Journal of Immunological Methods 339 (2008) 220–227

Contents lists available at ScienceDirect

Journal of Immunological Methods

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

Research paper

Functionally fused antibodies—A novel adjuvant fusion system

Martin Larsen [a], Kim Bak Jensen [a], Peter Astrup Christensen [b], Eduardo Suarez [c], Dominique Paris [d], Laura Sanz [c], Peter Ravn [b], Delphine Sauce [d], Philippe Saas [d], Steffen Goletz [b], Luis Álvarez-Vallina [c], Peter Kristensen [a,*]

[a] Department of Molecular Biology, University of Aarhus, Denmark
[b] NEMOD Biotherapeutics GmbH & Co. KG, Berlin-Buch, Germany
[c] Department of Immunology, Hospital Universitario Clínica Puerta de Hierro, Madrid, Spain
[d] INSERM U645, Besançon, France, University of Franche-Comte, Etablissement Français du Sang- Bourgogne/Franche-Comte, Besancon, France

ARTICLE INFO

Article history:
Received 15 August 2008
Revised 19 September 2008
Accepted 22 September 2008
Available online 12 October 2008

Keywords:
Adjuvant
Anti-idiotypic
Bacteriophage
scFv
Vaccine

ABSTRACT

Antibodies capable of recognizing key molecular targets isolated e.g. by phage display technology have been used in the pursuit of new and improved therapies for prevalent human diseases. These approaches often take advantage of non-immunogenic antibody fragments to achieve specific toxin-, radioactivity- or effector-domain delivery. There is now a growing interest in using *anti*-idiotypic antibodies or other antigen mimics to induce potent immune responses against antigen structures in question. We have earlier reported on the functional rescue of antibodies that are active when fused to the phage, but inactive as soluble protein [Jensen, K.B., Larsen, M., Pedersen, J.S., Christensen, P.A., Alvarez-Vallina, L., Goletz, S., Clark, B.F. and Kristensen, P. (2002) Functional improvement of antibody fragments using a novel phage coat protein III fusion system. Biochem. Biophys. Res. Commun. 298, 566–73.]. The rescue was accomplished by maintaining the fusion between the antibody fragment and portions of the filamentous bacteriophage coat protein 3, as present in the original antibody-displaying phage. In the present study, we have applied this system in an attempt to improve immunogenicity of *anti*-idiotypic antibodies isolated by phage display. Here we demonstrate that by preserving linkage between phage antibody and the N-terminal domain of phage coat protein 3, we induce multimerization of the antibody fragments, and improve their immunogenicity. This immunization approach allows induction of *anti*-idiotypic antibodies in mice, and facilitates the use of antibodies that are non-functional as non-fused soluble protein.

1. Introduction

Combating human diseases by vaccination can be achieved by the administration of live attenuated pathogens, whole inactivated organisms or inactivated toxins. Such vaccines act by presenting a plethora of epitopes to the immune system, which subsequently activate an array of biological mechanisms aiding the clearance of the disease causing agent. In a large number of human diseases the disease causing agent may unfortunately not be capable of eliciting an effective immune reaction. This is observed in progressive tumors where tumor associated antigens (TAA) do not induce spontaneous efficient *anti*-tumor responses. The application of vaccines using live attenuated pathogens, whole inactivated organisms or inactivated toxins may undesirably pose a risk factor to weak patients, and may be difficult to administer. Several studies have focused on alternative vaccine models, such as peptide vaccines, subunit vaccines and chimeric live

Abbreviations: DI, Domain I; scFv, Single-chain fragment variable; FDA, The Food and Drug Administration; FuncFAb, Functionally fused antibody; CFA, Complete Freund's adjuvant; IFA, Incomplete Freund's adjuvant; i.m., Intra muscular; i.p., Intraperitoneally; PBS, Phosphate buffered saline; MPBS, Low fat milk PBS; BPBS, Bovine serum albumin PBS; EHS, Engelbreth-Holm-Swarm.

* Corresponding author. Department of Molecular Biology, University of Aarhus, Gustav Wieds Vej 10c, 8000 Århus C, Denmark. Tel.: +45 89 42 52 75; fax: +45 86 12 31 78.
E-mail address: pk@mb.au.dk (P. Kristensen).

0022-1759/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jim.2008.09.015

vector vaccines that frequently need the addition of an immune adjuvant. Adjuvants can improve the adaptive immune response in several different ways: (i) increase imunogenicity towards weak antigens; (ii) improve blood clearance and transportation of the antigen; (iii) modulate antibody avidity to the antigen, antibody specificity, isotype or subclass distribution; (iv) stimulate cell-mediated immunity and (v) enable beneficial routes of administration (Singh and O'Hagan, 1999).

Severe difficulties arise in the quest for the best vaccine adjuvants, as the fine tuning of the elicited immune response should be of a nature, where specific antibodies are induced without inducing any self intolerance (Moingeon, 2001; O'Hagan et al., 2001). Most classical adjuvants are administered mixed with antigen, but without covalent attachment between adjuvant and antigen. Different alternatives to classical adjuvants have been reported such as protein fusions of immunostimulatory cytokines to antigens like scFv or peptides (Hakim et al., 1996; Batova et al., 1999; McCormick et al., 2001; Penichet and Morrison, 2001) as well as antigens fused to heat-shock proteins (HSP) such as pathogenic proteins *Mycobacterium bovis* strain BCG HSP65 (Anthony et al., 1999), HSP70 (Rico et al., 1998), HSP71 (Anthony et al., 1999), tetanus toxin (Spellerberg et al., 1997), serum albumin-binding region of streptococcal protein G (Sjolander et al., 1997), Hepatitis B virus core antigen (Schodel et al., 1996), *E. coli* heat-labile enterotoxin B subunit (Schodel et al., 1990) and B-subunit of cholera toxin (Lebens et al., 2003).

