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Anti-epitope antibody, a novel site-directed antibody against human acetylcholinesterase[1]

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KEY WORDS acetylcholinesterase; butyrylcholinesterase; epitopes; antibodies; enzyme-linked immunosorbent assay; Western blotting

ABSTRACT

AIM: To construct synthetic antigens using the epitope of human brain acetylcholinesterase (hbAChE) for induction and detection of the specific antibody against the epitope, and to analyse the immunogenicity of the antibody. **METHODS:** The epitope (RTVLVSMNYR, amino acids 143-152) of hbAChE was chemically synthesized, coupled with the carrier protein keyhole limpet hemocyanin (KLH) to construct an artificial immunogen (KLH-epitope), and injected into rabbits to raise antibody. The epitope conjugated with bovine serum albumin (BSA) was used as the detection antigen. The specificity of the antibody was tested by enzyme-linked immunosorbent assay (ELISA) and Western blotting. The immunoreaction between the anti-recombinant human butyrylcholinesterase (rhBChE) polyclonal antibody and the biotinylated-epitope was examined by indirect ELISA. **RESULTS:** The erythrocyte AChE, the hbAChE, rhBChE and the BSA-epitope all immunoreacted with the anti-epitope antibody against the epitope (143-152) of hbAChE, whereas the *torpedo* AChE did not. **CONCLUSION:** The hbAChE, the human erythrocyte AChE and hBChE share the conservative antigenic epitope RTVLVSMNYR, hence they can all immunoreact with the anti-epitope antibody. Since the epitope of hbAChE is less similar with the aligned amino acid sequences of AChE of *Torpedo californica* or *Torpedo marmorata*, there is not any immunoreactivity between them. The R, M, and N residues in the epitope seem to be necessary radicals for the conservation of antigenicity.

INTRODUCTION

Acetylcholinesterase (AChE, EC 3.1.1.7) is one of the essential components in the nervous system. Acetylcholine is hydrolyzed immediatelly by AChE after accomplishment of chemical transmission, thus maintaining the normal function of nerves. Therefore, great attention has been paid to study the structure and function of AChE[1].

There are two types of antibody preparations, polyclonal and monoclonal. The polyclonal antibody against a certain antigen contains actually a set of different antibodies that recognize various epitopes of the antigen, whereas the monoclonal antibody directs exactly to a definite epitope of it. The monoclonal antibody is usually produced by using a single population of identical hybridoma cells, thus consists of homogeneous antibodies recognizing the same epitope. Quite a few of sensitive analytical assays have been developed from monoclonal antibodies versus various proteins, and the antigen-antibody interaction is widely used to qualify

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and localize proteins. In the usage of monoclonal antibody, though it specifically binds to the protein therefrom it is induced, however it does not indicate which part of the protein molecule has been bound to. Recently, the B-cell epitopes on the human brain acetylcholinesterase (hbAChE) has been thoroughly studied, and 11 antigenic epitopes have already been identified from a combinatorial decapeptide library[2,3]. In this paper, we attempted to produce an anti-epitope antibody against the No 2 epitope (RTVLVSMNYR, amino acids 143-152) of hbAChE, using chemically synthetic antigens. A novel anti-epitope antibody binding to a definite sequence of protein will be made use of a sophisticated probe in protein chemistry.

MATERIALS AND METHODS

Animals White rabbits (Japanese species, male, 1.8-1.9 kg) were obtained from the Animal Breeding Center affiliated to the Academy of Military Medical Sciences.

Chemicals Keyhole limpet hemocyanin (KLH), *m*-maleimidobenzoyl-*N*-hydrosuccinimide ester (MBS), diamino benzidine tetrahydrochloride (DAB) and bovine serum albumin (BSA) were purchased from Sigma Chemical Co (USA). Recombinant human butyrylcholinesterase (rhBChE), hbAChE, human erythrocyte AChE and anti-rhBChE polyclonal antibody were prepared in our lab[4]. Nitrocellulose (NC) membrane was purchased from Bio-Rad Inc (USA). Microtiter plates were obtained from Nunc Co (Denmark). Horseradish-peroxidase-lablled sheep-anti-rabbit IgG (HRP-IgG) was from the Institute of Microbiology and Epidemiology (Beijing, China). All other chemicals were of AR grade.

