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Research paper

Functionally fused antibodies—A novel adjuvant fusion system

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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.

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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™,

Nunc, Roskilde, Denmark). Next, residual non-specific binding to the wells was blocked by incubation with PBS containing 2% w/v low-fat milk powder (2% MPBS) for 2 h. Following the blocking step, heteromultimeric antibody fusion protein was added at varying concentrations in 2% MPBS and incubation continued for 1 h. Plates were subsequently washed 6 times in PBS, and bound antibody was detected with either A76-A/C7 when fibronectin was coated or fibronectin (DAKO, Glostrup, Denmark) when A76-A/C7 was coated. Each protein was added at a concentration of 0.5 μg/ml. After washing six times in PBS the wells were incubated with a 1:1000 dilution of an HRP-conjugated rabbit *anti*-mouse antibody (DAKO) when fibronectin was coated. When A76-A/C7 was coated the soluble fibronectin was detected with a 1:1000 dilution of a polyclonal rabbit *anti*-fibronectin antibody (DAKO) followed by an HRP-conjugated goat *anti*-rabbit antibody (DAKO) in 2% MPBS. The reaction was developed with *o*-phenylenediamine (OPD)-tablets (DAKO) according to the manufacturer's instructions after additional six washes in PBS, and read at 490 nm with an ELISA reader (Bio-Rad, Herlev, Denmark).

2.3. Gel filtration analysis of higher order multimers

The heteromultimer consisting of R5 scFv-DI and D4 scFv-DI was analyzed with a TSK-gel G2000 SW column with a precolumn (ToSoHaas) at a flow rate of 0.8 mL/min with PBS. A low molecular weight protein marker (Amersham Biosciences, Hillerod, Denmark) was used for standardization and protein was detected using a Diode array detector 540+ (Biotek Instruments, Brondby, Denmark). Fusion protein was applied in as high concentration as possible.

2.4. Animals and immunizations

The immunological properties of the scFvs and DI were examined in 10–12 weeks old BALB/c female mice, which were immunized two times with: L36 (50 μg), L36 (50 μg)+IFA (Sigma-Aldrich,), L36-DI (75 μg), L36+DI (50 μg+25 μg), PACA17 (50 μg), PACA17 (50 μg)+IFA, PACA17-DI (75 μg) and PACA17+DI (50 μg+25 μg). Protein was dissolved in PBS and when mixed with IFA this was done by extensive vortexing of equal volumes of protein and IFA (140 μl of protein and 140 μl of IFA). Each group consisted of 5 identically treated mice. Injections of 280 μl of sample (volume kept identical for all antigen combinations) were given intraperitoneally (i.p.) at Day 0 and Day 14, and blood samples were collected at Day-1 and Day 28.

2.5. IgG1 and IgG2a antibody immune responses

The immunoassays were performed in Maxi-sorp™ plates (Nunc) coated overnight at 4 °C with 250 μg/well scFv in 0.1 M sodium carbonate pH~9.5. After blocking for 2 h at room temperature with PBS supplemented with 1% Bovine serum albumin (BPBS) 0.05% Tween-20, 50, 250, 1250 and 6250 fold dilutions of sera in 1% BPBS 0.05% Tween-20 were added to the wells. After 1 wash the IgG subclasses were detected by incubation with 1:1000 dilution of polyclonal Rabbit IgG1 and IgG2a (Abcam, Cambridge, UK) respectively. After additional washing, a HRP-conjugated goat *anti*-rabbit antibody (DAKO) was used for detection. The ELISAs were developed using 3′.3′.5′.5′.-tetramethylbenzidine (TMB) substrate (Tebu-bio, Roskilde, Denmark), and subsequently quenched with 1 M HCl. Absorbance of light with wavelength 450 nm and 655 nm were measured. Antibody titers have been determined by monitoring serial dilutions of antibody isotype controls corresponding to the two subclasses IgG1 and IgG2a.

2.6. Inhibition of L36 binding to Laminin-1

The ability of diluted sera to prevent L36 scFv binding to Laminin-1 (Becton Dickinson, Heidelberg, Germany) was studied by competition ELISA using Maxi-sorp™ plates coated over night at 4 °C with Laminin-1 (0.1 μg/well). After blocking for 2 h at room temperature with PBS supplemented with 4% BSA (BPBS), a mixture of L36 at 1.5 μg/ml preincubated with different dilutions of each serum in 1% BPBS supplemented with 0.05% Tween-20 was added and incubation continued for 1 h. Plates were washed three times and bound scFv was detected with HRP-conjugated *anti*-*c*-myc antibody (Invitrogen, Taastrup, Denmark). After washing, the chromogenic substrate TMB was added and the absorbance was measured at 450 nm.

