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Biochemistry

Anti-idiotypic antibodies as probes of protein active sites: Application to cholera toxin subunit B

(ganglioside GM1/labile toxin/ligand/receptor binding domain/structure–activity relationships)

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ABSTRACT Since Jerne proposed a "network" theory of immune regulation, the properties of anti-idiotypic antibodies (anti-IdAb) have been investigated widely. Anti-IdAb raised against antibodies to a variety of ligands have been shown to bind the ligands' receptors. Thus, the combining site of an anti-IdAb may contain information regarding the three-dimensional structure of an antigen. However, this remarkable property of "internal imagery" has not been exploited for structural investigation at the molecular level. In the present report, a monoclonal "auto"-anti-IdAb was raised against ganglioside GM1 (a cell-surface glycolipid that binds cholera toxin) and was shown to crossreact with the B subunit of cholera toxin. This antibody was presumed to recognize amino acid residues located within the GM1 binding domain. To identify these residues, the antibody was screened against homologous toxins purified from enterotoxigenic strains of *Escherichia coli* and chimeric peptides produced by recombinant methods. Amino acid variation at position 4 from the N terminus of these proteins was found to disrupt antibody binding. Since the toxins and chimera are all closely related in structure and function, the residue at position 4 (an asparagine in cholera toxin B subunit) appears to be in the epitope of the antibody and, by implication, in the GM1 binding site. Of particular significance, this structural detail could not be deduced with GM1 alone. It would seem that ligand and anti-ligand anti-IdAb encode similar stereochemical information but do so with different "chemical alphabets," giving rise to distinct binding specificities.

Cholera toxin, produced by *Vibrio cholerae* (1, 2), is composed of two subunits, A and B (3). The A subunit contains two polypeptide chains, A_1 (M_r, 22,000) and A_2 (M_r, 5000) linked by a disulfide bridge (4). A_1 can penetrate the plasma membrane of susceptible cells and therein activate adenylate cyclase (5, 6). The cholera toxin B subunit (CT-B) consists of identical polypeptide chains noncovalently associated as pentamers (4, 7). Each polypeptide (M_r, 11,600) contains 103 amino acids (8, 9); an intrachain disulfide bond links cysteine-9 and cysteine-86. CT-B specifically binds the monosialoganglioside GM1 [galactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)galactosylglucosylceramide] with an association constant of 10^9 M^{-1} (10). This glycolipid, present on many mammalian cell surfaces, provides an attachment site for cholera toxin during enteric infection. The oligosaccharide moiety of GM1 devoid of ceramide (OS-GM1) carries the determinants for interaction with CT-B (11). Bio- and physiochemical studies have suggested that several amino acids—tryptophan-88 (12–14), glycine-33 (15), the cystine residue (14), and possibly lysine (14, 16), arginine (14, 17), and histidine (18) residues—are important to the GM1 binding properties of CT-B. However, most of these studies cannot distinguish amino acid residues located within the GM1 binding domain from those residues that (*i*) indirectly contribute to function, (*ii*) undergo conformation change upon GM1 binding, or (*iii*) are in general proximity to, but not in contact with, bound GM1. In an attempt to circumvent these interpretive difficulties, we sought anti-idiotypic antibodies (anti-IdAbs) to GM1 that crossreact with CT-B and, therefore, recognize amino acid residues that participate directly in the B subunit–GM1 interaction.