Antigen The epitope (143-152) was synthesized according to Houghten[5], and conjugated to KLH or BSA to form two synthetic antigens (peptide-KLH for immunization and peptide-BSA for detection) according to Good *et al*[6].

KLH 5 mg was reacted with MBS 2 mg. The reaction product KLH-MBS was passed through a Sephadex G-25 column equilibrated and eluted with sodium phosphate buffer (PBS, pH 7.4) 50 mmol/L to remove the free MBS. Recovery of the conjugate KLH-MBS from the eluate (monitored by absorbance at 280 nm) was estimated at 80 %. The pooled effluent was lyophilized, redissolved, and reacted with 5 mg of the epitope (143-152). The reaction was carried out for 3 h at 20 ºC with stirring, and then dialyzed against PBS to remove the uncoupled peptide. The immunogen KLH-epitope was kept frozen at -20 ºC. The antigen BSA-epitope was also synthesized likewise.

Antibody Two rabbits were injected intradermally 600 μg KLH-epitope of each in complete Freund's adjuvant (1:1) on d 1, 600 μg KLH-epitope in incomplete Freund's adjuvant (1:1) on d 14 and week 9, and 400 μg KLH-epitope on week 14. Small amounts of rabbit blood were taken 10 d after each injection, and the rabbits were exsanguinated 2 weeks after the final booster.

Enzyme-linked immunosorbent assay (ELISA) The microtiter plate was coated with peptide-epitope 4 mg/L in sodium carbonate buffer (pH 9.6) 50 mmol/L at 4 ºC overnight, and blocked with 1 % BSA (100 μL/well) for 1 h at room temperature. The rabbit antiserum diluted in blocking buffer was added to the plate and incubated at 37 ºC for 1 h. The diluted HRP-IgG was then added to each well, followed by incubation at 37 ºC for 1 h. Intermittent washings of the microplate with buffer were carried out before each addition. The chromogenic enzyme reaction was initiated by adding the substrate solution (0.04 % *O*-phenydiamine, citric acid 35 mmol/L, sodium phosphate buffer 65 mmol/L, pH 5.0, 0.02 % H_2O_2), and reacted for 15 min at room temperature in dark. The reaction was stopped by adding 50 μL of H_2SO_4 2 mmol/L. The absorbance was read at 492 nm.

Competitive immunoassay The microtiter plate was coated with the BSA-epitope solution (400 ng/well) and blodked by BSA as above-mentioned. The diluted antiserum (1:1000) was first mixed with the same volume of serially diluted BSA-epitope , incubated at 37 ºC for 30 min, and then added to the plate for 1 h incubation. After washing of the plate, the following reactions were carried on as in ELISA.

Western blotting The rhBChE samples were analysed by SDS-PAGE[7]. The transferred NC membrane was blocked by 1 % BSA in PBS (pH 7.4) 0.05 mmol/L, incubated with anti-epitope antibody (diluted 1:200 in blocking buffer), washed and incubated with diluted HRP-IgG. After washing , the substrate solution (DAB 0.6 g/L, 0.03 % H_2O_2 in Tris-HCl 0.05 mmol/L, pH 7.6) was added and shaken at room temperature. Then the reaction mixture was discarded, and the NC membrane was washed with PBS.

Dot blotting Dot-blot immunoassays were carried out as described by Sorensen *et al*[8]. Aliquots of 0.5 U of each ChE sample in 5 μL of sodium phosphate

buffer (pH 7.4) 10 mmol/L containing NaCl 144 mmol/L and 0.1 % Triton X-100 were spotted onto NC membrane, denatured by incubation in buffer containing 10 % SDS at 95 ºC for 5 min, and then blocked for 2 h with 2 % BSA in PBS. The following procedures were the same as Western blotting.

