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In Vivo Molecular Imaging Analysis of a Nasal Vaccine That Induces Protective Immunity against Botulism in Nonhuman Primates

Yoshikazu Yuki,*† Tomonori Nochi,*† Norihiro Harada,† Yuko Katakai,‡ Hiroaki Shibata,§ Mio Mejima,* Tomoko Kohda,¶ Daisuke Tokuhara,* Shiiro Kurokawa,* Yuko Takahashi,* Fumiko Ono,‡ Shunji Kozaki,¶ Keiji Terao,¶ Hideko Tsukada,‡ and Hiroshi Kiyono*†

Nasal administration is an effective route for a needle-free vaccine. However, nasally administered Ags have the potential to reach the CNS directly from the nasal cavity, thus raising safety concerns. In this study, we performed real-time quantitative tracking of a nasal vaccine candidate for botulism, which is a nontoxic subunit fragment of Clostridium botulinum type A neurotoxin (BoHc/A) effective in the induction of the toxin-neutralizing immune response, by using [18F]-labeled BoHc/A–positron-emission tomography, an in vivo molecular imaging method. This method provides results that are consistent with direct counting of [18F] radioactivity or the traditional [111In]-radiolabel method in dissected tissues of mice and nonhuman primates. We found no deposition of BoHc/A in the cerebrum or olfactory bulb after nasal administration of [18F]-labeled BoHc/A in both animals. We also established a real-time quantitative profile of elimination of this nasal vaccine candidate and demonstrated that it induces highly protective immunity against botulism in nonhuman primates. Our findings demonstrate the efficiency and safety of a nasal vaccine candidate against botulism in mice and nonhuman primates using in vivo molecular imaging. The Journal of Immunology, 2010, 185: 000–000.
PET imaging agents usually consist of small-molecule tracers, such as glucose analogs or neurorceptor ligands (9), and rarely consist of proteins (11). In this study, we attempted to develop a PET imaging system that uses a protein tracer for evaluating nasal vaccine candidates in multiple species. For this approach, we chose to evaluate a recombinant form of the nontoxic fragment of the C-terminal half of the H chain of *Clostridium botulinum* type A neurotoxin (BoHc/A) as a subunit vaccine candidate against botulism (12). It has been shown that systemic immunization of primates with a similar preparation of type B (BoHc/B) resulted in the generation of neutralizing Abs (response (13). Because of the potential for botulinum neurotoxin to be disseminated by bioterrorists via airborne or oral routes and to result in a high mortality rate, it was classified as a category A agent by the U.S. Centers for Disease Control and Prevention. Thus, the development of an effective nasal vaccine against botulism is considered a potent strategy to prevent inhalation and gastrointestinal botulism (14).

In this paper, we show that a nasal BoHc/A vaccine candidate provided full protective immunity against toxin-based neurologic complications in nonhuman primates. To assess the safety issue of redirection of the Ag to the CNS, we developed PET with [18F]-labeled BoHc/A and evaluated the ADME of the nasal BoHc/A vaccine in both mice and nonhuman primates. In contrast with traditional whole-body autoradiography, the use of PET in combination with CT or MRI allowed noninvasive and dynamic in vivo quantitative imaging of [18F]-BoHc/A. Both PET and direct tissue counting showed no evidence of CNS deposition of the vaccine Ag in the cerebrum or OB after nasal administration of [18F]-BoHc/A or the more traditional [111In]-BoHc/A in mice and nonhuman primates.

### Materials and Methods

#### Preparation of *C. botulinum* neurotoxin type A

*C. botulinum* neurotoxin type A (BoNT/A) from *C. botulinum* type A was purified from the culture supernatant as previously described (15). The toxicity of purified BoNT/A (1.1 × 10^10^ mouse i.p. LD50/mg protein) was assayed by time to death after i.v. injection into mice (16).

#### Preparation of botulinum mucosal vaccine (BoHc/A)

The gene encoding the nontoxic C-terminal fragment of H chain of type A botulinum neurotoxin (strain 62A; aa 872–1296), BoHc/A, was synthesized by PCR with Phusion High-Fidelity polymerase (Daiichi Pure Chemicals, Tokyo, Japan) and specific primers (sense, 5′-CGTCGGGATCCATAATTTAGGATTGGATTG-3′; antisense, 5′-TAGTATGGCACTTACAGTGGCCTTCTCCTCCATCC-3′; BamHI and SalI restriction enzyme sites shown by underlining). After digestion with BamHI and SalI, the gene fragment was inserted into the pGEX-6P3 (GE Healthcare, Piscataway, NJ). After DNA sequencing, the plasmid was used to transform Rosetta 2(DE3) pLysS cells (Novagen, Madison, WI), and the cells were grown on LB agar plates containing 34 μg/ml chloramphenicol (Cm) for selection of Cm-resistant colonies. A single colony was used to inoculate a 5-ml culture in LB containing 34 μg/ml Cm. The overnight culture (1 ml) was used to inoculate 100 ml of LB containing 34 μg/ml Cm and 100 μg/ml ampicillin (Ap). The culture was grown at 37°C until the OD600 was 0.8, at which point the bacteria were induced with 0.1 mM isopropyl-p-b-D-1-thiogalactopyranoside (IPTG) (Roche, Basel, Switzerland) for 4 h at 28°C to induce BoHc/A expression. The cells were then suspended in PBS and sonicated on ice. After centrifugation, the supernatant was loaded onto an Octyl-Sepharose column (GE Healthcare) and eluted with PBS. The fusion protein on the column was treated with PreScission Protease (GE Healthcare) overnight at 4°C to cleave between GST and BoHc/A, and the digested fragment containing BoHc/A was collected. Finally, purification by gel filtration on a Superdex 5-100 (GE Healthcare) column was performed. Purified BoHc/A was confirmed by liquid chromatography/mass spectrometry (LC/MS-MS). A GLP-compliant Bioassay Study after treatment with trypsin gold (Promega, Madison, WI). The level of LPS contamination in the purified BoHc/A (<10 endotoxin units/mg protein) was measured by using the Limulus A Single Test (Wako, Osaka, Japan).