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EXPERIMENTAL PROCEDURES

Preparation of Monoclonal Antibody. The strategy for the preparation of monoclonal anti-IdAb follows Cleveland *et al.* (19). Eight-week-old female BALB/c mice were immunized i.p. with 50 μg of GM1 coated onto acid-treated, boiled *Salmonella* (20) in complete Freund's adjuvant (0.2 ml) and booster injections were given 6 weeks later i.p. with GM1–*Salmonella* in incomplete Freund's adjuvant. On days 4, 3, and 2 prior to fusion (7 months after initial injection), mice were injected i.v. with 0.1 ml of GM1–*Salmonella* in saline. Splenocytes were fused with P3X63 AG8.653 myeloma cells essentially according to Sugasawara *et al.* (21). Two weeks later, cell culture supernatants were screened for activity against CT-B by solid phase RIA (see below). This one-step method for producing "auto"-anti-IdAb was employed, instead of conventional techniques, for two reasons. Idiotypic (anti-GM1) antibody produced by direct antigen stimulation is perhaps more immunologically accessible than injected antibody that might bind endogenous GM1 (22) present in peripheral tissue. In addition, the screening procedures allow for selection of anti-idiotype producing clones with highest affinity for CT-B.

Preparation of OS-GM1 and a Radioiodinated Derivative. The oligosaccharide moiety was cleaved from GM1 by ozonolysis and alkaline fragmentation, purified by ion-exchange chromatography, and assessed by TLC according to the method of Wiegandt and Bücking (23) as modified by Fishman *et al.* (24). The concentration of OS-GM1 was determined by the thiobarbituric acid assay of Aminoff (25) as adapted by Ledeen and Yu (26). A radiolabeled derivative was synthesized by reductive amination (27) and Bolton–Hunter iodination (28).

Assays. Crossreaction of anti-IdAb with CT-B (Sigma), the B subunit of human or porcine labile toxin (hLT-B or pLT-B),

Abbreviations: anti-IdAb, anti-idiotypic antibody; CT-B, cholera toxin B subunit; GM1, galactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)galactosylglucosylceramide; OS-GM1, the oligosaccharide moiety of GM1; hLT-B or pLT-B, the B subunit of human or porcine labile toxin, respectively.

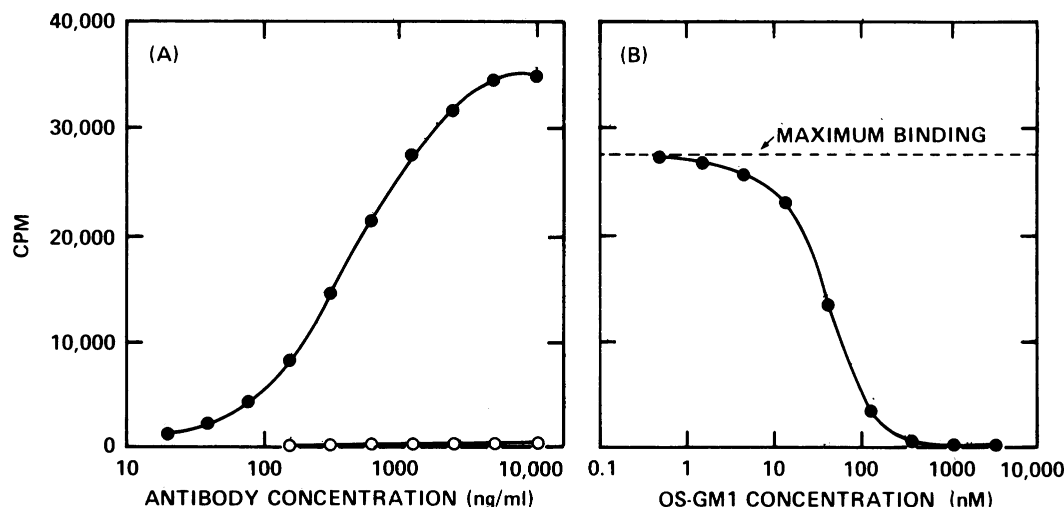

FIG. 1. Characterization of Ab9B6. (*A*) Binding to CT-B. Polyvinyl microtiter wells were incubated overnight with 100 μl of CT-B at 7μg/ml or, by way of control, bovine serum albumin at 7 μg/ml, gelatin at 7 μg/ml, thyroglobulin at 7 μg/ml, or GM1 at 7 μg/ml (○), in PBS, pH 7.4, and then washed with PBS containing 0.05% gelatin. Subsequently, serial dilutions of Ab9B6 were added to the wells, and bound immunoglobulin was determined. (*B*) Competition with OS-GM1. Serial dilutions of OS-GM1 were mixed with 100 ng of Ab9B6, and the resulting solution was allowed to compete for binding to CT-B in the solid phase of microtiter wells.

