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Generation of llama single-domain antibodies against methotrexate, a prototypical hapten

Nidia Alvarez-Rueda [a], Ghislaine Behar [b], Virginie Ferré [c], Martine Pugnière [d], Françoise Roquet [d], Louis Gastinel [e], Catherine Jacquot [f], Jacques Aubry [g], Daniel Baty [b], Jacques Barbet [a], Stéphane Birklé [g,*]

[a] *Inserm, Université de Nantes, Nantes Atlantique Universités, U601, Département de Recherche en Cancérologie, 9 quai Moncousu, F-44000 Nantes, France*
[b] *CNRS, UPR9027, Laboratoire des Systèmes Macromoléculaires, GDR2352, 31 chemin Joseph Aiguier, F-13009 Marseille, France*
[c] *Université de Nantes, Nantes Atlantique Universités, Faculté de Pharmacie, JE2437, 1 rue Gaston Veil, F-44000 Nantes, France*
[d] *CNRS, UMR5160, Centre de Pharmacologie et Biotechnologie pour la Santé, 15 avenue Charles Flahault, F-34093 Montpellier, France*
[e] *Université de Limoges, Faculté des Sciences, EA3176, Glycobiologie Végétale et Biotechnologie, 123 avenue Albert Thomas, F-87060 Limoges, France*
[f] *Université de Nantes, Nantes Atlantique Universités, Laboratoire de Pharmacologie Marine, EE0603, 1 rue Gaston Veil, F-44000 Nantes, France*
[g] *Université de Nantes, Inserm, Nantes Atlantique Universités, U601, Faculté de Pharmacie, 1 rue Gaston Veil, F-44000 Nantes, France*

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Abstract

Single-domain antibodies specific to methotrexate (MTX) were obtained after immunization of one llama (*Llama glama*). Specific VHH domains (V–D–J-REGION) were selected by panning from an immune-llama library using phage display technology. The antibody fragments specific to MTX were purified from *Escherichia coli* (C41 strain) periplasm by immobilized metal affinity chromatography with an expression level of around 10 mg/L. A single band around 16,000 Da corresponding to VHH fragments was found after analysis by SDS-PAGE and Western blotting, while competition ELISA demonstrated selective binding to soluble MTX. Surface plasmon resonance (SPR) analysis showed that anti-MTX VHH domains had affinities in the nanomolar range (29–515 nM) to MTX-serum albumin conjugates. The genes encoding anti-MTX VHH were found by IMGT/V-QUEST to be similar to the previously reported llama and human IGHV germline genes. The V–D and D–J junction rearrangements in the seven anti-MTX CDR3 sequences indicate that they were originated from three distinct progenitor B cells. Our results demonstrate that camelid single-domain antibodies are capable of high affinity binding to low molecular weight hydrosoluble haptens. Furthermore, these anti-MTX VHH give new insights on how the antigen binding repertoire of llama single-domain antibody can provide combining sites to haptens in the absence of a VL. This type of single-domain antibodies offers advantages compared to murine recombinant antibodies in terms of production rate and sequence similarity to the human IGHV3 subgroup genes.

Keywords: Methotrexate; Llama; Single-domain antibody; Hapten

1. Introduction

Since the discovery in Camelidae of IgG2 and IgG3 antibodies devoid of light chain and of CH1 domain (Hamers-Casterman et al., 1993), and the characterization of their single-domain antibodies (VHH), many reports have described the isolation of specific and high-affinity VHH directed against protein antigens (Omidfar et al., 2004; van der Linden et al., 2000). Crystal structures of VHH–antigen complexes have been determined. The major advantage of VHH domains is that they are of smaller size and thereby easier to manipulate genetically as compared to Fab, Fv or scFv derived from other mammals, because only one domain has to be cloned and expressed (Muyldermans et al., 2001). In contrast to VH domains of conventional antibodies, the corresponding VHH (MW ∼ 14,000 Da) are expressed at higher level in bacteria, bind to the antigen with high affinities and were shown to overcome, to a large extent, the stability, aggregation and degradation problems often present with scFv (Arbabi Ghahroudi et al., 1997; Frenken et al., 2000; van der Linden et al., 1999). Interestingly, the V genes expressed in VHH domains have a close homology with the human IGHV3 subgroup

* Corresponding author. Tel.: +33 2 40 08 47 17; fax: +33 2 40 35 66 97.
E-mail address: Stephane.Birkle@univ-nantes.fr (S. Birklé).

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genes, which may result in less immunogenicity (Conrath et al., 2003).

However, the capacity of VHH to bind to haptens has been questioned (Lange et al., 2001). Only two types of anti-hapten VHH have been obtained so far and their structure solved by X-ray (Spinelli et al., 2000, 2001, 2004; Yau et al., 2003). A few VHH specific for the azo-dye reactive red RR6 (728 Da) and RR120 (1335 Da) haptens have been produced that showed affinities in the nanomolar range (18–83 nM). This demonstrates that VHH from llama can provide cavities to accommodate hydrophobic haptens. By contrast, VHH directed against the low molecular weight hapten picloram (241 Da) have an affinity in the μM range. However, whether the reported binding characteristics are related to the VHH binding to the free or to the carrier protein-conjugated form of hapten is not clear in these reports.