#### Synthesis of [18F]-BoHc/A and [18F]-cholera toxin

[18F]-BoHc/A was labeled with N-succinimidyl-4-[18F]fluorobenzoate ([18F]SFB). In brief, [18F]fluoride was produced by the 18O(p, n)[18F] nuclear reaction by the use of a cyclotron (HM-18; Sumitomo Heavy Industries, Tokyo, Japan) at Hamamatsu Photonics PET Center. [18F]SFB was isolated from 4-tert-butylammonium triflate (17) and [18F]KF/K[2,2,2] according to the procedure of Tanaka et al. (18) with some modifications. [18F]KF/K[2,2,2] complex was added to 5 mg of the precursor in 1.5 ml acetonitrile (CH3CN) and reacted at 80°C for 10 min. After cooling the reaction mixture, 20 μl 1M tetra-n-propylammonium hydroxide (Pr4NOH) in 0.5 ml CH3CN was added and hydrolyzed at 120°C for 5 min. N,N,N′-N′-tetramethyl-O-(N-succinimidyl)urea tetrfluoroacetate (15 mg) in 0.5 ml CH3CN was added to the reaction mixture and reacted at 80°C for 5 min to produce [18F]SFB. The reaction mixture was diluted with 1.5 ml of 5% acetic acid (CH3COOH) and transferred to an HPLC injector. An extra 1.5 ml of 5% CH3COOH was used to rinse the reaction vessel. The combined crude product was purified by semi-preparative HPLC (HPLC conditions: Inertsil ODS-3 column, CH3CN: H2O = 300:700, 6 ml/min, detection: 254 nm). The radioactive peak that eluted at 13.5 min was collected, diluted with 30 ml H2O, and processed through a Sep-Pak C18 cartridge (Nihon Waters) and transferred to a vial through a Sep-Pak Dry cartridge (Nihon Waters). The decay-corrected radiochemical yield of [18F]SFB was 28.1–38.3% (average, 33.5%; n = 4), based on [18F]fluoride, and the radiochemical purity was ≥95%. The concentration of [18F]SFB was then concentrated at 60°C under He flow (200 μl/min) and used directly for next step. [18F]SFB was added to a solution of 0.5 M borate buffer (pH 8.5, 200 μl), PBS (100 μl), BoHc/A (4.1 mg/ml, 200 μl), and allowed to react at room temperature for 30 min. Purified BoHc/A was successfully radiolabeled by conjugation with [18F]SFB, which reacted with free amino groups including an N-terminal and e-Lys amino groups in the protein. The product was purified by gel-permeation chromatography (Superose 12, PBS, 1 ml/min), and the radioactive peak eluted at 13.5 min was collected. The 373–698 MBq (average, 548 MBq, n = 4) [18F]-BoHc/A was obtained in 140 min from the end of bombardment (EOB). The radiochemical purity from EOB was 100%, and the decay-corrected radiochemical yield from EOB was 2.8–4.5% (average, 3.7%; n = 4). The sp. act. of [18F]-BoHc/A was 1.91–2.83 MBq/μg protein (average, 2.46 MBq/μg protein; n = 4). Cholera toxin (List Biological Laboratories, Campbell, CA) was similarly radiolabeled by conjugation with [18F]SFB and purified by the HPLC. The 126 MBq [18F]-cholera toxin was obtained in 150 min from EOB. The radiochemical purity from the EOB was 100%, and the decay-corrected radiochemical yield from EOB was 0.85%. The sp. act. of the [18F]-cholera toxin was 1.42 MBq/μg protein.

#### Preparation of [111In]-BoHc/A and [111In]-cholera toxin

BoHc/A and cholera toxin (List Biological Laboratories) were labeled with indium chloride ([111In], half-life 2.805 d; Nihon Medi-Physics, Tokyo, Japan) anhydride (Dojindo, Kumamoto, Japan) via N-terminal and e-Lys amino groups using DTPA as described previously (19). The sp. act. of [111In]-BoHc/A and [111In]-cholera toxin was 638,000–915,000 (average, 775,000 cpn/μg protein; n = 4) and 825,000–909,000 cpn/μg protein (average, 867,000 cpn/μg protein; n = 2), respectively.

