A) (DTA), the 21,250 MW NH$_2$-terminal enzymically active fragment, and (B) (DTB), the 32,240 MW COOH terminal fragment where the specific cell receptor binding site is located (Collier & Kandel, 1971; Gill & Pappenheimer, 1972).

Several immunodiminant regions have been determined on DT from the interactions of polyclonal antisera or monoclonal antibodies with (i) immunologically cross-reactive mutant toxins (CRMs) (Pappenheimer, Uchida & Harper, 1972; Pappenheimer, 1977), (ii) proteolytic fragments, DTA and DTB (Bazaral, Groscienski & Hamburger, 1973; Cryz, Welkos & Holmes, 1980), and (iii) synthetic peptides (Audibert *et al.*,

1971). Most of the serologically defined antigenic determinants seem to be located in the A fragment and in the 17,000 MW COOH-terminal region of the B fragment.

The developments of hybridoma technology and of T-cell cloning have led to a better delineation of B- and T-cell epitopes on several native proteins such as myoglobin (Berzofsky *et al.*, 1982), lysosyme (Smith-Gill *et al.*, 1984) and tetanus toxoid (Sheppard, Cussel & Hughes, 1984). These studies have shown that the surface of a protein is a continuum of multiple, overlapping antigenic determinants. Most of the monoclonal antibodies recognize conformational antigenic determinants assembled from amino-acid residues distant in the primary structure of the protein, whereas T-cell determinants seem to be contained in a single segment of amino-acid sequence (Benjamin *et al.*, 1984).

Recently, we reported the fine specificity of human B- and T-cell responses in DT-vaccinated volunteers (Triebel *et al.*, 1986). Our results suggested that B- and T-cell determinants were differently distributed on the native toxoid: most of the polyclonal B-cell response was directed against DTA; in contrast, T-cell clones reacted predominantly against DTB. Moreover, analysis of their fine specificity revealed that some cyanogen bromide (CNBr) peptides of DTB had cross-reactive T-cell determinants.

In order to describe monoclonal B responses against DT,

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gen peroxide in citrate/phospahte buffer was added as the substrate. The reaction was stopped after 30 min by the addition of 12·5% H_2SO_4. The optical densities were read at 492 nm.

Anti-DT solid-phase RIA competition assay

Two purified moAbs (50 µg) (H_9 and H_{16}) were radio-iodinated with 0·3 mCi ^{125}I (Amersham) using the chloramine T method. Four anti-DT moAbs in ascitic fluids were added in serial dilutions in a PBS/Tween/1% BSA solution to DT-coated plates, prepared as described above, and incubated for 90 min at 37°. After washing with NaCl, 100,000 c.p.m. of iodinated anti-DT moAbs were added to each well and incubated at 37° for 2 hr. After washing, the specific binding of ^{125}I-labelled anti-DT moAbs in each well was measured in a gamma counter (Packard, Lombard, IL). Results were expressed as:

% inhibition =

$$1 - \frac{\text{experimental c.p.m.} - \text{background c.p.m.}}{\text{uninhibited c.p.m.} - \text{background c.p.m.}} \times 100.$$

Competition ELISA assay

MoAbs in ascitic fluids were diluted at serial dilutions from 1×10^{-3} to 1×10^{-6} in a PBS/Tween solution containing inhibitors with 1% BSA. After an overnight incubation at 4°, the mixtures were added to solid-phase DT., CB1- and CB3-coated wells, prepared as described for the ELISA assay. After a 2 hr incubation at 37°, the ELISA was subsequently performed as described above. Results were expressed as:

% inhibition =

$$1 - \frac{\text{experimental A 492 nm} - \text{background values}}{\text{uninhibited A 492 nm} - \text{background values}} \times 100.$$

Preparation of anti-idiotypic sera

The anti-idiotypic sera were obtained from BALB/c mice immunized with purified moAbs. Briefly, equal amounts of a 1 mg/ml solution of the protein A–Sepharose purified moAb and a 1 mg/ml solution of solubilized keyhole limpet haemocyanin (KLH) (Calbiochem, La Jolla, CA) were mixed with 0·1%

glutaraldehyde in PBS at room temperature. The reaction was stopped with lysine (0·2 M) and the mixture was dialysed overnight in PBS. BALB/c mice were immunized in footpads with 100 µg of the KLH-coupled moAb in complete Freund's adjuvant at 2-week intervals. Sequential bleedings were tested for their anti-idiotypic activity after the third injection in a competition ELISA. Sera obtained from mice before the first injection served as controls.

