Molecular Immunology 46 (2009) 1718–1726

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Pentabody-mediated antigen delivery induces antigen-specific mucosal immune response

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ARTICLE INFO

Article history:
Received 11 December 2008
Received in revised form 29 January 2009
Accepted 2 February 2009
Available online 9 March 2009

Keywords:
Single domain antibody
Mucosal immunization
CT
CTB

ABSTRACT

An efficient immunization system is essential for the development of mucosal vaccine. Cholera toxin (CT) and *Escherichia coli* heat labile toxin (LT) are among the strongest adjuvants tested in experimental animals but their use in humans has been hindered by their toxicity. On the other hand, the role of their non-toxic B-subunits, CTB or LTB, in enhancing mucosal immune response is not clear. We propose here a novel strategy for the induction of mucosal immune responses. Single domain antibodies (sdAbs) against a model antigen bovine serum albumin (BSA) were raised from the antibody repertoire of a llama immunized with BSA, pentamerized by fusing the sdAbs to CTB, generating the so-called pentabodies. These pentabodies were used to deliver the antigen by mixing the two components and administering the mixture to mice intranasally. One construct was equivalent to CT in helping induce mucosal immune response. It was also found that this ability was probably due to its high affinity to BSA, providing some insight into the controversial role of CTB in mucosal immunization: at least for BSA, the model antigen BSA employed in this study, CTB has to be tightly linked to the antigen to have adjuvant/immune-enhancing effect.

1. Introduction

Despite the early success of mucosal vaccines such as polio vaccine, induction of effective and long-lasting antigen-specific mucosal immune response remains a challenge (Holmgren and Czerkinsky, 2005). Among many tested mucosal delivery systems such as liposomes (Sakaue et al., 2003), lipo-structures and virus-like particles (Kang et al., 2004) and mucosal adjuvants such as CpG DNA (McCluskie and Davis, 1999), cholera toxin (CT) (Walsh, 1993), *Escherichia coli* heat labile toxin (Guy et al., 1998) and their derivatives, CT- or LT-based adjuvants are among the most potent mucosal adjuvants in experimental animals (Holmgren et al., 2003).

Much effort has been made to develop CT- or LT-based adjuvants which are equivalent to CT in terms of adjuvanticity but with reduced toxicity to human. Since both CT and LT are composed of a B-subunit pentamer, which binds to the cellular receptor G_M1 on nucleated cells (Revesz et al., 1976), and an A subunit monomer, which is an ADP-ribosyltransferase (Vaughan and Moss, 1978) and the toxic entity, it is hoped that the separation of adjuvanticity from toxicity is possible and CT- or LT-variants with preserved adjuvanicity but much reduced toxicity can be obtained. Although reduction in toxicity in CT and LT is often associated with loss of adjuvancity, promising CT variants have been generated (Dickinson and Clements, 1995; Douce et al., 1997; Giuliani et al., 1998; Tsuji et al., 1997; Yamamoto et al., 1997) and are being tested.

Another strategy for raising mucosal immune response is to employ the receptor binding B-subunit pentamer of CT or LT, CTB or LTB. CTB and LTB have been reported to have preserved the adjuvanticity of the holotoxins in some cases. Influenza subunit antigen adjuvanted by LTB was found to induce protective intranasal IgA after *i.n.* immunization (Haan et al., 2001). However, addition CTB or LTB to the antigen did not induce significant mucosal IgA in many reported cases. Fibronectin-binding domain of the SfbI protein of *Streptococcus pyogenes* was one of them (Schulze et al., 2003).

A more efficient way is to fuse the antigen to CTB or LTB. A T-cell and a B-cell epitope of the 28 kDa glutathione-*S*-transferase of *Schistosoma mansoni*, after fused to CTB, was found to form a pentamer and induce antigen-specific IgA in mice (Lebens et al., 2003). However, CTB-antigen fusion proteins were reported to induce lower level of immune response in comparison to CT-adjuvanted anti-

Abbreviations: *i.n.*, intranasal; k_a, association rate constant; k_d, dissociation rate constant; K_D, dissociation constant.

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0161-5890/$ – see front matter.
doi:10.1016/j.molimm.2009.02.007

gen (Matoba et al., 2006). In another case, no mucosal immune response was reported (Gockel and Russell, 2005). In addition, fusion of antigen to CTB may encounter problems due to the size and hydrophobicity of the antigen.

We propose here a novel strategy of mucosal immunization based on pentameric single domain antibody (sdAbs), or, pentabody.

sdAbs refer to variable regions of heavy chains (V_H) or light chains (V_L) of immunoglobulins. sdAbs from conventional IgGs tend to aggregate because of the hydrophobic portions of the molecular surface of V_H or V_L which are used to pair with their V_L and V_H counterparts. Heavy-chain antibodies (HCAbs) naturally devoid of light chains were discovered in camelids (Hamers-Casterman et al., 1993) such as camel, llama and alpaca and sharks (Greenberg et al., 1995). The variable regions of these heavy-chain-only Ig molecules, or V_HHs, are solely responsible for antigen binding. Unlike sdAbs from conventional IgGs, sdAbs from camelid HCAbs are not required to interact with V_L and the C_H1 domains. Accordingly, more hydrophilic residues were seen (Phe37, Glu44, Arg45 and Gly47) where more hydrophobic ones (Val37, Gly44, Glu45 and Tyr47) are usually used in conventional IgGs. As a consequence, camelid sdAbs usually exist as monomeric proteins when expressed alone (Zhang et al., 2004b). The non-aggregating feature of camelids sdAbs, together with their small sizes (~13 kDa) high thermostability, high detergent resistance, relatively high proteolytic resistance (Dumoulin et al., 2002), high affinity by isolation from an immune library (Arbabi Ghahroudi et al., 1997) or by *in vitro* affinity maturation (Davies and Riechmann, 1996; De Genst et al., 2004) make sdAbs ideal reagents when high affinity and high stability are required (Revets et al., 2005).