or the chimeric B subunits (purified from *Escherichia coli*—see below) were assessed by solid-phase RIA: Polyvinyl microtiter wells were incubated overnight with 100 μl of B subunit at 7 μg/ml, of bovine serum albumin at 7 μg/ml, of gelatin at 7 μg/ml, of thyroglobulin at 7 μg/ml, or of GM1 at 7 μg/ml, in PBS (50 mM Na_2HPO_4/100 mM NaCl, pH 7.4) and then washed with PBS containing 0.05% gelatin. Subsequently, serial dilutions of antibody were added. After 2 hr, the wells were washed, and rabbit anti-mouse antibodies (Miles-Yeda), 1:2000 dilution, was introduced. One hour later, the wells were washed, and 75,000 cpm of ^{125}I-labeled protein A (Amersham) was added. Following a 1-hr incubation, bound radioactivity was measured in a γ counter. An apparent avidity constant K_{av} for the antibody was calculated by Scatchard analysis (29) of solid-phase RIA data. Competition between anti-IdAb and OS-GM1 was demonstrated as follows: Serial dilutions of OS-GM1 were mixed with 100 ng of antibody, and the resulting solution was added to microtiter wells containing CT-B in the solid phase. After 2 hr, the wells were washed and then incubated with rabbit anti-mouse antibodies and ^{125}I-labeled protein A as described above.

Preparation of the Chimeric B Subunits. Chimeric B subunits, containing the pLT-B amino acid sequence substituted with hLT-B residues as indicated (see Fig. 3), were purified to homogeneity (30) by agarose affinity chromatography (31, 32) from *E. coli* carrying recombinant plasmids. Genes for the chimeric B subunits were constructed by fusing the following DNA fragments [see Leong *et al.* (33) for restriction map] from plasmids encoding hLT-B and pLT-B: pDL2, *Eco*RI to *Sma* I (pLT-B) and *Sma* I to *Hin*dIII (hLT-B); pDL3, *Eco*RI to *Cla* I (pLT-B) and *Cla* I to *Hin*dIII (hLT-B); pDL5, *Eco*RI to *Sma* I (pDL3, described above) and *Sma* I to *Hin*dIII (pLT-B); and pDL7, *Eco*RI to *Cla* I (hLT-B) and *Cla* I to *Hin*dIII (pLT-B). The genes were sequenced by the methods of Chen and Seeburg (34).

RESULTS AND DISCUSSION

Mice were immunized with GM1 to induce anti-GM1 antibody that would, in turn, elicit an immune response in the original animals. Hybridomas producing these "auto"-anti-IdAb antibodies were identified by screening medium for CT-B-binding antibodies. Of 1955 hybridomas grown, 13 tested positive. Antibody from one cell line (designated Ab9B6, isotype IgM) was purified from ascites fluid by ammonium sulfate precipitation and gel filtration chromatography. Ab9B6 binds CT-B specifically (Fig. 1*A*), with an apparent avidity constant (K_{av}) of 8×10^9 M^{-1}. To determine whether this clone recognizes the GM1 binding site, we conducted a competition RIA with the oligosaccharide moiety of GM1 (Fig. 1*B*). Nanomolar concentrations of OS-GM1 (M_r, 1000) inhibited the binding of Ab9B6 to CT-B [but not the binding of polyclonal antisera (data not shown)]. Since this antibody that was raised by immunization with GM1 binds CT-B specifically and competes with OS-GM1, we propose that Ab9B6 is an anti-IdAb.