RESULTS

Immune reactivities of 54 anti-diphtheria toxoid monoclonal antibodies

Fifty-four stable anti-DT antibody-producing hybridomas were obtained after subcloning. Their antigenic specificity was determined with a solid-phase ELISA using as antigens: (i) the DT, (ii) the isolated A and B fragments of the toxin (DTA and DTB), (iii) the five CNBr peptides of the B fragment (Table 1), and (iv) a synthetic peptide of 16 amino acids corresponding to the loop between the COOH region of DTA and the NH_2 region of DTB. Results are presented in Table 1.

Twenty-five anti-DT moAbs did not react with the A or B fragments, nor with the CNBr peptides or the 16.AA loop, while they did recognize the entire diphtheria toxoid, indicating that these moAbs bound to conformational determinants present on the native DT. None of the twenty-nine other moAbs recognized both the A and B framgents. Twenty moAbs were directed against DTA, and nine against DTB. Neither the anti-DTA nor the anti-DTB moAbs reacted with the 16.AA loop, while a polyclonal mouse anti-DT serum, used as a control, bound weakly to the 16.AA loop (data not shown). Among the nine anti-DTB moAbs, we could distinguish three patterns of reactivities:

(1) only two moAbs reacted with a single CNBr polypeptide which was CB1 in both cases;

(2) the seven other moAbs recognized several CNBr polypeptides: three anti-DTB moAbs reacted with both the CB1 and

Table 1. Immune reactivities of 54 moAbs against DT, DTA, DTB and five CNBr peptides of DTB

Number of moAbs*	Antigens†							
	DT	DTA	DTB	CB1	CB2	CB3	CB4	CB5
25	+‡	−	−	−	−	−	−	−
20	+	+	−	−	−	−	−	−
9	+	−	+					
2	+	−	+	+	−	−	−	−
3	+	−	+	+	−	+	−	−
4	+	−	+	+	−	+	+	−

* Hybridoma supernatants (at 1:50 to 1:100 dilutions) were tested at least twice in duplicate solid-phase ELISA.

† Indicated antigens were coated onto ELISA wells at concentration of 5 µg/ml.

‡ Absorbance values were considered positive when they were three times above the background values.
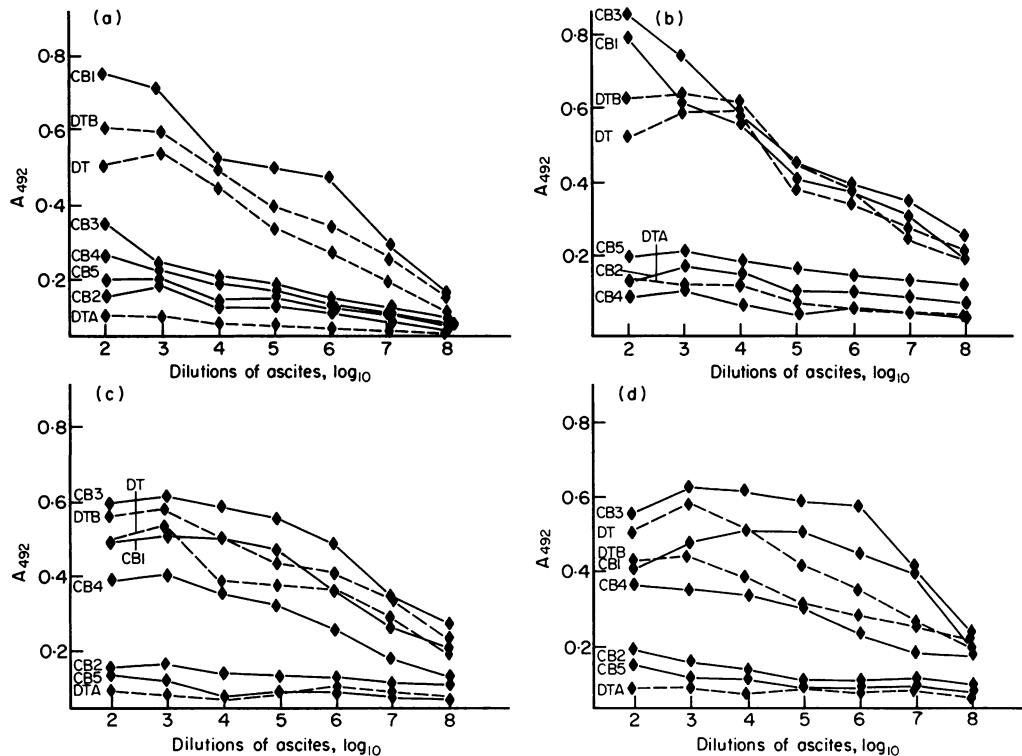
Figure 2. Direct anti-DT ELISA. The indicated antigens (5 μg/ml) were applied to the wells of an ELISA plate followed by serial dilutions of four moAb containing ascites: H_{11} (a), H_9 (b), H_2 (c) and H_{16} (d), beginning with a 1:100 dilution.