Raising and screening high-affinity antibodies to low molecular weight molecules (haptens) is sometimes more complex than developing antibodies to large immunogenic proteins. However, many monoclonal anti-hapten antibodies (Mabs) are produced from hybridoma cell lines. Most are currently applied to commercial immunoassay kits that are often specific for environmental targets, diagnostic detection of drugs, drug monitoring or disease therapy. High-affinity recombinant anti-hapten antibodies from various animal species have also been isolated by phage display technologies (Charlton et al., 2001; Jorgensen et al., 2002; Kobayashi et al., 2005; Moghaddam et al., 2001; Strachan et al., 2002). The inherent problems associated with producing anti-hapten Mabs are well documented (Tuomola et al., 2000). Mabs with high affinity for non-immunogenic haptens with molecular weight of <500 Da can only be generated using the hapten conjugated to a carrier protein. The selection of anti-hapten Mabs of the desired specificity largely depends on the hapten design (physico-chemical characteristics and spatial conformation), selection of the appropriate carrier and probe protein, and the conjugation method (Morel et al., 1990; Singh et al., 2004, 2006). An additional potential problem exhibited by some of these antibodies is their cross-reactivity with structurally related compounds and metabolites (Chames and Baty, 1998).

In the present work, we describe the generation by phage display and the binding characteristics of VHH domains specific to methotrexate (MTX), which is a low molecular weight (454 Da) hydrosoluble molecule. MTX could be considered a prototypical hapten: large enough to minimize the influence of conjugation to the carrier protein and not too hydrophobic to generate adventitious interactions with hydrophobic protein pockets. This drug is a well-known and extensively characterized antineoplastic agent widely used in the treatment of cancer as a competitive inhibitor of the enzyme dihydrofolate reductase (Dellapasqua and Castiglione-Gertsch, 2005; Hoang-Xuan et al., 2004). Several polyclonal and monoclonal antibodies have been generated against MTX to measure the drug and its metabolites in patient serum, but most anti-MTX antibodies developed so far are mouse or rabbit whole IgGs (Bore et al., 1984; Kato et al., 1984). Anti-MTX VHH may thus be useful reagents in biomedical research and comparison of germline and cDNA sequences of the VHH may shed some light on how the antigen-binding repertoire of llama antibodies could provide combining sites to haptens in the absence of a VL.

2. Materials and methods

2.1. Reagents and solutions

Methotrexate (MTX), folic acid, folinic acid, bovine serum albumin (BSA), Tween 20, glycerol, incomplete Freund's adjuvant, dimethylsulfoxide (DMSO), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbo-diimide (EDC), *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (HEPES), ampicillin, kanamycin, polyethylene glycol (PEG), NaCl, isopropyl-β-D-thiogalactopyranoside (IPTG), β-mercaptoethanol (ME), glycerol and sodium dodecyl sulfate (SDS) were purchased from Sigma Aldrich Inc., St. Louis, MO, USA. Blue carrier immunogenic protein™ (BIP) was obtained from Pierce Chemicals, Rockford, IL, USA. 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was purchased from Roche Diagnostics France, Meylan, France, and polyvinylidene fluoride (PVDF) membranes were purchased from Millipore Corporation, Bedford, MA, USA.

2.2. Immunogen and llama immunization

The immunogenic form of MTX was synthesized by coupling MTX (0.16 M) to BIP (20 mg/ml) using EDC (0.26 M) in *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer pH 8.0 (Becker et al., 1998). The conjugate was purified by gel filtration on a Sephadex G-25 column (PD 10, GE Healthcare, Uppsala, Sweden) and used for immunizations. MTX was also coupled to BSA and the conjugate was used as antigen in antibody selection and immunoassays. The extent of MTX coupling per mole of carrier protein was determined spectrophotometrically with the absorption of the hapten-protein conjugate at 365, 495 and 280 nm compared with that of protein and MTX. One female llama was immunized by a single intramuscular injection of MTX–BIP conjugate (2.5 ml, 1 mg) mixed with an equal volume of Freund's incomplete adjuvant followed by three booster injections every 2 weeks. The blood of immunized animal was collected, treated by centrifugation and the sera were stored in aliquots at −20 °C. Peripheral blood lymphocytes were obtained by centrifugation on a Ficoll cushion (Eurobio, les Ulis, France) and stored at −70 °C until further use. The immune response was monitored by titration of sera by an enzyme-linked immunosorbent assay (ELISA) with MTX–BIP, MTX–BSA and blue carrier protein immobilized on NuncImmuno™ 96 Microwell™ Maxisorp™ plates (Nunc A/S, Roskilde, Denmark) (the coating solution contained 1 μg of protein per ml diluted in phosphate buffered saline PBS (100 μl/well)). Wells were blocked with 1% BSA–PBS (200 μl/well) and incubated with serum dilutions in 0.1% BSA–PBS. After extensive washings, the bound llama antibodies were detected with a goat anti-human γ chain antibody conjugated to horseradish peroxidase (HRP) (Clinisciences, Montrouge, France). Peroxidase enzyme activity was determined by adding 1 mg/ml ABTS as substrate and read out at 405 nm.