Cholera toxin bears close resemblance—in primary structure, subunit arrangement, antigenicity, physiochemical properties, and ligand binding function—to hLT and pLT, heat-labile toxins produced by enterotoxigenic strains of *E. coli* isolated from humans or swine, respectively (31, 32, 35–38). Solid-phase RIA (Fig. 2) and immunoblot analysis (data not shown) indicated that Ab9B6 crossreacts with hLT-B (K_{av}, 7×10^8 M^{-1}) but not with pLT-B. These proteins differ in amino acid sequence at only 4 of 103 positions, suggesting that residues 4, 13, 46, and/or 102 of hLT-B and CT-B constitute part of the combining site of Ab9B6 (Fig. 3).

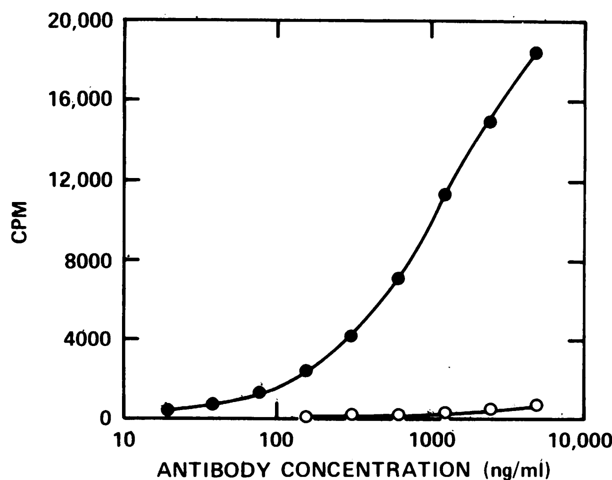

FIG. 2. Crossreaction of Ab9B6 with hLT-B and pLT-B. The binding of Ab9B6 to hLT-B (●)- or pLT-B (○)-coated microtiter wells (at 7 μg/ml) was assessed.

hLT-B	Ala			**Ser**			Glu			Ser								Tyr		Ile
CT-B	Thr	Pro	Gln	Asn	Ile^{5}	Thr	Asp	Leu	Cys	Ala^{10}	Glu	Tyr	His	Asn	Thr^{15}	Gln	Ile	His	Thr	Leu^{20}
pLT-B	Ala			**Thr**			Glu			Ser			**Arg**					Tyr		Ile

hLT-B		Asp			Leu						Met							Val		
CT-B	Asn	Asn	Lys	Ile	Phe^{25}	Ser	Tyr	Thr	Glu	Ser^{30}	Leu	Ala	Gly	Lys	Arg^{35}	Glu	Met	Ala	Ile	Ile^{40}
pLT-B		Asp			Leu						Met							Val		

hLT-B				Ser																
CT-B	Thr	Phe	Lys	Asn	Gly^{45}	Ala	Thr	Phe	Gln	Val^{50}	Glu	Val	Pro	Gly	Ser^{55}	Gln	His	Ile	Asp	Ser^{60}
pLT-B				Ser		**Glu**														

hLT-B										Asp					Thr					Thr
CT-B	Gln	Lys	Lys	Ala	Ile^{65}	Glu	Arg	Met	Lys	Asn^{70}	Thr	Leu	Arg	Ile	Ala^{75}	Tyr	Leu	Thr	Glu	Ala^{80}
pLT-B										Asp					Thr					Thr

hLT-B		Ile	Asp											Asn	Ser					
CT-B	Lys	Val	Glu	Lys	Leu^{85}	Cys	Val	Trp	Asn	Asn^{90}	Lys	Thr	Pro	His	Ala^{95}	Ile	Ala	Ala	Ile	Ser^{100}
pLT-B		Ile	Asp											Asn	Ser					

hLT-B		**Glu**	
CT-B	Met	Ala	Asn
pLT-B		**Lys**	

Chimera	Residues Changed
pDL2	102
pDL3	46 and 102
pDL5	46
pDL7	4 and 13

FIG. 3. Amino acid sequence of the hLT-B/pLT-B chimera. Chimeric B subunits, containing the pLT-B amino acid sequence substituted with hLT-B residues as indicated, were purified from *E. coli* carrying recombinant plasmids.