Table 2. Antibody-mediated neutralization of DT cytotoxicity

Antibody	Specificity	VERO cell assay	Guinea-pig skin test
A5	DTA	±*	−
H_{11}	DTB (CB1)	−	−
H_9	DTB (CB1/CB3)	−	−
H_2	DTB (CB1/CB3/CB4)	−	−
H_{16}	DTB (CB1/CB3/CB4)	±	−
Polyclonal antiserum	DT	+†	+

* ±denotes ≥ 50% inhibition of cytotoxicity.
† +denotes > 90% inhibition of cytotoxicity.

CB3 peptides, and four anti-DTB moAbs recognized the CB1, CB3 and CB4 polypeptides;

(3) the CB2 and CB5 polypeptides, although recognized in the same ELISA assay by the mouse anti-DT control serum (data not shown), did not react with any moAb.

In order to analyse cross-reactivities between the CNBr peptides, four representative independently derived moAbs were selected for further cross-inhibition experiments. Their A492 values in the direct ELISA are shown in Fig. 2.

Neutralization of the diphteria toxin cytotoxicity

The capacity of some of these anti-DT moAbs to neutralize the toxicity of the toxin was tested, both *in vitro*, using the diphtheria toxin-sensitive VERO cells, and *in vivo*, using the guinea-pig skin test. As shown in Table 2, only two moAbs reacting with DTA (A5) or the CB1, CB3 and CB4 peptides of DTB (H_{16}) partially inhibited the DT cytotoxicity *in vitro* but not *in vivo*. On the other hand, the mouse anti-DT control serum neutralized both *in vitro* and *in vivo* the DT cytotoxicity at a 2000-fold greater dilution.

Monoclonal antibodies reacting with the CB1 and CB3 CNBr peptides recognize distinct epitopes

The cross-reactivity of several anti-DTB moAbs with the CB1, CB3 and CB4 peptides raised the question of whether the cross-reactive epitopes were identical. Therefore, we performed a RIA competition assay between four moAbs that recognized both CB1 and CB3.

Their specific binding to DT was studied using a solid-phase radioimmunoassay. H_{11} (a IgG2a anti-CB1 moAb), H_9 (a IgG1 anti-CB1/CB3 moAb), H_2 and H_{16} (both IgG1 anti-CB1, CB3 and CB4 moAbs) were used as competitors with ^{125}I-labelled H_9 and H_{16} (Table 3 and Fig. 3). Three patterns of reactivity could be distinguished:

(1) H_2 and H_{16} moAbs recognize common or close epitopes on CB1, CB3 and CB4 since they both inhibit the ^{125}I-H_{16} binding;

(2) H_9 may react with epitopes on CB1 and CB3 distinct from those recognized by H_2 and H_{16} since H_9 and H_{16} did not compete with each other for their DT binding;

