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Antibody and T cell recognition of the light chain of botulinum neurotoxin A in two high-responder mouse strains

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ABSTRACT

We investigated in two inbred mouse strains the submolecular recognition of botulinum neurotoxin type A (BoNT/A) by Abs (B cells) and by T lymphocytes. For mapping, we employed a set of overlapping synthetic peptides that encompassed the entire light (L) chain of BoNT/A. After 3 BoNT/A toxoid injections, BALB/c T cells responded *in vitro* to challenge by peptides L18 (residues 239–257), L23 (309–327), L27 (365–383), L29 (393–411), or L31 (421–439) and more weakly to peptides L3 (29–47), L9/L10 (113–145), L15 (197–215), L17 (225–243), or L26 (351–369). The other peptides stimulated little or no T cell responses. SJL mice mounted, after 3 BoNT/A injections, stronger T cell responses that were medium-to-strong to peptides L2/L3 (15–47), L10/L11 (127–159), L19 (253–271), or L23 (309–327) and low to peptides L17 (225–243), L21 (281–299), L27 (365–383), or L30/L31 (407–439). After 3 BoNT/A injections, BALB/c and SJL antisera protected mice against lethal BoNT/A doses, but displayed restricted epitope profiles compared to outbred (ICR) mice Abs. BALB/c Abs displayed medium-to-high binding to peptides L4/L5 (43–75), L10/L11 (127–159), L18 (239–257) or L27 (365–383). SJL Abs were high to peptides L4/L5 (43–75), L14 (183–201), L16 (211–229), or L18/L19 (239–271), and medium to peptides L10 (127–145), L11 (141–159), L12 (155–173) or L29 (393–411). The other peptides had little or no binding. Responses to each T cell or Ab epitope were under separate genetic control. T and B (antibody) cell recognition regions may coincide, but there were also regions recognized only by Abs or by T cells.

Introduction

Botulinum neurotoxins (BoNTs) are produced by strains of the anaerobic bacterium Clostridium botulinum in seven serologically distinct serotypes (A–G). The toxin is synthesized as an inactive, 150 kDa single polypeptide chain, which is activated by proteolytic cleavage of a peptide bond to give a heavy (H, 100 kDa) and a light (L, 50 kDa) chain that are held together by a disulfide bond. BoNTs have three functionally distinct domains that play different roles in cell poisoning (Montecucco and Schiavo 1995; Simpson 1989). The L chain is a Zn^{2+}-dependent endopeptidase (Schiavo et al. 1992a,b; Fu et al. 1998), while the H chain is involved in binding to nerve cell receptor at the presynaptic neuromuscular junction and in intracellular translocation.

Botulinum neurotoxins (BoNTs) are the most toxic substances known. Active protection against toxin poisoning may be achieved by vaccination or by transfer of protective Abs (Aoki et al. 2010). Protective Abs are directed primarily against the H chain. But there are also regions on the L chain that are found to stimulate protective Abs (Atassi et al. 2011; Dolimbek et al. 2011a). It is essential to understand the role of T-cell recognition in the development of antibody responses against the toxin and in protection against BoNT poisoning. Identification of the regions of recognition by T cells and by Abs in the same host and how these responses collaborate and interact is crucial for the development of synthetic anti-toxin vaccines and for the design of strategies for manipulation of anti-toxin immune responses.

We have mapped human Ab responses to the toxin (Dolimbek et al. 2007; Atassi et al. 2011), but there are difficulties in studying the human T lymphocyte responses. Therefore we have performed these studies in mice. There are similarities in the regions recognized by anti-BoNT/A Abs of human and other species (Atassi and Dolimbek 2004; Atassi et al. 1996, 2011; Dolimbek et al. 2007, 2011a). Previously, we had mapped the Ab and T cell recognition on the H_C domain (C-terminal domain, residues 855–1296, of the

Abbreviations: Ab, Antibody; BoNT, Botulinum neurotoxin; BoNT/A, Botulinum neurotoxin serotype A; BoNT/B, Botulinum neurotoxin serotype B; BSA, Bovine serum albumin; L chain, The light chain (residues 1–448) of BoNT/A; H chain, The heavy chain (residues 449–1296) of BoNT/A; LNC, Lymph node cells; PBS, 0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.20; SI, Stimulation index = mean cpm incorporated by stimulated cells/mean cpm incorporated by unstimulated cells.