2.7. Determination of anti-idiotypic responses

To study the presence of *anti*-idiotypic antibodies in the different sera, an ELISA was carried out by coating Maxi-sorp™ plates with rabbit *anti*-EHS (Engelbreth-Holm-Swarm) Laminin polyclonal antibody (Sigma-Aldrich) over night at 4 °C in PBS (0.3 μg/well). Plates were blocked with 4% BPBS for 2 h at room temperature and then incubated with serum diluted 1:25 in 1% BPBS supplemented with 0.05% Tween-20 for 1 h. After washing, *anti*-idiotypic antibodies were detected using HRP-conjugated goat-*anti*-mouse IgG antibody (Sigma-Aldrich).

3. Results

3.1. Construction of pKBJ vectors and derivatives thereof

The previously described family of pKBJ vectors (Jensen et al., 2002) enables fusion of scFvs to domains of the filamentous bacteriophage gene III (Fig. 1). In order to study the ability of domain I (DI) fusion proteins to heteromultimerize we constructed two pKBJ3 vectors having distinct antibiotic resistance markers. Accordingly, the fusion gene scFv-DI was subcloned from the original pKBJ3 vector encoding ampicillin resistance into an analogous vector harboring resistance to chloramphenicol.

3.2. Expression and activity analysis of recombinant scFv

Four scFv antibodies, R5, D4, L36 and PACA17, were cloned into the FuncFAb fusion vectors and a non-fusion expression plasmid. R5 and PACA17 (obtained by proteolytically selective phage display and specific elution as described in (Goletz et al., 2002)) bind in a competitive manner to the antigen combining site of the murine antibodies A76-A/C7 (Karsten et al., 1998; Price et al., 1998) and A46-B/B10 (Karsten et al., 1988), respectively. A76-A/C7 recognizes a MUC1 conformational peptide epitope of the immunodominant tandem repeat region of MUC1 which is induced by a Tn or Thomsen-

Fig. 1. Schematic representation of FuncFAb domain structure. Schematic representation of the domain structure of filamentous bacteriophage protein III and the constructed fusion protein using the following abbreviations: DI, domain I, DII, domain II, DIII domain III, SP, signal peptide—pelB leader, Tags, 6xHIS and *c*-Myc.

Friedenreich glycosylation of the DTR motif, whereas A46-B/B10 recognizes the H type 2 trisaccharide (Fucα1-2Galß2-4GlcNAcß-). When the study was initiated R5 and PACA17 were potentially structural mimicking *anti*-idiotypic scFvs, displaying the internal image of the combining sites of the murine antibodies against which they were selected. D4 scFv and L36 scFv recognizes fibronectin (Jensen et al., 2003) and laminin-1 (Sanz et al., 2001), respectively.

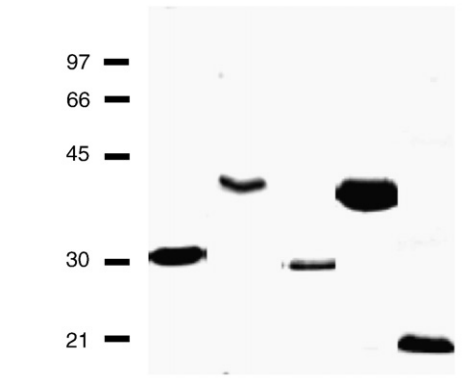
3.3. Immunization with FuncFAb protein

BALB/c mice were immunized with L36 and PACA17 scFvs alone, mixed with either soluble DI or IFA as well as fused to DI of protein III using the FuncFAb system (Fig. 2). ELISA show that the IgG responses towards L36 scFv and PACA17 scFv were increased by fusion to DI compared to the scFvs alone (student t-tests comparing scFv alone with scFv-DI fusion gives *p*-values 0.027 and 0.052 for PACA17 and L36 scFvs respectively) (Fig. 3). The observed responses were as expected lower than the responses of L36 scFv and PACA17 scFv injected with the strong adjuvant IFA. However, whereas the response for the fusion system seems to be independent of the scFv, the size of the response to the scFv supplemented with IFA varies greatly dependent on the particular scFv (data not shown). This is most likely due to the non-linked nature of the IFA adjuvant. The response against scFvs administered in a mixture with DI was similar to the scFvs alone, once again high-lighting the importance of the covalent fusion between antigen and adjuvant (student t-tests comparing combined injection of scFv and soluble DI with scFv-DI fusion give *p*-values 0.013 and 0.038 for PACA17 and L36 scFvs respectively) (Fig. 3).

The CD4 T cell dependent antibody class switch to IgG suggests that the DI fusion augments the uptake of antigen by DCs and macrophages and enhances presentation of antigen on MHC class II. The differentiation of the antibody responses into IgG1 and IgG2a responses shows a complete skewing of the response towards IgG1. The IgG2a levels are below 2.5 µg per ml of serum in all mice except two mice immunized with L36 and IFA (data not shown). The dominant presence of IgG1 antibodies indicates that the Ig class switching is due to Th2 type T cell help.