Table 3. Anti-diphtheria toxoid RIA competion assay between four anti-CBI and anti-CB3 moAbs

| Competing moAbs (specificity) | Target ^{125}I moAbs† | | | |
| | H9 | | H16 | |
	c.p.m.‡	% of inhibition	c.p.m.	% of inhibition
None	5938	—	8583	—
H9 (CB1/CB3)	1530	75	6647	23
H11 (CB1)	5095	15	6659	23
H2 (CB1/CB3/CB4)	4993	16	2071	76
H16 (CB1/CB3/CB4)	4287	28	2755	68

* Each unlabelled monoclonal antibody in ascitic fluid was applied onto DT-coated plates (5 μg/ml) in eight serial log$_{10}$ dilutions beginning with a 1:150 dilution.

† Purified ^{125}I moAbs in a 1:1000 dilution.

‡ C.p.m. values obtained with the maximum inhibiting dilution (1:1000) of the unlabelled moAbs.
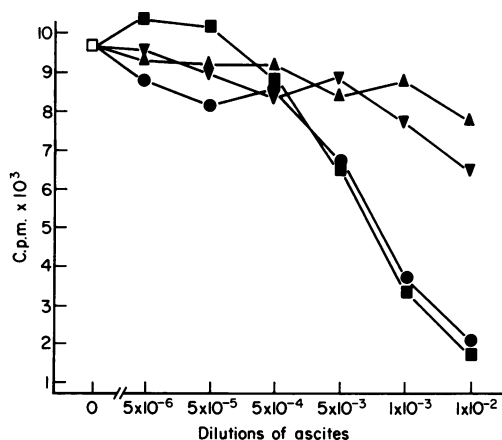
Figure 3. Competition assay between moAb H$_{16}$ and the moAbs H$_{11}$, H$_9$, H$_2$ and H$_{16}$ for binding to DT. Serial dilutions of four anti-DTB moAb containing ascites: H$_{11}$ (▲), H$_9$ (▼), H$_2$ (●), and H$_{16}$ (■), beginning with a 1×10^{-2} dilution, were applied to the wells of a DT-coated ELISA plate (5 μg/ml). 100,000 c.p.m. of the radio-iodinated purified H$_{16}$ moAb (□) were then added, and the RIA competition assay was subsequently performed as described in the Materials and Methods. Each point is the mean of duplicates of a representative experiment.

(3) H$_{11}$, an anti-CB1 moAb, failed to compete with either H$_9$ or H$_{16}$.

Altogether, these results indicate that these moAbs, although reacting with common CNBr fragments of the toxoid, recognize several distinct epitopes on the CB1, CB3 and CB4 peptides of DTB.

The CB1 and CB3 CNBr peptides share cross-reactive epitopes with different immune reactivities

The above results indicate that both the CB1 and CB3 peptides are diversely recognized by the cross-reactive moAbs. In order to investigate these immune cross-reactivities, we used the CB1 and CB3 peptides as inhibitors in a solid-phase ELISA competition assay. Results of the inhibition of binding of H$_{11}$ and of three cross-reactive moAbs H$_9$, H$_2$ and H$_{16}$ showed two patterns of reactivities.

The soluble CB3 peptide repeatedly competed with the solid-phase CB3 for the binding of the cross-reactive H$_9$ (67% of inhibition), H$_2$ (90%) and H$_{16}$ (90%) moAbs (Fig. 4). In addition, the soluble CB3 peptide inhibited the H$_9$ binding (39%), the H$_2$ binding (93%) and the H$_{16}$ binding (91%) to CB1 (Fig. 4).

In contrast, the soluble CB1 gave only a 32–33% inhibition of the H$_9$ binding to CB1 and CB3, and failed to compete with CB1 and CB3 for their H$_2$ and H$_{16}$ binding. It could not inhibit the binding of H$_9$, H$_2$ and H$_{16}$ to the entire diphtheria toxoid (data not shown). However, the capacity of the soluble CB1 to bind the anti-CB1 moAbs was demonstrated by the 61% inhibition of the H$_{11}$ binding (data not shown).

