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Vaccine 23 (2005) 4926–4934
Vaccine
www.elsevier.com/locate/vaccine

Prolonged in vivo residence times of llama single-domain antibody fragments in pigs by binding to porcine immunoglobulins

Michiel M. Harmsen *, Conny B. Van Solt, Helmi P.D. Fijten, Marga C. Van Setten

Animal Sciences Group (ID-Lelystad BV) of Wageningen University and Research Centre, Edelhertweg 15, P.O. Box 65, 8219 PH, Lelystad, The Netherlands

Received 21 September 2004; received in revised form 21 March 2005; accepted 20 May 2005
Available online 13 June 2005

Abstract

The therapeutic parenteral application of llama single-domain antibody fragments (VHHs) is hampered by their small size, resulting in a fast elimination from the body. Here we describe a method to increase the serum half-life of VHHs in pigs by fusion to another VHH binding to porcine immunoglobulin G (pIgG). We isolated 19 pIgG-binding VHHs from an immunized llama using phage display. Six VHHs were genetically fused to model VHH K609 that binds to *Escherichia coli* F4 fimbriae. All six yeast-produced genetic fusions of two VHH domains (VHH2s) were functional in ELISA and bound to pIgG with high affinity (1–33 nM). Four pIgG-binding VHH2s were administered to pigs and showed a 100-fold extended in vivo residence times as compared to a control VHH2 that does not bind to pIgG. This could provide the basis for therapeutic application of VHHs in pigs.

Keywords: Single-domain antibody; Vaccination; Clearance

1. Introduction

Antibodies are useful therapeutic agents for treatment of human and animal diseases. However, the costs of conventional antibodies are often prohibitively high, especially for treatment of livestock. In contrast, antibody fragments such as Fab, $F(ab')_2$ and single chain Fv (scFv) retain full antigen binding capacity and can be produced at low-cost in microorganisms. A disadvantage of antibody fragments is their lack of functions encoded by the Fc fragment, such as the recruitment of effector functions involved in pathogen elimination. Furthermore, antibody fragments are rapidly removed from the circulation of mammals with an elimination half-life of several hours [1,2], whereas intact antibodies have a half-life of about 3 weeks [3]. This rapid elimination limits their therapeutic parenteral application. It is predominantly caused by the small molecular size of antibody fragments (15–60 kDa) as compared to intact IgG (150 kDa), resulting in their passage of the glomerular filter in the kidney and disposal in the urine [2]. Furthermore, due to their lack of the Fc fragment, antibody fragments are unable to interact with FcRn. This receptor protects intact antibodies from catabolism by binding them after pinocytosis of blood by vascular epithelium and recycling them into the circulation. The importance of this mechanism was shown by transgenic mice lacking this receptor, which had a 10-fold decreased in antibody half-life [4,5].

Extension of the half-life of antibody fragments has been achieved by several methods, which predominantly rely on increasing molecular size. Chemical coupling to polyethylene glycol is widely used [6,7]. Coupling to long-lived serum proteins such as albumin, immunoglobulin or portions of these proteins may also retard antibody fragment clearance. Such coupling can be accomplished by chemical means, direct genetic fusion or by fusion to a second antibody fragment specific for these proteins [8–10]. All these methods have been developed either for use in humans or laboratory animals, but not for use in pigs.

* Corresponding author. Tel.: +31 320 238436; fax: +31 320 238961.
E-mail address: michiel.harmsen@wur.nl (M.M. Harmsen).

0264-410X/$ – see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2005.05.017

To enable the commercial passive immunization of pigs with antibody fragments we aimed to develop a method for increasing serum residence time of antibody fragments in pigs. Genetic fusions were constructed between a model antibody fragment and a second antibody fragment which was able to bind porcine IgG (pIgG). In mice this method was previously shown to have the additional advantage of recruitment of effector functions such as antibody-dependent cellular cytotoxicity, complement fixation and antibody-mediated phagocytosis [10]. We used single-domain antibody fragments (VHHs) derived from camelid heavy chain antibodies because these minimum sized antigen binding moieties have several advantages for biotechnological applications [11], such as a high physicochemical stability [12], high production level in microorganisms [13], and the facile construction of bispecific VHH dimers by genetic fusion of two VHH domains [14]. pIgG-binding VHHs were selected from an immunized llama by phage display. Six of these pIgG-binding VHHs were subsequently genetically fused to a model VHH K609 that binds to *E. coli* F4 fimbriae. The clearance of four of such VHH2s in pigs was analyzed after parenteral application.