3.4. Anti-idiotypic response after L36 immunization

The specificity of the sera obtained from mice immunized with scFv L36 in the various formulations was further examined by testing their ability to inhibit the binding of L36 to its ligand, laminin-1. L36 and sera were preincubated and screened for L36 binding to coated laminin-1 by ELISA (Fig. 4A). In sera from mice either immunized with L36 scFv supplemented with IFA or fused to DI but not in sera from mice immunized with L36 scFv alone, components are induced, which were able to compete with L36 binding to laminin-1. Moreover, the observed effect was directly correlated to the observed level of humoral immune response against L36 scFv (data not shown). Therefore, the L36 fusion to the N-terminal domain of protein III seems to



Lane	1	2	3	4	5
Fusion		DI		DI	DI
ScFv	PACA17		L 36		

Fig. 2. SDS-PAGE of purified protein used for immunizations. Purified protein separated on a 12% SDS-PAGE gel shows the purity and size of the two scFvs, PACA17 and L36, either alone (lane 1 and 3) or fused to DI (lane 2 and 4). Also purified DI is included (lane 5).

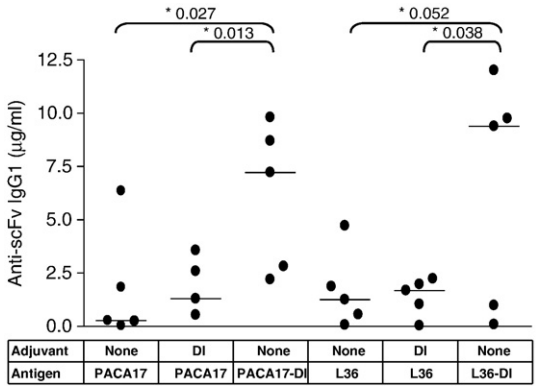


Fig. 3. IgG titer of mice immunized with FuncFAb protein. The scatter diagram shows the immune response for individual mice immunized with L36 scFv, PACA17 scFv, DI and derivatives thereof. One representative example out of two identical but independent experiments is presented. For each mouse we determined the titer of the antibodies recognizing the non-fused scFv from which the immunized protein originated. The immune responses were analyzed for their IgG1 and IgG2a subclass distribution, but since no IgG2a responses were found, only IgG1 responses are shown. The median values are indicated with horizontal lines, and the different subsets are analyzed statistically for their difference using a non-paired student t-test. Abbreviation: DI, domain I.

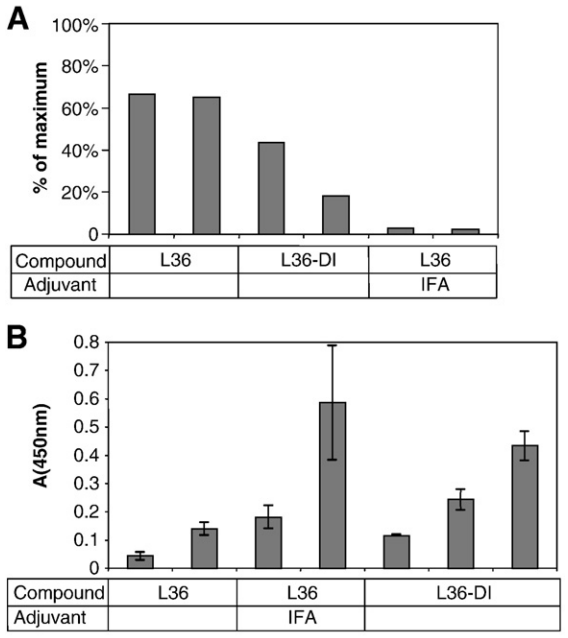


Fig. 4. *Anti*-idiotypic response towards L36 scFv and derivatives. Diagram of a mouse sera ELISA competition assay (A) is pictured as a bar diagram, each bar showing the ability of different sera to compete for the binding of L36 to Laminin-I. Optimized sera concentrations were found to be 1:15 dilution. The maximum is defined by the binding of L36 when mixed similarly with pre-immune serum (100%). In (B) an assay determining the presence of structural mimics of Laminin-I among the antibodies in the sera is depicted. Structural mimics of Laminin-I were recognized in ELISA by the rabbit *anti*-EHS Laminin-I polyclonal antibody. Each bar represents one serum, and all sera were analyzed in triplicates and standard deviations are indicated. Abbreviation: DI, domain I.

present the scFv binding site as immunological accessible as the non-fused scFv.