Cross-reactive idiotopes on the anti-DT response

It seemed difficult to analyse further the cross-reacting epitopes on CB1 and CB3 using the soluble CB1 peptide as an inhibitor in a solid-phase competition assay. Therefore, we investigated the existence of an inverse cross-reactive immunogenicity between the combining sites of the moAbs reactive with CB1 and CB3.

Figure 4. Cross-inhibition assay of four anti-DT moAbs binding with the CB3 peptide. Serial dilutions of three anti-DTB moAb containing ascites: H9 ($\triangledown$), H2 (O) and H16 ($\square$), beginning wi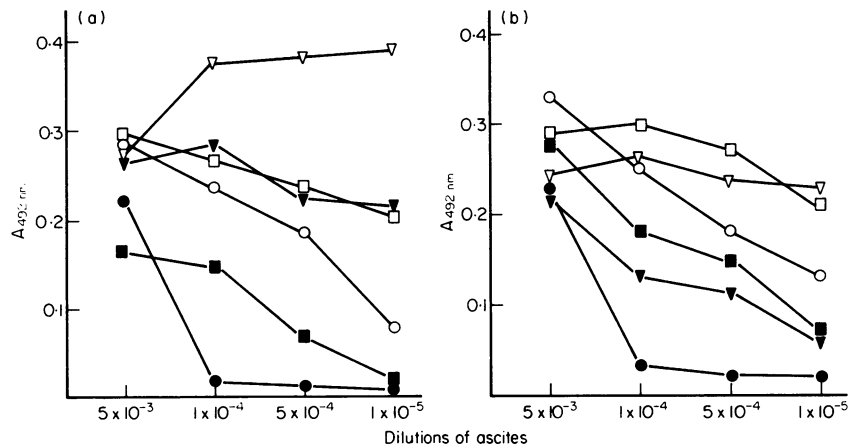th a 5×10^{-3} dilution, were applied directly or after a preincubation with the CB3 peptide in PBS/BSA (5 μg/ml) [respectively, ($\blacktriangledown$), ($\bullet$), ($\blacksquare$)] on a CB1- (a) or a CB3- (b) coated ELISA plate (5 μg/ml). The ELISA was subsequently perfomed as described in the Materials and Methods. Each point is the mean of a representative experiment performed in duplicate.
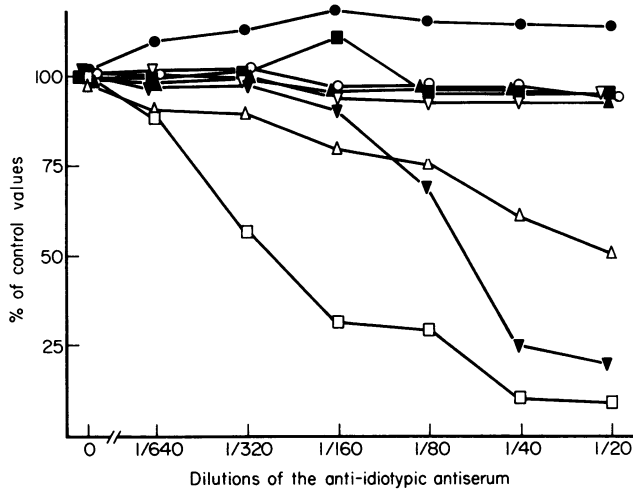

Figure 5. Competition assay between the anti-idiotypic anti-H11 antiserum and the anti-DT moAbs and antiserum. Anti-DT moAbs, respectively, H11 ($\square$), H9 ($\blacktriangledown$), H2 (O), H16 ($\bullet$), A5 ($\blacktriangle$), and mouse anti-DT antiserum ($\triangle$), were preincubated overnight at a dilution of 1×10^{-4} with serial 1:2 dilutions of the anti-H11 antiserum and with the negative mouse control serum for H11 ($\blacksquare$) and H9 ($\triangledown$). Mixtures were then tested in duplicate experiments for their residual anti-DT activity in the solid-phase ELISA as described in the Materials and Methods. Results are expressed as a percentage of each control A 492 nm OD value (100%). Respective 100% OD values were: H11 0·869; H9 0·633; H2 0·548; H16 0·874; A5 0·408; anti-DT mouse antiserum 0·595.