Cross-immunoreaction Indirect ELISA was previously described by Zhang *et al*[2]. Then the biotinylated peptides (60 mg/L) was coated on the plate. The purified rabbit anti-rhBChE polyclonal antibody (7.2 mg/L) was added to the wells. The mouse anti-human AChE polyclonal antibody was used as the positive control. Absorbance at 492 nm (A_{492}) higher than two folds of the negative control were judged as positive reaction.

RESULTS

Generation of the specific anti-epitope antibody The titers of the anti-sera from two KLH-epitope-immunized rabbits against the epitope (143-152) of human brain AChE were assessed by ELISA. The results showed that the titer of the rabbit sera came to 32000 and 16 000, respectively (Tab 1).

Tab 1. ELISA titer of rabbit anti-epitope (143-152) antiserum. *n*=3. Mean±SD.

Dilution	A_{492} Antiserum of Rabbit 1	Antiserum of Rabbit 2	Normal Rabbit Serum
1:1000	2.03±0.08	1.70±0.05	0.184±0.001
1:2000	1.65±0.08	1.17±0.06	0.160±0.003
1:4000	1.185±0.028	0.719±0.028	0.139±0.002
1:8000	0.795±0.016	0.44±0.03	0.124±0.004
1:16000	0.496±0.004	0.286±0.019	0.116±0.002
1:32000	0.316±0.001		0.104±0.011

Titer is the reciprocal of dilution. Immunogen: KLH-epitope (143-152); Detection antigen: BSA-epitope (143-152).

Competitive ELISA The immunoreaction between the antibody and the antigen BSA-epitope was measured by competitive ELISA. It could be seen that along with the increase of the concentations of the synthetic antigen, the absorbances gradually decreased (Fig 1). It implies that the anti-epitope (143-152) antibody immunoreacts well with the antigen.

Western blotting The Western blotting showed

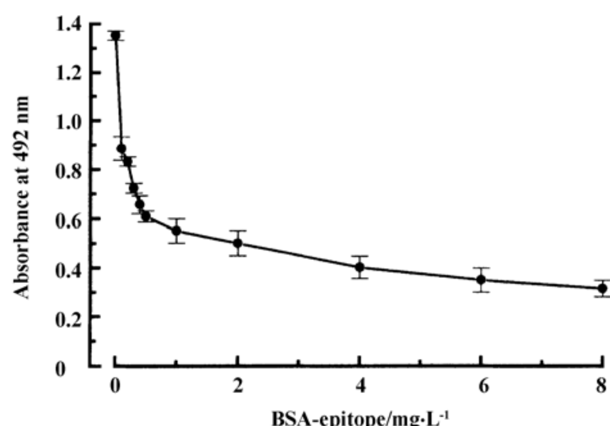

Fig 1. Competitive ELISA between anti-epitope antibody and different concentrations of synthetic antigen BSA-epitope. *n*=3. Mean±SD. Dilution of the rabbit-anti-epitope (143-152) antibody used in ELISA was 1:1000.

that the rabbit anti-epitope antibody could bind to the rhBChE (71 kDa and 65 kDa, two types of rhBChE with different glycosylations) (Fig 2), which indicates that the anti-hAChE epitope (143-152) cross-immunoreacts

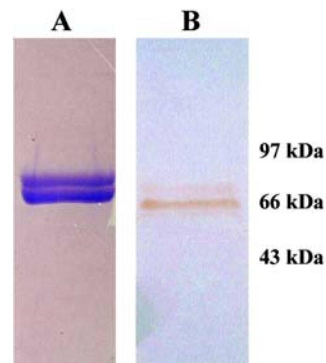

Fig 2. Western blotting of rhBChE *vs* anti-epitope (143-152) antibody. A) SDS-PAGE (8 %) was performed under reducing condition; B) Western blot of rhBChE.

with the hBChE.