#### Animals

Female BALB/c mice (4–7 wk old) purchased from Japan SLC (Shizuoka, Japan) were used for the direct radioactive counting in tissues after nasal administration of [111In]-BoHc/A at the Institute of Medical Science of The University of Tokyo, and for PET/CT imaging and direct radioactive counting in tissues after nasal administration of [18F]-BoHc/A at PET Center of Hamamatsu Photonics K.K. For the primate study, two groups of monkeys housed in two different research centers were used. Four female naive cynomolgus macaques (*Macaca fascicularis*, 5 y old, ∼3 kg) were used for the immunization/challenge study and were maintained at the Tsukuba Primate Research Center for Medical Science in National Institute of Biomedical Innovation (NIBIO, Ibaraki, Japan). In the separate experiment, two male and naive rhesus macaques (*Macaca mulatta*, 5–6 y old, ∼5 kg) were used for the PET imaging study at PET Center of Hamamatsu Photonics K.K. All experiments were performed according to the guidelines provided by the Animal Care and Use Committee of the University of Tokyo, Hamamatsu Photonics K.K., and NIBIO.

#### PET/CT imaging in mice

After nasal administration of 0.90 MBq (0.34 μg) per 20 μl [18F]-BoHc/A (10 μl, each nostril), mice were anesthetized by i.p. injection of chloral hydrate (300 mg/kg i.p.) and placed in a small-animal PET scanner.
(Clairvivo-PET; Shimadzu, Kyoto, Japan) (20). Scans were conducted for 10 h. A CT scan was obtained by using a small-animal CT scanner (Clairvivo-CT; Shimadzu) after the PET scan to identify organs and tissues.

**Planar positron whole-body imaging system imaging in mice**

Mice (n = 2) were fixed on an acrylic plate with thread and surgical tape (two animals on each acrylic plate) after nasal administration of 0.90 MBq (0.34 µg) per 20 µl [18F]-BoHc/A. Mice with an acrylic plate were placed at the center position between two planar positron whole-body imaging system (PPIS) detectors and scans with 10-min time frame were performed for 10 h with 60 × 10-min frame using a planar positron imaging system (PPIS-4800; Hamamatsu Photonics, Hamamatsu, Japan) (21). PPIS data were analyzed by using ImageJ software and expressed as counts per pixel. Time-activity curves (TACs) of planar images were expressed as total counts in region of interest.

**Analyses of radiolabeled-BoHc/A and cholera toxin in dissected tissues in mice**

After nasal administration of 0.90 MBq (0.34 µg) per 20 µl [18F]-BoHc/A or 0.56 MBq (0.39 µg) per 20 µl [18F]-cholera toxin to mice (n = 3 at indicated time), the radioisotope counts in various tissues were directly measured by a gamma counter (1480 Wizard; PerkinElmer, Waltham, MA), and the values were adjusted for tissue weight to provide standardized uptake values (SUVs), at the times indicated in Fig. 2A–C. A similar procedure was carried out after nasal administration of mice (n = 3 at indicated time) with 1 × 10^6 cpm (1.1 µg) per 18 µl [111In]-BoHc/A or 1 × 10^6 cpm (1.1 µg) per 20 µl [111In]-cholera toxin, at the times indicated in Fig. 2D–F.

**Immunization and sample collection**

The cynomolgus macaques were nasally immunized with 500 µg per 500 µl BoHc/A (250 µl, each nostril), on four different occasions, at 2-wk intervals. Before immunization and 7 d postimmunization, serum and nasal wash samples were collected for the determination of BoNTA-specific Ab titers.

**ELISA**

BoNTA (5 µg/ml, 100 µl) was coated onto 96-well microtiter plates overnight at 4°C. Nonspecific binding to the plates was blocked with 1% BSA, and then 2-fold serial dilutions of samples were added and incubated for 2 h at room temperature. After washing, HRP-conjugated goat anti-monkey IgG (Nordic Immunological Laboratory, Tilburg, Netherlands) or HRP-conjugated goat anti-monkey IgA (Cortex Biochem, San Leandro, CA) diluted 1:1000 were added and incubated for 2 h at RT together with 1% normal goat serum (Vector, Burlingame, CA). The reaction was finally developed with the use of the TMB Microwell Peroxidase Substrate System (XPL, Gaithersburg, MD). End-point titers were expressed as the reciprocal log_2 of the last dilution, which gave an OD450 of 0.1 greater than the negative control.

**PET/MRI imaging in nonhuman primates**

After nasal administration of [18F]-BoHc/A (34.5 MBq [18.1 µg] per 315 µl, each nostril) to male rhesus macaques (n = 2), each macaque’s head was tilted back for 10 min and then scanned in an upright position after anesthesia. PET scans were conducted for 345 min with frames of 25 × 3 min, followed by 27 × 10 min, with the use of a high-resolution animal PET scanner (MR-MicroPET HR+/R200-300; Hamamatsu Photonics, Shizuoka, Japan) (22). An MRI scan was obtained by using a MAGNETOM Allegra (3T; Siemens, Munich, Germany) to identify cerebrum regions. After PET imaging, the [18F] radioactivity within each dissected organ of the macaque, including brain and OB, was directly measured by a gamma counter (1480 Wizard).