To further characterize this epitope, we employed engineered genes encoding pLT-B substituted with hLT-B DNA fragments. The resulting chimeric proteins, containing various combinations of hLT-B and pLT-B residues (Fig. 3), were screened for crossreactivity with Ab9B6. Fig. 4 depicts the results of this study. Ab9B6 did not bind pDL2, pDL3, or pDL5 that have hLT-B residues at position 102, positions 46 and 102, or position 46, respectively; however, the anti-IdAb did recognize pDL7 (K_{av}, 8×10^{8} M^{-1}), substituted at positions 4 and 13. This interaction is blocked by nanomolar concentrations of OS-GM1. The OS-GM1 derivative

$$\text{Gal}-\text{GalNAc}-\underset{\text{NANA}}{\text{Gal}}-\text{Glu}-\text{NH}-\overset{\text{O}}{\overset{\|}{\text{C}}}-\text{CH}_2-\text{CH}_2-\text{C}_6\text{H}_3(^{125}\text{I})-\text{OH}$$

(NANA,*N*-acetylneuraminic acid) was used to control for possible differences in the concentration and in the orientation of B subunit molecules attached to the solid phase in these RIAs. CT-B, hLT-B, pLT-B, and the chimera bound labeled OS-GM1 identically under saturating and nonsaturating conditions, indicating that the number of accessible GM1 and, by implication, anti-IdAb binding sites were equivalent in the assays described in Figs. 1*A*, 2, and 4. Therefore, the epitope recognized by Ab9B6 appears to encompass residues 4 and/or 13.

Another enterotoxigenic *E. coli* strain pathogenic for humans (generously provided by Tatsuo Yamamoto of Juntendo University, Japan) has been isolated, and its toxin (hLT_2) has been purified, sequenced (38), and shown to bind OS-GM1. The B subunit of this toxin (hLT_2-B) differs from hLT-B by only one amino acid, containing the pLT-B residue at position 13. Ab9B6 binds hLT_2-B, as determined by RIA (data not shown), indicating that residue 13 does not interact with the antibody. Instead, these data imply that asparagine-4 constitutes part of the epitope recognized by Ab9B6. Since Ab9B6 carries an "internal image" of GM1, we conclude that residue 4 is also located within the GM1 binding domain of toxins in the cholera/labile toxin family. This finding supports a model proposed by Ludwig *et al.* (14) wherein the intramolecular disulfide bridge juxtaposes in space residues near cysteines-9 and -86, which jointly participate in ligand binding.

Our characterization of the epitope recognized by Ab9B6 rests upon an assumption that amino acid substitutions that affect antibody binding do so directly, rather than through allosteric interaction. We base this assumption on the close structural similarity of hLT to pLT, both native, active toxins. Moreover, since the proteins exhibit equivalent affinities for GM1, the receptor binding domain and, thus, the region specifying the epitope for Ab9B6 are presumed to be conformationally intact.