By fusing an sdAb to the B-subunit of shiga toxin 1 (stx1-B), a pentameric sdAb, or a pentabody, was generated (Zhang et al., 2004b). The pentabody formed a homogenous pentamer, was relatively resistant to trypsin and chemotrypsin digestion, and had a relatively good thermostability. It also retained the binding ability of stx1-B to its cellular receptor G_b3 (Zhang et al., 2004b).

Although Stx1-B and CTB/LTB have relatively low sequence identity, the three proteins share striking structural similarity and they all belong to the AB_5-toxin family. It is therefore interesting to build pentabodies using CTB as the pentamerization domain and utilize its G_M1 binding for mucosal antigen delivery.

We present here the construction of CTB–pentabodies against a model antigen bovine serum albumin (BSA) and uses thereof for the induction of BSA-specific mucosal immune response in a mouse model.

2. Materials and methods

2.1. Materials

E. coli TG1 and M13KO7 helper phage were purchased from New England Biolabs (Mississauga, Ont.). Expression vector pSJF2H, which expresses 6× His-tagged protein instead of 5× His-tagged protein, as the vector pSJF2 (Tanha et al., 2003) does, was kindly provided by Dr. J. Tanha. DNA encoding CTB was a gift from Dr. D. Miller (U. of Toronto). CT was purchased from Sigma (St. Louis, Missouri) and recombinant CTB from SBL Vaccine AB (Stockholm, Sweden).

2.2. Animals

Six to eight-week-old female Balb/c mice were purchased from Charles River Laboratory (St. Constant, Quebec). The animals were housed in the Animal Facility of the Institute for Biological Sciences, National Research Council of Canada, Ottawa in accordance with the recommendations of the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals and the experimental protocols were approved by the institutional animal care committee.

2.3. Phage display screening

A female llama was immunized with BSA. An sdAb phagemid display library was constructed from the V_HH repertoire of this llama (immunization and library construction will be published separately by S.M. and T.F. and colleagues) and this library was used for the isolation of sdAbs against BSA.

The llama immune phage display library was panned against 1 mg/ml BSA that was preadsorbed to a Reacti-Bind™ maleic anhydride activated microtiter plate well. About 10^{11} phage were added to the well and incubated at 37 °C for 2 h for antigen binding. After disposal of unattached phage, the wells were washed six times with phosphate buffered saline supplemented with 0.05% Tween 20 (PBST) for round one and washes were increased by one for each additional round. Phage were eluted by 10 min incubation with 100 μl 100 mM triethylamine and the eluate was subsequently neutralized with 200 μl 1 M Tris–HCl (pH 7.5). Phage were rescued and amplified using M13KO7 and used for the next round of panning. After three rounds of panning, eluted phage were used to infect exponentially growing *E. coli* TG1 and rescued by M13KO7. The produced phage were used in phage ELISA.

For phage ELISA, wells of a 96-well plate were coated overnight with 5 μg/ml BSA and then blocked with 1% casein for 2 h at 37 °C. Phage were preblocked with casein overnight, added to the preblocked wells and incubated for 1 h. Positive phage clones detected by standard ELISA procedure were sent for sequencing.

2.4. Construction and expression of sdAbs and pentabodies

DNA encoding four sdAbs (BSA7, BSA8, BSA12 and BSA16) was amplified by PCR and flanked with BbsI and BamHI restriction sites. The products were cloned into the BbsI and BamHI sites of pSJF2H to generate pBSA7, pBSA8, pBSA12 and pBSA16.

CTB-based pentabodies were constructed by standard molecular cloning procedures. DNA encoding CTB was amplified by PCR and franked with BbsI restriction site and DNA encoding linker sequence GGGGSGGGGSGGGGS at 5′- and 3′-ends, respectively. DNA encoding BSA8, BSA12 and BSA16 was amplified by PCR and flanked with DNA encoding the linker sequence GGGGSGGGGSGGGGS and BamHI restriction site at 5′- and 3′-ends, respectively. CTB and the three sdAbs were fused at DNA level by overlap extension PCR. The final PCR product was digested by BbsI and BamHI and ligated into pSJF2 digested with the same enzymes to generate clones pC3C-BSA8, pC3C-BSA12 and pC3C-BSA16 (Fig. 1).

All clones were inoculated in 25 ml LB-Ampicillin (Drake et al., 2004) and incubated at 37 °C with 200 rpm shaking overnight. The next day, 20 ml of the culture was used to inoculate 1 l of M9 medium (0.2% glucose, 0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.1% NH_4Cl, 0.05% NaCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$) supplemented with 0.4% casamino acids, 5 mg/l of vitamin B1 and 200 μg/ml of ampicillin, and cultured for 24 h. Next, 100 ml of 10× TB nutrients (12% Tryptone, 24% yeast extract and 4% glycerol), 2 ml of 100 mg/ml Amp and 1 ml of 1 M isopropyl-beta-D-thiogalactopyranoside (IPTG) were added to the culture and incubation was continued for another 65–70 h at 28 °C with 200 rpm shaking. *E. coli* cells were harvested by centrifugation and lysed with lysozyme. Cell lysates were centrifuged, and clear supernatant was loaded onto 5 ml immobilized metal affinity chromatography (IMAC) High-Trap™ chelating affinity column (GE Healthcare, Uppsala, Sweden). High-Trap™ chelating affinity columns and His-tagged proteins were purified.