**Data analysis of images**

PET data were analyzed by means of the PMOD software package (PMOD Technologies, Zurich, Switzerland). Each PET image was superimposed on the corresponding MRI or CT data to identify the volume of interest. SUVs were calculated from the level of radioactivity in the volume of interest. PET images were presented as a maximum intensity projection (MIP) image. TACs of PET images were based on SUVs.

**Results**

In vivo quantitative imaging and whole-body imaging of nasally administered [18F]-BoHc/A in mice

[18F]-BoHc/A was synthesized after expression of recombinant BoHc/A in Escherichia coli and purification by chromatography (Supplemental Figs. 1 and 2). Mice were nasally administered 0.9 MBq per 20 µl [18F]-BoHc/A (10 µl per nostril) and were then subjected to anesthesia after 5 min to allow immediate analysis by PET together with CT (PET/CT) or by means of a PPIS. The MIP images of PET data, which were obtained from 10 min to 10 h after nasal administration, were coregistered with a CT surface rendering image, and sites of [18F]-BoHc/A accumulation were identified by image fusion analysis (Fig. 1A). PET data obtained 50–60 min after nasal administration of [18F]-BoHc/A were also visualized as an MIP image coregistered with a CT image to produce a three-dimensional view of a mouse (Supplemental Video 1). Quantitative TACs of [18F]-BoHc/A from the PET data showed that the nasal cavity and urinary bladder were major sites of accumulation of radioactive material after 10 h, whereas the throat, stomach, and intestine were temporary sites of accumulation of radioactive material (Fig. 1B). The whole-body imaging by PPIS (Supplemental Video 2 and Fig. 3) showed dynamic imaging of [18F]-BoHc/A in the nasal cavity, throat, stomach, intestine, and urinary bladder in mice from 10 min to 10 h after nasal administration. [18F]-BoHc/A was detected in the nasal cavity throughout the entire examination period.

**Tissue distribution of nasally administered [18F]-BoHc/A analyzed by direct tissue counting in mice**

When the tissue distribution of [18F]-BoHc/A was quantified by direct counting of radioactivity (Fig. 2A), [18F]-BoHc/A was found to be preferentially located in urine and in the nasal cavity and, in decreasing order of abundance, in the esophagus, stomach, intestine, feces, nasopharynx-associated lymphoid tissue, and kidney. These data are consistent with the quantitative TACs of [18F]-...
BoHc/A in mice obtained by PET/CT imaging (Fig. 1B). Furthermore, we could not detect [18F]-BoHc/A in the CNS, including the OB and cerebrum (Fig. 2B). The accumulated [18F]-BoHc/A in the OBs but not in the cerebrum at 6 h on the enlarged SUV scale (n = 3 per time point) was measured directly and displayed as SUV. C, Nasal administration of [18F]-cholera toxin was used as a positive control; this toxin accumulated in the OBs but not in the cerebrum at 6 h on the enlarged SUV scale (n = 3 per time point). D, After nasal administration of [111In]-BoHc/A, the isotope activity of each mouse tissues at the indicated time (n = 3 per time point) was measured directly and displayed as SUV. E, To further check whether there is an accumulation of [111In]-BoHc/A in the CNS, we enlarged the SUV scale for cerebrum and OBs to focus on levels near the detection limit in the experiment above. F, Nasal administration of [111In]-cholera toxin was used as a positive control; this toxin accumulated in the OBs but not in the cerebrum at 6 h on the enlarged SUV scale (n = 3 per time point). Error bar shows SD. NALT, nasopharynx-associated lymphoid tissue.

Analysis of the longevity of the nasal vaccine in mouse tissues by using [111In]-BoHc/A

We used the traditional [111In]-radiolabel method to examine the persistence of the nasal BoHc/A vaccine in mouse tissues. Because the half-life of [111In] is 67.3 h, we counted the radioactivity in 6 h after nasal administration of 0.56 MBq per 20 μl [18F]-cholera toxin (Fig. 2C). When the radioactivity samples extracted from nasal cavity and esophagus were further examined by size-exclusion chromatography on prepacked disposable (PD-10) columns (GE Healthcare UK, Buckinghamshire, U.K.), [18F]-BoHc/A was found in the high m.w. fractions. The radioactivity detected in samples from the digestive tract, which included stomach, small intestine, and feces, was present in both high and low m.w. forms, whereas that in the urine sample was present only in low m.w. forms (Fig. 3A).