In order to raise a syngeneic anti-idiotypic response, we selected H11, which is an IgG2a moAb reactive only with CB1. The anti-serum obtained specifically inhibited 91% of the H11 DT binding (Fig. 5) in a competition ELISA assay, while the control serum gave no inhibition. The inhibition obtained was not isotype related since the anti-H11 anti-serum could also inhibit 75% of the fixation on DT of H9, and IgG1 moAb reactive with both CB1 and CB3. Moreover, the anti-H11 anti-idiotypic serum also gave a 59% inhibition of the fixation on DT

of a syngeneic anti-DT polyclonal serum, but could not inhibit the other tested anti-DTA, anti-CB1, CB3 and CB4 moAbs (Fig. 5). Finally, the anti-H11 anti-idiotypic serum failed to compete with CB3 for the CB3 binding of H9 (data not shown).

DISCUSSION

Studying the human B- and T-cell responses against diphtheria toxoid, we have detected, even at the clonal T-cell level, numerous cross-reactivities that could not be explained by repetitive sequences on the primary structure of DT (Triebel *et al.*, 1986).

In the present study, we have studied the fine antigenic specificity of moAbs derived from mice immunized with the native DT. Our results show that: (i) most of the isolated moAbs are directed against native conformational determinants present in the toxoid, (ii) 35% of the remaining moAbs react against DTA, while, (iii) moAbs reacting with DTB represent only 20% of the isolated moAbs, (iv) most of the anti-DTB moAbs reacted with at least three distinct epitopes of the CB1 and the CB3 peptides in the 21,000 MW C-terminal region of DTB, and (v) these anti-DTB moAbs predominantly recognized cross-reacting determinants spread on several CNBr peptides of DTB but not on DTA.

Previous workers (Hayakawa *et al.*, 1983; Zucker & Murphy, 1984) reported murine moAbs reacting with either DTA or DTB. They could distinguish three immunodominant regions located within the C-terminal portion of DTA, the central region and the C-terminal region of DTB. In contrast, they reported the absence of immune reactivity of the NH2-portion of DTB and suggested that this region could be masked in the native protein. However, we isolated four moAbs reacting with CB4, the C-terminal peptide of DTB, indicating that this region could be immunogenic in the same strain of mice. On another hand, like Zucker & Murphy (1984), we could not isolate any moAb reacting with the 16-AA loop overlapping on DTA and the CB4 peptide. Altogether, these data suggest that the whole

native toxoid is immunogenic with several constant immunodominant domains.

Only two moAbs specific for DTA or DTB could neutralize *in vitro* but not *in vivo* the cytotoxicity of the diphtheria toxin. Our results contrast with those obtained by Zucker & Murphy (1984), who reported an effective *in vitro* neutralization of the toxin with several anti-DT moAbs. Audibert *et al.* (1971) also obtained a consistent neutralizing effect both *in vitro* and *in vivo* with antisera obtained after immunization with the 16-AA loop.

Most of the anti-DTB moAbs recognized, even after subcloning, several highly purified CNBr peptides of DTB. No moAb reacted with both the DTA and the DTB fragments. Results of the cross-inhibition assay between the CB1 and CB3 peptides revealed differences of inhibition induced by the soluble CB1 peptide that could be related to the co-existence of some accessible and masked epitopes in this portion of the native molecule. Indeed, a striking feature of the plot of hydrophilicity of the B fragment of the diphtheria toxin is the alternation of segments of inverse polarity (Fig. 1) (Falmagne *et al.*, 1984). One could suggest that putative accessible and masked epitopes on the CB1 peptide might be related to the hydrophilic and hydrophobic segment of this portion of the molecule as demonstrated in several proteic models (Pu Tao Shi *et al.*, 1984; Benjamin *et al.*, 1968; Hopp & Woods, 1981; Berzofsky, 1985).