Dot blotting The reactions of anti-epitope antibody with native and heat-/SDS-denatured enzymes from various species were further assessed by the dot-blot method. Native and denatured human erythrocyte AChE, human brain AChE, and rhBChE reacted well with the anti-epitope antibody on dot-blots, whereas no reaction could be seen in case of *Torpedo* AChE (Tab 2).

Cross-immunoreaction of anti-rhBChE polyclonal antibody with the biotinylated epitope (143-152) The rabbit anti-rhBChE polyclonal antibody

Tab 2. Dot-blot (on nitrocellucose membrane) immuno-analysis of anti-epitope (143-152) antibody *vs* various ChEs.

Source	Native enzyme	Denatured enzyme
Human erythrocyte AChE	+	+
Human brain AChE	+	+
rhBChE	+	+
Torpedo AChE	−	−

Titer is the reciprocal of dilution. Immunogen: KLH-epitope (143-152); Detection antigen: BSA-epitope (143-152).

showed obvious cross-immunoreaction with the epitope (143-152) (Fig 3). The alligned sequence RVIVVSMNYR of hBChE shares 70 % of the amino acid residues with that of hbAChE (Fig 4).

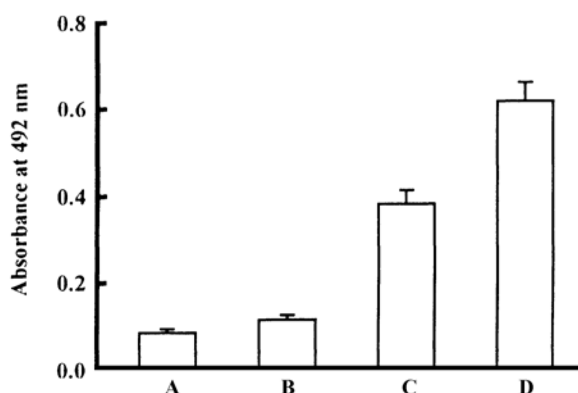

Fig 3. Indirect ELISA between hBChE polyclonal antibody and epitope (143-152) of human AChE. A) Normal rabbit serum control; B) Peptide 10-19 (VTVRGGRLRG) located at the non-epitopic region of the hbAChE; C) Rabbit anti-hBChE polyclonal antibody; D) Mouse anti-hAChE polyclonal antibody. *n*=3. Mean±SD. Ratio (A_{492} of sample/ A_{492} of negative control) ≥2 denotes positive cross-immuno-reaction.

DISCUSSION

In this paper, we successfully produced an anti-epitope antibody directed to a definite sequence in the antigenic region of a protein by immunization with a chemically synthesized immunogen using the epitope as a haptene.

The anti-epitope (143-152) antibody exclusively cross-immunoreacted with human cholinesterases of different types other than *Torpedo* AChEs. In comparison of the amino acid sequence of the No 2 epitope of hbAChE with that of the other cholinesterases from various sources[9-12], it seems that the R,M,N residues are critical in the conservation of the immunogen specificity to humans (Fig 4).

Anti-epitope immunoglobulin functions itself as a specific antibody similar to monoclonal antibody with better recognition to the targeted site on the protein molecule. Site-directed antibodies will be quite useful in the study of the structure and function of proteins. Preparation of a series of anti-epitope antibodies against the other ten epitopes of hbAChE has been undertaken in our lab. The inhibitive, if any, or the discriminative antibodies versus human brain, erythrocyte acetylcholinesterase and butyrylcholinesterase will be expected to be useful in study of human diseases.