A disease specific component (i.e., the antigen) is in addition to the adjuvant essential for a vaccine. Subunit vaccines consist of a fragment of the disease causing agent, selected based on properties such as immunogenicity, specificity, toxicity and stability. Several carbohydrate TAA such as Thomsen-Friedenreich (TF), Tn-antigen (Springer, 1997) and (sialylated)-Lewis Y/X/a are very weak immunogens (Le Pendu et al., 2001). Molecular mimicry by peptides or *anti*-idiotypic antibodies has been suggested as one solution to increase the immunogenicity of these carbohydrates. To further increase the effect of such antigens, the simultaneous use of several different peptides or *anti*-idiotypic antibodies as well as specific targeting may prove effective.

Multivalent display of disease causing antigens in vaccination strategies as a beneficial carrier property is well known to have a beneficial effect on the evoked immune response. Studies are now turning to different display formats that will prove useful for human therapies such as display of antigens on cell surfaces (Rode et al., 1999; de Ines et al., 2000; Paul et al., 2000) and virus particles (Jiang et al., 1997; McInerney et al., 1999; Mottershead et al., 2000). In addition a few fusion protein systems have been investigated, such as the pentameric cholera toxin B subunit (Liljeqvist et al., 1997) as well as the closely related pentameric *E. coli* heat-labile enterotoxin B subunit (Schodel et al., 1990). The two latter examples both perform well in oral administration, although complete Freund's adjuvant (CFA) or incomplete Freund's adjuvant (IFA) was added to evoke the desired immune response. Other multivalent display systems have been applied in immunizations without directly investigating the possible beneficial effect of multimerization e.g. dimeric IFN-γ (McCormick et al., 2001) and dimeric glutathione S-transferase (Anthony et al., 1999; Yip et al., 2001). Another attractive scaffold for immunization is the filamentous bacteriophage which very early was used for immunization purposes in rabbits and mice (de la Cruz et al., 1988; Greenwood et al., 1991; Frenkel et al., 2003). These studies demonstrated that the filamentous phage on its own is capable of eliciting an adjuvant like effect.

In this work we investigate the adjuvant and carrier properties of the FuncFAb (**Func**tionally **F**used **A**nti**b**ody) system (Jensen et al., 2002). Here, we demonstrate that the immune response to scFv fragments is significantly improved by fusion to the N-terminal domain of coat protein 3 of the filamentous bacteriophage. Further, we observe that fusion leads to the formation of higher order multimers. Here we show that such multimers can be composed of scFvs with, different functional properties thus paving the way for the construction of multifunctional complexes enabling e.g. (i) simultaneous display of several disease associated epitopes, (ii) display of a mixture of adjuvant components as well as disease associated epitopes or (iii) tissue targeting of disease associated epitopes by tissue specific scFvs.

2. Materials and methods

2.1. Construction of pKBJ vectors and expression of protein

Vectors were constructed as described previously (Jensen et al., 2002). Briefly, filamentous phage gene III fragments from pHEN2 (http://www.mrc-cpe.cam.ac.uk) were subcloned into pUC119 His6MycXbaI and a chloramphenicol resistant version of pHEN2 in order to facilitate expression of antibody fragments fused to DI (pKBJ3). Furthermore, a DI vector was constructed which enables expression of DI without a fused antibody fragment. The oligonucleotides 5′-CAT GGC CGG GGC-′3 and 5′-GGC CGC CCC GGC-′3 were annealed by mixing 1 μM of each oligonucleotide, heating to 100 °C and subsequently cooling to 4 °C. The annealed oligo was ligated into the NcoI/NotI cut pKBJ3 vector. Sequencing was done with primer (M13 rev Sequence) by SeqLab Sequence Laboratories Göttingen GmbH (Göttingen, Germany).

All expressions and immobilized metal ion affinity chromatography (IMAC) purifications of FuncFAb antibodies, non-fused antibodies and non-fused DI were performed as previously described (Jensen et al., 2002). Protein concentrations were subsequently determined according to Bradford (Bradford, 1976) and purity by SDS-PAGE (Sambrook et al., 1989). All yields and purities of expression and purification of the scFvs R5 and D4 in their respective constructs were similar to the previously reported data (Jensen et al., 2002). However, for the immunization experiments we added an additional purification step using a salt gradient over a MonoQ column. This resulted in slightly reduced yields but very pure protein. For PACA17, PACA17-DI and L36-DI we obtained yields of 1 mg of protein per liter of culture. For L36 and DI alone the yields were 0.5 mg of protein per liter of culture.

2.2. Activity measurements using ELISA

Activity of the R5/D4 pKBJ3 heteromultimer was measured by sandwich ELISA. The ELISA was performed by coating of fibronectin (Sigma-Aldrich, Copenhagen, Denmark) or A76-A/C7 overnight at 4 °C in phosphate buffered saline (PBS) at a concentration of 0.1 μg/well in ELISA-plates (MAXI-sorp™,