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Isolation of a Human Monoclonal Antibody with Strong Neutralizing Activity against Diphtheria Toxin

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We isolated a human monoclonal antibody against diphtheria toxin (DT). It bound to fragment B with a binding activity (K_d) of 3.01 nM. The neutralizing activity assayed by the rabbit skin test was estimated to be 73,600 IU/g. This could be used as a therapeutic drug against DT in place of the traditional equine sera.

Antisera prepared from hyperimmune horse blood are still used as drugs against diphtheria toxin (DT) in emergency situations. Since equine antisera could induce serious side effects such as serum sickness, there is a strong need to develop a human monoclonal antibody (Ab) against DT. DT excreted by *Corynebacterium diphtheriae* has been well characterized (12). It is a single polypeptide chain (M_r, 58,000) composed of two structurally distinct regions with three functional domains and contains a protease-sensitive site. The nicked toxin produced upon limited proteolysis consists of two polypeptides that are held together by a disulfide bond. The NH_2-terminal region, fragment A, catalyzes the transfer of the ADP-ribose moiety from NAD to elongation factor 2 and thus blocks protein synthesis (4). The COOH-terminal region, fragment B, binds to a specific receptor on the cell surface and mediates transfer of fragment A to the cytoplasm (6, 11, 14). DT is lethal for susceptible animals, including humans, in doses of 100 ng/kg or less (12). Mass immunization of children has been performed on a worldwide scale since the 1940s. The degree of immunity to DT in the serum of each person should be critical for determination of susceptibility to diphtheria. There is a good correlation between clinical protection and the presence of serum antitoxin, whether this results from disease or immunization. According to internationally accepted definitions, an antitoxin concentration of less than 0.01 IU/ml indicates susceptibility, 0.01 to 0.09 IU/ml indicates basic protection, and >0.1 IU/ml indicates full protection (2). Once the symptoms of this disease start to appear, the antiserum should be given to the patient as soon as possible. The amount of Abs required for curing is much larger than that required to prevent infection. It ranges from 5,000 to 50,000 IU, depending on the degree of disease progress (2).

A human Ab library was screened with DT and diphtheria toxoid (DTD) as the antigen (Ag) by the panning method (3, 5). DT and DTD were kindly given to us by Kunio Ohkuma (Kaketsuken, Kumamoto, Japan). DTD is inactivated toxin that is used for vaccination. It has been prepared by treatment with formaldehyde (13). The Abs were initially prepared in the form of an Ab fused with truncated cp3 (Fab-cp3) and converted to immunoglobulin G1 (IgG1) (3). In this paper, we report data obtained with IgG. Fifty-five different clones were isolated. Four of them, DTD4, DTD8, DTD10, and DTD76, distinctively showed neutralizing activities. The amino acid sequences of these four clones are shown in Fig. 1. Western blotting with separated fragments A and B indicated that DTD4 and DTD76 bound to fragment B and DTD8 and DTD10 bound to fragment A. The rate constants, and thus the binding constants, of these four clones against DTD and DT were measured with the BIAcore instrument (5) (Table 1). Abs were coupled to the sensor chip, and Ags were injected to avoid the influence of divalency. Clones DTD4, DTD8, and DTD10 bound to DT more strongly than to DTD, whereas DTD76 bound to DTD more strongly than to DT.

In vitro DT-neutralizing activities were estimated by the pH color change method (9, 10). When the cells were metabolically active, the color of the medium changed to yellow. When cellular metabolism was stopped by toxin action, it remained red. Thus, the titration endpoint for anti-DT neutralizing activity was taken at the highest dilution of anti-DT Ab to be tested in the well in which the color of the medium was orange. The results are indicated in the left column of values in Table 2. The antitoxin titers are expressed in international units by comparison with the result obtained with equine sera. The in vivo neutralizing activities of Abs against DT were determined by the rabbit skin test as described previously (1, 7). In brief, DT

TABLE 1. Rate constants (k_a, k_d) and dissociation constant (K_d) of IgG form of Abs with DTD and DT assayed by the BIAcore instrument