Previously, anti-IdAb have been raised against antibody to a variety of protein-binding ligands. Anti-IdAb against insulin or retinal binding protein bound insulin or retinal binding protein receptors, respectively (39). Anti-IdAb against alprenolol recognized β-adrenergic receptors and stimulated

FIG. 4. Crossreaction of Ab9B6 with the hLT-B/pLT-B chimera. The binding of Ab9B6 to pDL2 (△)-, pDL3 (●)-, pDL5 (○)-, or pDL7 (■)-coated microtiter wells was assessed.

basal adenylate cyclase activity (40). Further, anti-IdAb to the chemoattractant peptide *N*-formyl-Met-Leu-Phe crossreacted with its receptor on neutrophils (41). These studies produced immunoglobulin molecules that manifested the same pharmacologic properties as ligands. Such antibody, however, cannot provide more information about a receptor, in structure–function analysis, than the ligand does. But, ligands and the anti-IdAb they elicit, owing to potential differences in size and chemical nature, need not necessarily exhibit identical pharmacologic specificity, a phenomenon that may be termed "dissociability." Along these lines, Couraud *et al.* (42) found that anti-IdAb to substance P, a naturally occurring neuropeptide, had both agonist and antagonist properties. Their investigation poised a fundamental question: Did the polyclonal antibody examined actually distinguish between two substance P receptors with different structures or were at least two kinds of antibodies present in the sera?

The present study directly demonstrates dissociability between the binding requirements of a ligand and a monoclonal anti-IdAb to the ligand (i.e., GM1 binds pLT-B but Ab9B6 does not). This dissociability was attributable to a single amino acid substitution and allowed identification of an active site residue. In this sense, antigen and internal image-bearing anti-IdAb encode similar stereochemical information but with different "chemical alphabets," giving rise to distinct binding specificities (Fig. 5). By utilizing appropriate screening procedures for hybridoma banks, it should be possible to isolate anti-IdAb that define receptor classes as, for example, muscarine identifies a subset of acetylcholine receptors. Anti-IdAb might also be selected for agonist or antagonist action. Further, these antibodies may prove useful in distinguishing receptor conformation, channel state (i.e., cis or trans), or enzyme isotype. Thus, anti-IdAb and ligands or substrates constitute alternative and complementary probes of protein active sites.

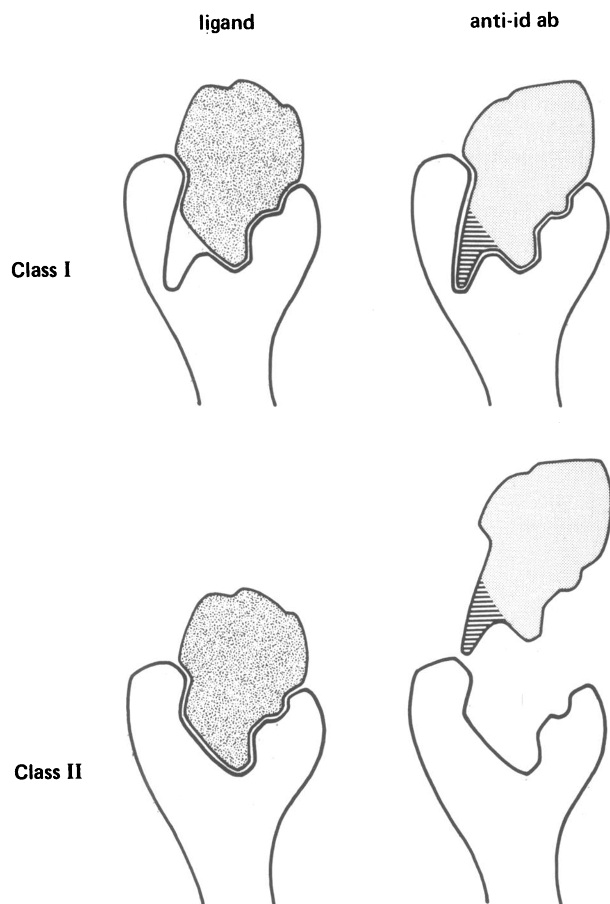

FIG. 5. Dissociation of binding specificity between ligand and "internal image"-bearing anti-IdAb. Ligand and antibody, due to differences in size and chemical nature, may exhibit subtle stereochemical dissimilarities (striped area of antibody). Here, the ligand binds both receptor classes, but the antibody interacts with class 1 only.

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