FIGURE 2. SUVs of tissues after nasal administration of [18F]-BoHc/A or [111In]-BoHc/A in mice. A, After nasal administration of [18F]-BoHc/A, the isotope activity in each mouse tissue at the indicated time (n = 3 per time point) was measured directly and displayed as SUV. B, To further check whether there is an accumulation of [18F]-BoHc/A in the CNS, we enlarged the SUV scale for cerebrum and OBs to focus on levels near the detection limit in the experiment above. C, Nasal administration of [18F]-cholera toxin was used as a positive control; this toxin accumulated in the OBs but not in the cerebrum at 6 h on the enlarged SUV scale (n = 3 per time point). D, After nasal administration of [111In]-BoHc/A, the isotope activity of each mouse tissues at the indicated time (n = 3 per time point) was measured directly and displayed as SUV. E, To further check whether there is an accumulation of [111In]-BoHc/A in the CNS, we enlarged the SUV scale for cerebrum and OBs. F, Nasal administration of [111In]-cholera toxin was used as a positive control; this toxin accumulated in the OBs but not in the cerebrum at 6 h on the enlarged SUV scale (n = 3 per time point). Error bar shows SD. NALT, nasopharynx-associated lymphoid tissue.

FIGURE 3. Degradation of [18F]-BoHc/A or [111In]-BoHc/A in tissues after nasal administration. A, The supernatants of homogenized tissues and feces, and urine at indicated time from mice or macaques taken after nasal administration of [18F]-BoHc/A were loaded onto a prepacked PD-10 column containing Sephadex G-10 (GE Healthcare) and fractionated; the [18F] radioactivity was then counted directly by using a gamma counter. B, The supernatants of homogenized tissues and feces, and urine at indicated time from mice or macaques taken after nasal administration of [111In]-BoHc/A were fractionated with PD-10 column, and the [111In] radioactivity was then counted directly by using a gamma counter. Tissue samples with low radioactivity were not determined (N.D.).
various tissues from 10 min to 72 h after nasal administration of \([111\text{In}]\)-BoHc/A. The \([111\text{In}]\)-BoHc/A was detected in the nasal cavity as early as 10 min and remained for at least 24 h after administration. The highest level of radioactivity was found in feces sampled 6 h after nasal administration (Fig. 2D). No evidence for the deposition of \([111\text{In}]\)-BoHc/A in the CNS was found. The accumulated BoHc/A in the cerebrum and OB were 0.01 and 0.1 SUV for the entire examination period from 10 min to 48 h after nasal administration of \(1 \times 10^6\) cpm per 18 ml (9 ml, each nostril) \([111\text{In}]\)-BoHc/A per mouse, respectively (Fig. 2E). On the contrary, \([111\text{In}]\)-cholera toxin was detected in the OB from 6 h after nasal administration of \(1 \times 10^6\) cpm per 20 ml (10 ml, each nostril) \([111\text{In}]\)-cholera toxin per mouse (Fig. 2F). This finding is in complete agreement with the data obtained with \([18\text{F}]\)-BoHc/A (Fig. 2B). Radioactive material in samples extracted from the nasal mucosa 10 min to 24 h after administration and subjected to size-exclusion chromatography analysis by using PD-10 columns was found mostly in the high m.w. fractions at 6 h and then gradually appeared in the low m.w. fractions (Fig. 3B). This finding suggests that intact BoHc/A molecules were attached to the nasal mucosa for at least the first 6 h after administration. Radioactivity was found in the low m.w. fractions of urine and feces samples at 6 h after nasal administration, suggesting that the vaccine Ags were degraded.

Nasal immunization with BoHc/A induces protective immunity against BoNT/A in nonhuman primates

Nasal immunization of mice, but not macaques, with BoHc/A can induce protective immunity against BoNT/A (12). To investigate whether the recombinant BoHc/A nasal vaccine induces protective immunity, we nasally immunized three cynomolgus macaques with 500 \(\mu\)g BoHc/A, and we administered to one cynomolgus macaque PBS as a control (Fig. 4A). Ag-specific Ab responses were assessed by using BoNT/A as a coating Ag for ELISAs. After nasal immunization, the BoNT/A-specific serum IgG Ab titers increased in all BoHc/A-vaccinated macaques, but not in the

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**FIGURE 4.** Nasal immunization with BoHc/A induces protective immunity in nonhuman primates. A, Details for cynomolgus macaques used in the experiment and the dose of BoHc/A used for immunization are shown in this table. B, Each cynomolgus macaque was nasally immunized with 500 \(\mu\)g BoHc/A at the times indicated with arrows, serum and nasal wash were collected (indicated by circles), and then Ag-specific IgG Ab in serum (left) and IgA Ab in nasal wash (right) were measured by the toxin-specific ELISA. x-axis indicates time (week) before and after immunization of BoHc/A. C, Nasally immunized cynomolgus macaques and one control were challenged systemically with 25 \(\mu\)g \((2.7 \times 10^6\) LD\(_{50}\)) BoNT/A/kg body weight and routinely monitored for 30 d after challenging. All cynomolgus macaques, except the control, were completely protected against the lethal toxin challenge without any clinical signs of disease for 30 d. One control macaque showed onset of the disease as early as 4 h after the challenge and was humanely euthanized.
control macaque administered PBS. In addition, the toxin-specific secretory IgA Ab titers in nasal washes increased in all the nasally immunized macaques, but not in the control macaque (Fig. 4B). These results indicated that BoHc/A is a highly immunogenic nasal vaccine capable of inducing toxin-specific Abs not only in systemic compartments but in mucosal compartments in cynomolgus macaques.