Clone	Anti-DTD			Anti-DT		
	k_a (10^4 M^{-1} s^{-1})	k_d (10^{-4} s^{-1})	K_d (10^{-9} M)	k_a (10^4 M^{-1} s^{-1})	k_d (10^{-4} s^{-1})	K_d (10^{-9} M)
DTD4	4.14	8.70	21.2	10.6	3.19	3.01
DTD8	4.99	3.82	7.66	16.2	0.831	0.513
DTD10	8.89	3.76	4.22	8.52	0.243	0.285
DTD76	11.9	2.29	1.93	7.8	4.83	6.19

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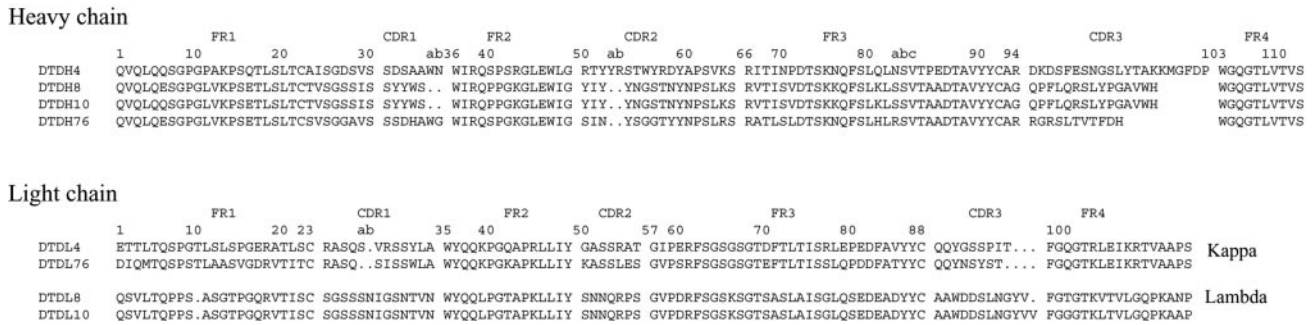

FIG. 1. Amino acid sequences of variable regions of the heavy and light chains of Abs that exhibited neutralizing activities against DT. The numbering of amino acid positions is according to the definition of Kabat et al. (8).

mixed with serially diluted Abs was injected into rabbit back skin. The diameter of local erythema was measured at the site of injection at 48 h postinjection. The results are shown in the rightmost column of Table 2. The antitoxin titers are expressed in international units as relative potency with respect to the result obtained with the standard antitoxin. The neutralizing activities of Abs assayed by the pH color change method and by the rabbit skin test were similar to each other in four cases, indicating a good correlation between the two systems (10).

In the case of DTD4, which showed the strongest neutralizing activity of the four clones, it was estimated to be 73,600 IU/g by the in vivo assay. The binding activity (K_d) with DT was 3.01 nM. On the other hand, while DTD76 bound to DTD with strong affinity, it showed very weak neutralizing activity. Although both clones bound to fragment B, they should recognize completely different epitopes. It is possible that clone DTD4 corresponded to the Ab that had matured in vivo by immunization with vaccine against DT. We propose that DTD4 be clinically tested for therapeutic use.

Nucleotide sequence accession numbers. The nucleotide sequences of the eight genes described in Fig. 1 have been registered in GenBank under accession numbers AB063724 (DTDH4), AB063723 (DTDH8), AB063729 (DTDH10), AB063743 (DTDH76), AB063937 (DTDL4), AB064049 (DTDL76), AB063977 (DTDL8), and AB064205 (DTDL10).

TABLE 2. Neutralizing activity against DT shown by IgG form of Abs

Clone	Neutralizing activity[a]	
	In vitro assay[b]	In vivo assay[c]
DTD4	52,000	73,600
DTD8	4,800	3,360
DTD10	2,300	1,612
DTD76	215	372

[a] In international units per gram.
[b] Measured by the pH color change method.
[c] Measured by the rabbit skin test.

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