An anti-idiotypic serum raised against H_{11}, a moAb specific for CB1 alone, could also inhibit the DT binding of the moAb H_9, reactive with the CB1 and CB3 peptides, and of a polyclonal mouse anti-DT serum suggesting the presence of common idiotypes in the murine response to DT. The common idiotype would be shared by two moAbs bearing distinct combining sites: H_{11}, reactive with CB1 alone, and H_9, cross-reactive with CB1 and CB3. Shared idiotypes have already been identified on moAbs specific for different antigenic determinants on such molecules as lipopolysaccharide (Hiernaux & Bona, 1982). I-E molecules (Devaux *et al.*, 1984), and sperm whale myoblobin (Kohno *et al.*, 1982), suggesting that these moAbs were specific for closely related epitopes, or that a common Id–anti-Id network regulates antibody responses to different antigenic determinants of a protein.

The data presented here showed two levels of cross-reactivity in the murine B-cell response to DT, in accordance with our previous results: a cross-reactivity between the CNBr peptides of DTB, and a cross-idiotypy between anti-DT moAbs. A contamination between the CNBr peptides, particularly between CB1 and CB3, could be eliminated by the high degree of purification of these peptides (Falmagne *et al.*, 1978). Moreover, in our first study, specific T-cell clones were able to proliferate, even after subcloning, against several CNBr peptides in very low concentrations (1 μg/ml). A contamination of less that = 1% could not have explained strong proliferation at these concentrations.

Cross-reactivities between CNBr peptides have been reported for several proteins such as albumin and glycophorin (Benjamin *et al.*, 1984; Barsoum, Bhavannandan & Davidson, 1985). These findings could be explained by repetitive amino-acid sequences or common oligosaccharides of glycosylated proteins. The intra-DTB cross-reactivities reported in the present study cannot be explained by one of these mechanisms. No repetitive amino-acid sequences could be found in the

primary structure of the non-glycosylated DT toxin, deduced from the nucleotide sequence of the structural gene (Kaczorek *et al.*, 1983; Greenfield *et al.*, 1983) and confirmed by the complete amino-acid sequence of the DT (Falmagne *et al.*, 1985).

However, the anti-DTB moAbs could recognize conformational determinants at least partly preserved in the CNBr peptides or induced after conformational modifications of the molecule. Indeed, the 3-dimensional structures of the diphtheria toxin and toxoid are not known, and the A and B fragments (respectively 21,250 and 32,240 MW) as the CNBr peptides (from 4200 to 13,070 MW) are large enough to present conformational determinants.

As demonstrated by Geysen (1985), the partial denaturation of the proteins during the *in vivo* recognition processes induces conformational changes which could be reproduced on proteins linked to solid surfaces. Then, the adsorption of CNBr peptides onto plastic surfaces could induce conformational modifications and expose masked epitopes or determine similar conformational structures despite their distinct amino-acid sequences. Both the common idiotype shared by moAbs specific for cross-reacting peptides and the cross-reactions found at the T-cell level suggest that these *in vitro* modifications could reproduce the alterations occurring *in vivo* during the antigen processing.

An alternative explanation could be that the conformational determinants of anti-DTB moAbs are partly preserved on the CNBr peptides of DTB, so that only one or two contact points could react with the antigen-binding sites. Then, the CNBr peptides epitopes would occupy only a part of the moAbs combining sites, which might be insufficient to block their binding to another CNBr peptide by steric hinderance. Alternatively, a moAb specific for one of the CNBr peptides could react with one or two identical contact points present on similar structures of the other CNBr peptides, indicating a structural similarity, as reviewed by Ghosh & Campbell (1986).

Finally, cross-reactivities of synthetic peptides with the native proteins have been reported in several systems. This characteristic could be widely used to prepare synthetic vaccine (Jacob, Sela & Arnon, 1983). The 16 amino-acid loop synthetic peptide, prepared by Audibert *et al.* (1971) for this purpose, was not immunoreactive in our hands when tested with moAbs directed against the native toxoid or the A and B fragments or the CNBr peptides. The intra-molecular cross-reactivities shown in our study could therefore provide new information with which to select new cross-reactive synthetic peptides as vaccines.

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