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Table 1
Synthetic peptides of the light chain of BoNT/A.[a]

Pept No.	Residue numbers	Amino acid sequence
L1	1–19	MPFVNKQFNYKDPV**NGVDI**
L2	15–33	**NGVDI**AYIKIPNVG**QMQPV**
L3	29–47	**QMQPV**KAFKIHNKI**WVIPE**
L4	43–61	**WVIPE**RDTFTNPEE**GDLNP**
L5	57–75	**GDLNP**PPEAKQVPV**SYYDS**
L6	71–89	**SYYDS**TYLSTDNEK**DNYLK**
L7	85–103	**DNYLK**GVTKLFERI**YSTDL**
L8	99–117	**YSTDL**GRMLLTSIV**RGIPF**
L9	113–131	**RGIPF**WGGSTIDTE**LKVID**
L10	127–145	**LKVID**TNCINVIQP**DGSYR**
L11	141–159	**DGSYR**SEELNLVII**GPSAD**
L12	155–173	**GPSAD**IIQFECKSF**GHEVL**
L13	169–187	**GHEVL**NLTRNGYGS**TQYIR**
L14	183–201	**TQYIR**FSPDFTFGF**EESLE**
L15	197–215	**EESLE**VDTNPLLGA**GKFAT**
L16	211–229	**GKFAT**DPAVTLAHE**LIHAG**
L17	225–243	**LIHAG**HRLYGIAIN**PNRVF**
L18	239–257	**PNRVF**KVNTNAYYE**MSGLE**
L19	253–271	**MSGLE**VSFEELRTF**GGHDA**
L20	267–285	**GGHDA**KFIDSLQEN**EFRLY**
L21	281–299	**EFRLY**YYNKFKDIA**STLNK**
L22	295–313	**STLNK**AKSIVGTTASLQYM
L23	309–327	**SLQYM**KNVFKEKYL**LSEDT**
L24	323–341	**LSEDT**SGKFSVDKL**KFDKL**
L25	337–355	**KFDKL**YKMLTEIYT**EDNFV**
L26	351–369	**EDNFV**KFFKVLNRK**TYLNF**
L27	365–383	**TYLNF**DKAVFKINI**VPKVN**
L28	379–397	**VPKVN**YTIYDGFNL**RNTNL**
L29	393–411	**RNTNL**AANFNGQNT**EINNM**
L30	407–425	**EINNM**NFTKLKNFT**GLFEF**
L31	421–439	**GLFEF**YKLLCVRGI**ITSKT**
L32	435–453	**ITSKT**KSLDKGYNK**ALNDL**

[a] Primary structure of the synthetic peptides of the L chain of BoNT/A. The peptides encompassed the entire 448-residue L chain and overlapped consecutively by 5 residues. The 5-residue overlaps between consecutive peptides are underlined and bolded. Peptide L 32 ends with a 5-residue overlap with the next peptide (i.e., first peptide of the H chain previously studied; Atassi and Dolimbek, 2004; Atassi et al., 2005; Dolimbek et al., 2007; Maruta et al., 2004).

H chain of BoNT/A) in BALB/c (H-2^d) and SJL (H-2^s), which are high responders to BoNT/A (Oshima et al. 1997; Rosenberg et al. 1997). In another study, we reported the localization of the regions on the H_N domain (residues 449–859 of the H chain of BoNT/A) that are recognized by Abs and T cells obtained after immunization with BoNT/A (Dolimbek et al. 2005). The current studies report the mapping of the L chain regions that are recognized by Abs and/or by T lymphocytes of BALB/c and SJL mice, and thus completing the elucidation of the Ab and T lymphocyte recognition profiles in these two mouse strains of the entire BoNT/A molecule.

Materials and methods

Animals and materials

Female BALB/c (H-2^d) (National Cancer Institute, Frederick, MD) and SJL/JCr (H-2^s) (Jackson Laboratory, Bar Harbor, ME) mice, 6–8 weeks old, were used in these studies. Active BoNT/A and formaldehyde-inactivated toxoids of BoNT/A and BoNT/B were purchased from Metabiologics (Madison, WI). Formaldehyde was removed from the toxoid by dialysis against 0.01 M sodium phosphate buffer, pH 7.2 containing 0.15 M NaCl (PBS) before use. Synthesis, purification and characterization of the 32 19-residue peptides (Table 1) that overlapped consecutively by five residues and corresponded to the entire L chain (residues 1–448) were described elsewhere (Dolimbek et al. 2011a).