Next, a challenge test with 25 μg (2.7 × 10^6 LD_{50}) BoNT/A per kilogram body weight was performed on all three BoHc/A-vaccinated macaques and on the one control macaque; the dosage required was estimated from the neutralizing activity in serum from the immunized macaques (Supplemental Fig. 4). The BoHc/A-vaccinated macaques, but not the control, were completely protected against the high lethal dose of i.p. injected BoNT/A (Fig. 4C). There were no clinical signs of toxin-associated disease over the 30-d observation period after the BoNT/A challenge test. The control macaque was euthanized 4 h after the challenge because of the development of several clinical signs of botulism. After 30 d, all BoHc/A-vaccinated macaques that had undergone the challenge test were sacrificed. Detailed examinations revealed the absence of any clinical signs of organ abnormality or botulism. Taken together, these results demonstrate that the nasal BoHc/A vaccine is a safe and effective mucosal vaccine against *C. botulinum* neurotoxin.

**Adaptation of PET/MRI in vivo molecular imaging for tissue distribution analysis of nasally administered [18F]-BoHc/A in nonhuman primates**

Because we confirmed that the nasal immunization of macaques with BoHc/A induced protective immunity, we then investigated whether the nasal BoHc/A vaccine is transported to the CNS via the OB in nonhuman primates using two naive rhesus macaques on the different experiment. To ensure that the [18F]-BoHc/A completely penetrated the nasal cavity and the olfactory epithelium, we laid two macaques (No. 5 and 6) on their backs for 10 min after the nasal administration of 34.5 and 500 MBq [18F]-BoHc/A in 315 and 500 μl PBS, respectively. After anesthesia, the macaque’s head was placed into the PET scanner system and real-time imaging was performed for 6 h. To confirm the exact position of the cerebrum, we performed an MRI scan and then superimposed the PET images onto the MRI images. Real-time PET images of macaque No. 6, from 15 min to 6 h after nasal administration, are shown in Fig. 5A. No CNS deposition of vaccine Ags in cerebrum was detected within 6 h after nasal administration of [18F]-BoHc/A in either macaque. To confirm the absence of [18F]-BoHc/A in the cerebrum of the two macaques, we directly counted radioactivity in each tissue, including cerebrum and OB, at 6 h after immunization (Fig. 5B,5C). We found that the accumulated [18F]-BoHc/A in the cerebrum and OB were <0.01 and <0.04 SUV, respectively. Taken together, these results demonstrated that there was no CNS deposition of nasally administered BoHc/A vaccine in the rhesus macaques. Six hours after nasal administration of [18F]-BoHc/A, radioactivity was detected in the urine, stomach, kidney, small intestine, and nasal mucosa (Fig. 5B). Among these samples, the highest level of radioactivity was found in the urine. The radioactivity in urine was found in the low m.w. fractions after size-exclusion chromatography analysis by using a PD-10 column (Fig. 3A), suggesting that degradation of [18F]-BoHc/A had occurred.

**Discussion**

Radio- or luminescence-labeling techniques have the capacity to track vaccine Ags and adjuvants (3, 23). However, these classical...
methods are generally end-point approaches because they require the use of multiple animals for diachronic analysis. In this paper, we showed that quantitative real-time analysis of the tissue distribution and degradation of nasal vaccines could be achieved in both rodents and nonhuman primates. To evaluate the safety of the nasal vaccine candidate against *C. botulinum* neurotoxin, BoHc/A, we developed an in vivo molecular imaging system for ADME by using $^{18}$F-BoHc/A and PET together with CT or MRI and the use of PPIS. Our data from in vivo molecular imaging with $^{18}$F-BoHc/A was consistent with the results obtained by classical direct counting to measure the radioactivity in various tissues in mice and nonhuman primates. Thus, our in vivo molecular imaging system is a potentially important strategy for the precise analysis of nasal vaccines and adjuvants for preclinical safety testing in individual animals.

After the nasal administration of $^{18}$F-BoHc/A to mice, a major portion of $^{18}$F-BoHc/A immediately adhered to the nasal mucosa and, simultaneously, some Ag was cleared from the nasal cavity by the mucociliary clearance mechanism (4) and was transported to the throat, esophagus, gastrointestinal tract (including the stomach and intestine), and urinary bladder in order of decreasing abundance (Fig. 1A, Supplemental Videos 1 and 2, and Supplemental Fig. 3). We used our PET imaging method to identify the sites of accumulated $^{18}$F-BoHc/A in real time after nasal administration and to quantify the amounts of accumulated $^{18}$F-BoHc/A (Fig. 1B). We confirmed the accuracy of this approach by comparing these data with those derived by the classical direct counting assay on various dissected tissues (Fig. 2).