2. Materials and methods

2.1. Animals

Twenty-four large, white cross-bred Piëtrain pigs weighing about 10 kg and a single one-year-old llama (*Lama glama*) were used for this study. They were provided with food and water ad libitum. Animal experiments were performed under the supervision of the Animal Experimental Committee according to the Dutch Act on Experimental Animal Use.

2.2. Phage display selection of pIgG-binding VHHs

A llama was immunized i.m. three times with 3-week intervals with 1 mg pIgG (Jackson ImmunoResearch, West Grove, PA) per immunization using Specol (Cedi Diagnostics, Lelystad, The Netherlands) as an adjuvant. A blood sample containing 10^8 peripheral blood lymphocytes was taken 1 week after the last immunization and the VHH repertoire was cloned as recently described [13]. The VHH repertoire was amplified by RT-PCR using primer VH1BACK and Lam07 or Lam08 [13] or BOLI192 (5′-AACAGTTAAGCTTCCGCTTGCGGCCGCTACTTCATTCGTTCCTGAGGAGACGGT-3′; *Hin*dIII site underlined, *Not*I site double underlined). The latter primer is based on the short hinge sequence GTNEVCKCPKCP identified in llamas [15]. PCR fragments digested with *Pst*I and *Not*I were inserted into phage display vector pRL144, resulting in a library of 10^7 independent clones. Vector pRL144 was derived from pUR5071 [16] by insertion of a synthetically-prepared fragment into *Not*I–*Bsm*I cut pUR5071. As a result pRL144 contains the following elements between the *Not*I cloning site and the p3 encoding region (amino acid sequence between parentheses): an enterokinase cleavage site (DDDDK), a c-myc tag (EQKLISEEDLN), a small flexible linker (GAA), a hexahistidine tag (HHHHHH), another small flexible linker (GAA), and the amber stop codon.

Phage display selections were performed by biopanning [17], using pIgG-coated 96-well microtiter plates. After two consecutive rounds of panning, individual colonies were picked and the VHH genes were induced with 1 mM isopropyl β-D-thiogalactopyranoside. Recombinant VHHs extracted from the periplasm were tested for binding to pIgG by ELISA. Clones producing a pIgG-binding VHH were further analyzed by fingerprinting [18].

2.3. Construction of bispecific VHH2s

The VHH encoding regions of 21 clones (Table 1) were isolated from the phage display vector by *Pst*I–*Bst*EII digestion and transferred into similarly cut yeast VHH expression vector pUR4585 [19] in order to introduce C-terminal c-myc and hexahistidine tags followed by a stop codon and a *Hin*dIII site. These clones were then sequenced as described previously [19].

For genetic fusion of pIgG-binding VHHs to the K609 VHH C-terminus we constructed plasmid pRL193 in a series of cloning steps. We first modified a pUR4585-derived plasmid encoding K609 by insertion of a synthetic DNA fragment (5′-GCGCGGTCACCGTCTCCTCACAGGTGCAGCTGCAGTAAGATCTAAGCTTGCGC-3′, and its complementary oligonucleotide) into the *Bst*EII (underlined) and *Hin*dIII (double underlined) sites, resulting in plasmid pK609D1. This introduces a second *Pst*I site (thick underlined) suitable for insertion of a second VHH encoding fragment into the *Hin*dIII and this second *Pst*I site, resulting in direct fusion of two VHH domains without a linker. A next generation of VHH2 expression vector containing a unique *Pvu*II site suitable for insertion of the second VHH was generated by deletion of three of the four *Pvu*II sites and one of the two *Pst*I sites of pK609D1 in two steps. Two fragments were amplified from pK609D1 by PCR using oligonucleotides 5′-TCATTAATGCAGTTGGCACGACAGGT-3′ in combination with 5′-CCCAGGTCACCGTCTCCTCACAGGTGCAGCTGAAGTAAGATCTAAG-3′ and 5′-ACCTGTCGTGCCAACTGCATTAATGA-3′ in combination with 5′-GGTCTCGCGGTATCATTGCAGCAC-3′. These fragments were then assembled in a splice overlap extension PCR to a 0.8 kb *Drd*I–*Bst*EII fragment that was inserted into the 6.0 kb *Drd*I–*Bst*EII fragment of pK609D1. Then the 0.4 kb *Kas*I–*Pst*I PCR fragment, obtained by amplification of pK609D1 with oligonucleotides 5′-GCGCGGCGCCGAATTCCGCCCGGGGGATCTAGCT-3′ and 5′-CCCTGACTCCTGCAGTTGCACCTGCGCAGA-3′, was inserted into the corresponding sites, resulting in plasmid pRL110. We next introduced a linker comprising the amino acids GGS between the two VHH