Preparation of anti-toxin antibodies

Mouse antisera were prepared by injection of the mice subcutaneously in the hind footpads with 5 μg of toxoid emulsified in Complete Freund's adjuvant. The mice were given 2 boosters at 4 and 8 weeks with a similar dose of toxoid, using incomplete Freund's adjuvant (Difco Laboratories, Detroit, MI) instead of Complete Freund's adjuvant. Sera were collected prior to immunization (pre-immune sera) and 2 weeks after each injection. For each mouse strain, antisera of the respective bleeds from 10 mice were pooled and kept at −20 °C until use. The antisera collected after the last injection (week 10), were employed for binding studies to the peptides. The presence of blocking Abs in the mouse antisera against BoNT/A was confirmed by a mouse protection assay as described (Dolimbek et al. 2005).

Lymphocyte proliferative assay

At the time of the last bleed, the inguinal and periaortic lymph nodes were removed and single cell suspensions were prepared in RPMI 1640 medium. The lymph node cells (LNC) were washed, re-suspended in RPMI 1640 containing 10 mM HEPES, 5×10^{-5} M 2-mercaptoethanol and 1% normal mouse serum (Rosenberg et al. 1997). The number of viable cells was obtained by vital staining with fluorescein diacetate. A fixed number of viable LNC (5×10^5 cells/well) was co-cultured in triplicates in flat-bottom 96-well plates (Corning, Corning, NY) with various concentrations (1.2–5 μg/ml) of inactivated BoNT/A, synthetic L-chain peptides (10–40 μg/ml) or inactivated BoNT/B (1.2–5 μg/ml). Controls included unstimulated cells and cells stimulated with ConA (1–2 μg/ml), PPD (2 μg/ml), unrelated protein (hen lysozyme and ovalbumin, 25–100 μg/ml; myoglobin, 6 μg/ml) or a synthetic peptide (Sequence, ESSGTGIESSGTGI; 10–40 μg/ml) that is unrelated to the BoNT/A peptides. After incubation for 3 days at 37 °C in a humidified, 5% CO_2 atmosphere, the lymphocytes were pulsed for 18 h with [^{3}H]thymidine (1 μCi/well) (MP Biomedicals, Santa Ana, CA) then harvested onto glass microfiber filters (Whatman, Clinton, NJ) and counted by liquid scintillation. Results were expressed in stimulation index (SI = mean cpm incorporated by stimulated cells/mean cpm incorporated by unstimulated cells) at the optimum challenge dose of each antigen. For the purpose of this study, an SI value >2.0 is considered as a positive response.

Solid-phase radioimmunoassay

Staphylococcal protein A (Pharmacia Biotech, Piscataway, NJ) was radiolabeled with ^{125}I (PerkinElmer, Billerica, MA) using the chloramine-T method (Hunter and Greenwood 1962). Unbound ^{125}I was removed from the radiolabeled protein A by gel filtration on a Sephadex G-25 (Pharmacia Biotech, Piscataway, NJ) column (0.8 cm × 20 cm), equilibrated with PBS containing 0.1% bovine serum albumin (BSA) (Sigma Chemicals, St. Louis, MO). Binding of mouse Abs to BoNT/A or to peptides was determined by a solid-phase radioimmunoassay. Polyvinylchloride 96-well plates (Becton Dickinson Labware, Oxnard, CA) were coated with each of the 32 overlapping L chain peptides (2.5 μg in 50 μl of PBS/well) or with BoNT/A (1 μg in 50 μl of PBS/well). Proteins and synthetic peptides unrelated to BoNTs were used as negative controls. The plates were incubated overnight at 4 °C then extensively washed with PBS and incubated (1 h at 37 °C) with 1% BSA in PBS (100 μl/well) to prevent nonspecific binding in subsequent steps. After washing with PBS, the plates were incubated (3 h at 37 °C) with mouse antisera (50 μl/well) that had been pre-diluted 1:500 (v/v) in 0.1% BSA in PBS. The wells were washed with PBS, incubated (37 °C, 2 h) with 50 μl of affinity-purified rabbit anti-mouse (IgG + IgM) antisera (Accurate Chem. Sci. Corp., Westbury, NY) prediluted 1:1000