Because radioactive material with a low m.w. was found in the stomach and intestine, as well as in the feces, we suggest that the portion of nasal $^{18}$F-BoHc/A or $^{111}$In-BoHc/A vaccine that reached the digestive tract was degraded and then absorbed in the intestines (Fig. 3A, 3B). Radioactive material with a low m.w. was also found in the urine. Because $^{18}$F-BoHc/A and $^{111}$In-BoHc/A were synthesized by labeling N-terminal and ε-Lys amino groups on BoHc/A, nasally administered $^{18}$F-BoHc/A or $^{111}$In-BoHc/A might be degraded in the gastrointestinal tract and converted to $^{18}$F-ε-Lys or $^{111}$In-ε-Lys, which undergo fast blood clearance via the kidneys. Many $^{111}$In-glycoproteins administered to rodents are degraded and converted to $^{111}$In-ε-Lys, which is then recovered in urine and feces (24). Most of the low m.w. compounds labeled with $^{111}$In were detected in feces at 6 h after nasal administration (Fig. 2D), whereas most of the low m.w. compounds labeled with $^{18}$F were present in urine (Fig. 2A). This finding suggests that the $^{18}$F-compounds are absorbed from the gastrointestinal tract and cleared by the kidneys into the urine at a faster rate than is the case for the $^{111}$In compounds. In addition, we found no indication of accumulated radioactivity in bones in mice nasally administered $^{18}$F-BoHc/A (Fig. 2A). Because $^{18}$F fluoride accumulates in bone (25), our observation suggests that degradation of $^{18}$F-BoHc/A to $^{18}$F fluoride did not occur.

The radioactive material detected in the nasal mucosa that was derived from $^{18}$F-BoHc/A or $^{111}$In-BoHc/A was high m.w., suggesting the maintenance of the intact nasal vaccine for as long as 6 h after nasal administration (Fig. 3A, 3B). Although BoHc/A degraded gradually on the surface of the nasal epithelium and/or within the nasal cavity beginning 6 h after administration (Fig. 3B) by contact with proteolytic enzymes (26, 27), intact BoHc/A can bind and penetrate the mucosal epithelial cells by a transcytosis mechanism mediated by an as yet unknown receptor (28).

Therefore, we propose that some of the BoHc/A taken up by and released from the nasal epithelium might stimulate the airway immune system and lead to the induction of Ag-specific immune responses. In fact, we demonstrated the induction of toxin-specific systemic IgG and mucosal IgA Ab responses with strong Ag neutralizing activity when macaques were nasally immunized with 500 μg BoHc/A (Fig. 4B). In addition, we performed a toxin challenge test to provide proof of efficacy of the nasal BoHc/A vaccine in nonhuman primates (Fig. 4C). All the macaques that received the nasal BoHc/A vaccine were protected from an i.p. challenge with a dose of BoNT/A (25 μg [2.7 × 10^6 LD_{50}/kg]), which is ~35,000 times the lethal dose of ~0.7 ng/kg (29).

Recent nasal vaccine studies have raised concerns about the deposition and accumulation of candidate vaccine Ags in the CNS through direct transport from nasal mucosa to the cerebrum via the OB (1, 3). For this reason, we investigated the deposition and accumulation of the nasal BoHc/A vaccine by direct tissue counting analyses of $^{18}$F-BoHc/A or $^{111}$In-BoHc/A in mice. We showed that there was no OB deposition or accumulation of BoHc/A in the CNS within 6 h ($\leq$0.04 SUV by $^{18}$F counts) or 48 h ($\leq$0.1 SUV by $^{111}$In counts) after nasal administration (Fig. 2B, 2D). These results demonstrate that the nasal BoHc/A vaccine did not reach the mouse CNS, despite the large surface area of the nasal cavity containing olfactory epithelium that is connected to the CNS (4), and thereby confirm the safety of the vaccine. On the basis of these promising findings for the efficacy of the nasal BoHc/A vaccine in mice, we used rhesus macaques as a nonhuman primate model to perform the preliminary experiments necessary to validate the approach for future human trials. Using the $^{18}$F-BoHc/A–PET in vivo molecular imaging method, we showed that there was no $^{18}$F-BoHc/A deposited in the cerebrum of rhesus macaques 6 h after nasal administration of 57.4 MBq $^{18}$F-BoHc/A (Fig. 5A). Furthermore, 6 h after nasal administration of $^{18}$F-BoHc/A, no $^{18}$F radioactivity was detected in the cerebrum or OB of the macaque by direct tissue counting (Fig. 5C). Thus, we showed that there is no deposition of the Ag in the CNS in nonhuman primates. This result confirms our results in mice and shows that nasal delivery of BoHc/A is safe with respect to its ADME profile. In addition, the radioactivity in various tissues of the macaques after nasal administration of $^{18}$F-BoHc/A were in complete agreement with the data obtained in mice (Figs. 2A, 5B). Degraded radioactive material with a low m.w. was also found in the urine of macaques (Fig. 3A). Taken together, our findings suggest that after nasal administration of $^{18}$F-BoHc/A to macaques, the $^{18}$F-compound is absorbed from the gastrointestinal tract and cleared by the kidneys into the urine.

In summary, we developed an in vivo molecular imaging system that combines PET with CT or MRI and is suitable as an ADME profiling system for biotechnology-derived pharmaceuticals, including nasal vaccines. Using this system, we demonstrated the safety and efficiency of a nasal vaccine candidate against botulism in mice and nonhuman primates. Our in vivo molecular imaging system can be used to replace the current whole-body autoradiography method for the preclinical ADME evaluation and may be suitable for use in human clinical studies.

Disclosures

The authors have no financial conflicts of interest.