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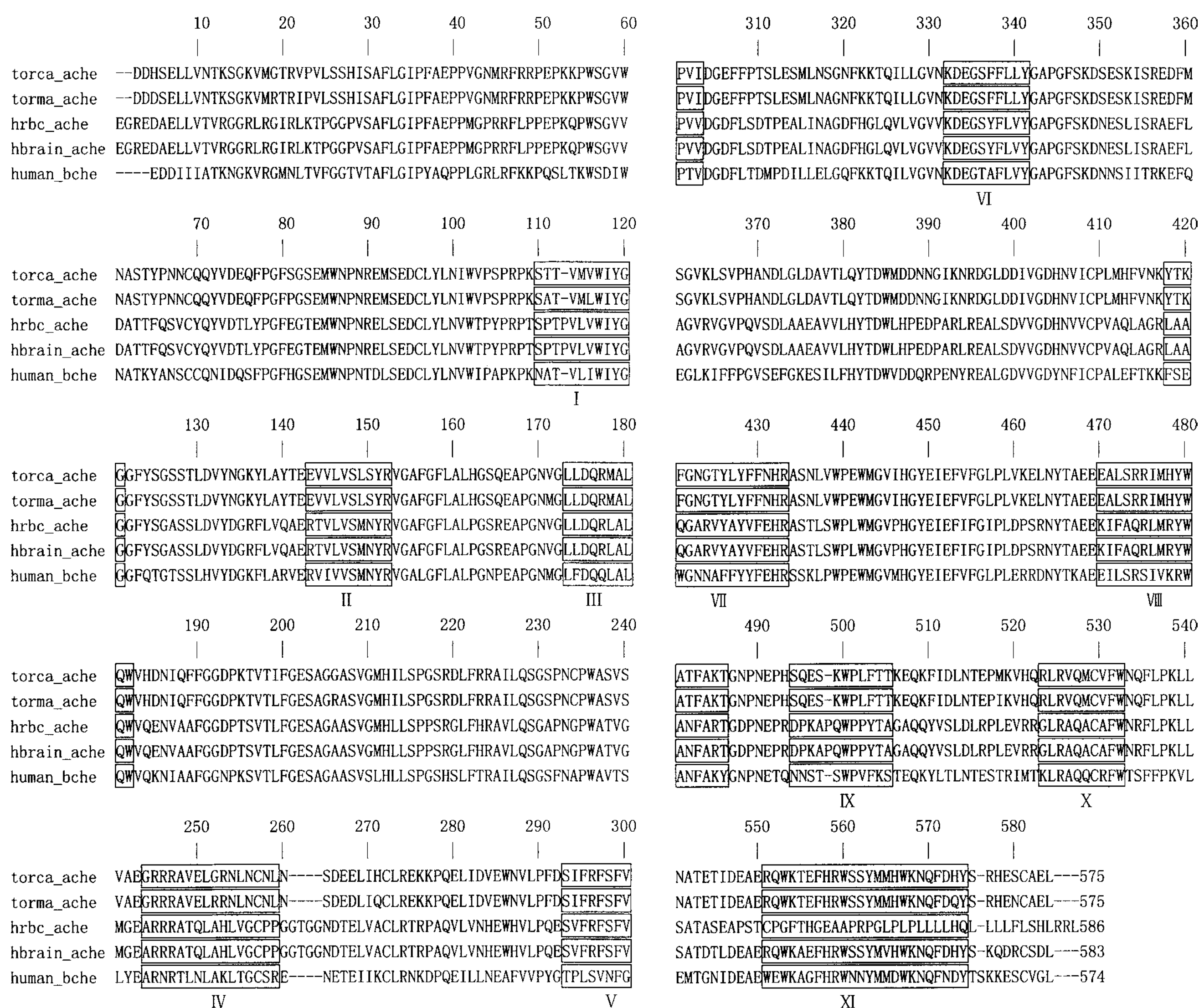

Fig 4. Sequence homology and similarity of cholinesterase. torca_ache: torpedo californica AChE; torma_ache: torpedo marmorata AChE; hrbc_ache: human erythrocyte AChE; hbrain_ache: human brain AChE; human_bche: human butyrylcholinesterase.

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