References


Data Supplement

*In vivo* molecular imaging analysis of a nasal vaccine that induces protective immunity against botulism in non-human primates.

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This PDF file includes:

Figure S1. Preparation of the botulinum mucosal vaccine (BoHc/A)
Figure S2. Synthetic route for [\(^{18}\text{F}\)]-BoHc/A
Figure S3. Dynamic images of mouse whole body.
Figue S4. Neutralizing activity in serum from Cynomolgus macaques immunized via the nasal route.
Video S1 and S2 legend.

Other Data Supplement for this manuscript includes the following:

Video S1and S2 (In MOV format).
**Fig. S1:**
Preparation of the botulinum mucosal vaccine (BoHc/A) (A) Construction of BoHc/A expression vector, pBoHc-GEX6P3. The promoter and the GST gene are represented by a hatched bar, and the sequence coding for BoHc/A is represented by a filled bar. Arrows indicate the direction of transcription. “Ori” signifies the origin of replication. The amino acid sequence of the BoHc/A fusion protein including the PreScission protease cleavage site is shown at the bottom of the figure. (B) Sodium dodecyl sulfate-polyacrylamide gel showing purified BoHc/A. The purified protein was visualized, following sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using Colloidal Coomassie Blue stain. Molecular weight markers are shown on the left side of the gel. (C) Mass spectrometry analysis of BoHc/A. Forty two percent (shown in red) of the deduced amino acid sequence of BoHc/A was identified by LC MS/MS analyses. The tandem MS spectrum of one major BoHc/A peptide sequence was identified as “YESNHLIDLSR”.

**Sequence coverage: 41.2%**
Synthetic route for $[^{18}\text{F}]$-BoHc/A (A) $[^{18}\text{F}]$-BoHc/A was synthesized by labeling BoHc/A with succinimidyl 4-$[^{18}\text{F}]$fluorobenzoate ($[^{18}\text{F}]$SFB) at the Hamamatsu Photonics PET Center. TSTU, $N,N,N',N'$-tetramethyl-\(O-(N\text{-succinimidyl})\)uronium
tetrafluoroborate. (B, C) After the final synthesis step, BoHc/A was purified by preparative HPLC. The purity of the protein was confirmed using Superose 12 HPLC by detecting the absorbance at 280 nm (B) and radioactivity (C) of the eluate.
Fig. S3.
Dynamic images of mouse whole body. After nasal administration of $[^{18}\text{F}]-\text{BoHc/A}$, two mice were placed on an acrylic plate at the center between two PPIS detectors. Multiple scans, each of 10 min duration, were performed over a 10 h period. Dynamic images of the mouse whole body are shown at the indicated time frames. See also movie S2.
Neutralizing activity in serum from Cynomolgus macaques immunized via the nasal route. To estimate the antigen neutralizing activity in serum from nasally immunized mice, 10 μl of serum diluted 1:10 were incubated with 0.2 ng (22 LD₅₀), 2 ng (2.2 × 10² LD₅₀) or 20 ng (2.2 × 10³ LD₅₀) of BoNT/A diluted in 90 μl of 0.2% gelatin/PBS for 30 min at room temperature. The mixture was then injected into mice via the intraperitoneal route. The mice were monitored for 1 week after the systemic challenge with BoNT/A. The sera from all macaques in the nasal immunization group, with the exception of the negative control, neutralized BoNT/A at 0.2-20 ng (22 - 2.2 × 10³ LD₅₀) in an antibody-titer-dependent manner. Neutralization of 0.2 ng BoNT/A (22 LD₅₀) required a maximum of 1 μl of sera (>14 reciprocal log₂ titer per ml, Fig. 4B)
from the immunized macaques. Based on this observation, we calculated that a
cynomolgus macaque with a body weight of 3 kg (300 ml as total serum) should be able
to neutralize at least 60 μg (6.6 × 10^6 LD_{50}), of BoNT/A if appropriately immunized
with nasal BoHc/A vaccine. Therefore, a challenge test with 75 μg (8.2 × 10^6 LD_{50}) of
BoNT/A (25 μg (2.7 × 10^6 LD_{50})/kg) was performed on all three cynomolgus macaques
that were nasally immunized with the BoHc/A vaccine and one control macaque that
was administered PBS (See Fig. 4C).
**Video Legends**

**Video S1:**
The three dimensional view of a mouse 50–60 min after nasal administration of $[^{18}\text{F}]-\text{BoHc/A}$. After nasal administration of $[^{18}\text{F}]-\text{BoHc/A}$ the mouse was placed in a small animal PET system and the whole body was scanned for 10 h. The PET/CT obtained at 50–60 min is shown as a maximum intensity projection (MIP) image for the three dimensional view. See also Fig. 1A.

**Video S2:**
Real-time images of a mouse whole body after nasal administration of $[^{18}\text{F}]-\text{BoHc/A}$. After nasal administration of $[^{18}\text{F}]-\text{BoHc/A}$, two mice were placed on an acrylic plate at the center between two PPIS detectors. Multiple scans, each of 10 min duration, were performed over a 10 h period. The whole-body imaging of one mouse is shown as a movie. See also Supplemental Fig. S3.