Anti-idiotypic antibodies competing for L36 binding to laminin-1 may be *anti*-idiotypic antibodies type Ab2γ (idiotopes close to the antigen binding site) or Ab2β (idiotopes in the antigen binding site, resembling the epitope recognized by L36). To test whether any Ab2β *anti*-idiotypic antibodies mimicking laminin-1 were present in the sera, we studied sera binding to a polyclonal rabbit antibody against laminin-1 (Fig. 4B). Immunization with either L36 scFv supplemented with IFA or L36 fused to the N-terminal domains of protein III induced significant *anti*-idiotypic Ab2β immune response, whereas mice immunized with L36-scFv alone generated no *anti*-idiotypic Ab2β immune response. Comparing the overall and the *anti*-idiotypic immune responses to L36 shows that the diverging strength and nature of the *anti*-idiotypic immune response are not dependent on the overall immune response (data not shown). Presumable the underlying difference can be found in the difference between the inherent immune system of the individual mice.

The generation of *anti*-idiotypic antibodies in L36 immunizations demonstrates that the technique is able to determine if *anti*-idiotypic antibodies are true structural mimics (Ab2β). Therefore, we determined whether the *anti*-idiotypic antibodies R5 and PACA17 were structural mimics of their corresponding carbohydrates. It was however not possible to detect *anti*-idiotypic antibodies in serum from mice immunized with R5 and PACA17 scFvs supplemented with IFA or fused to DI, that were capable of binding the two carbohydrate structures that the scFvs were supposed to mimic (data not shown). We therefore conclude that R5 and PACA17 are not structurally mimicking *anti*-idiotypic Ab2β antibodies, but merely Ab2γ antibodies binding idiotopes close to the antigen binding site.

3.5. Multimerization analysis by gel filtration

3.5.1. Homomultimers

As observed previously, FuncFAbs expressed in *E. coli* form dimers and higher multimers, which could have immense impact on their immunological properties; 2% dimer and 68% higher multimers in the case of L36-DI (Jensen et al., 2002). The stability and reversibility of multimers were studied by incubation of fractions containing either monomers, dimers or higher multimers for 36 h at 37 °C followed by gel filtration analysis. The monomers remained as monomers, the dimers dissociated into monomers, and about 50% of the higher multimers dissociated into monomers. No dimers were observed upon multimer dissociation at 37 °C, suggesting a leakage of monomers. These data suggest that the multimerization state equilibrium is shifted towards the monomeric state at body temperature; however, about 50% of the higher multimers are still present after 36 h.

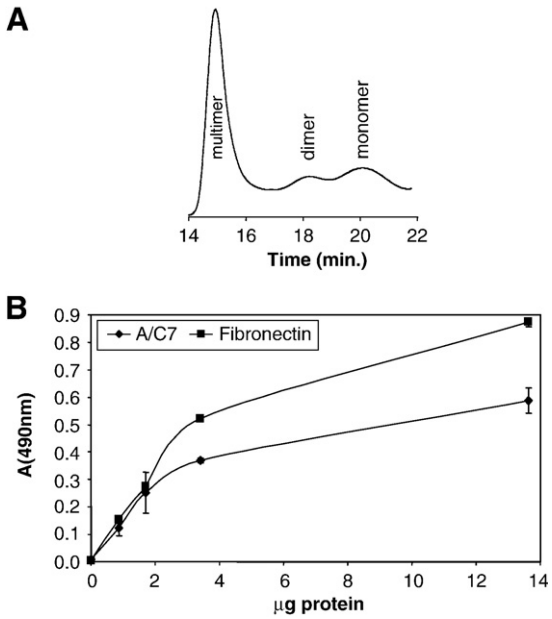


Fig. 5. Heteromultimerization of R5-DI and D4-DI. Heteromultimeric R5-DI and D4-DI IMAC purified protein was analyzed for multimerization on an HPLC gel filtration column with theoretical exclusion limit at 120 kDa (A) and for bispecific activity in a sandwich ELISA experiment (B). The sandwich ELISA was performed both with fibronectin and with A76-A/C7 coated to the wells as indicated in the figure key. The bispecific heteromultimer was subsequently challenged with the reverse binding partner in solution, A76-A/C7 when fibronectin was coated and fibronectin when A76-A/C7 was coated. Finally secondary antibodies enabled the read-out of whether bispecific heteromultimers are present that can form a bridge between the two antigens, fibronectin and A76-A/C7 and thus retain the conjugated secondary antibodies in the wells. Abbreviation: DI, domain I.

The multimers are much more stable at low temperatures as observed for the L36-DI fusion stored at 4 °C, which seems to retain multimerization at least for several weeks, reflecting the slower equilibrium dynamics at low temperatures. In order to test the ability to generate multimers in vitro, the monomers were concentrated from 0.3 mg/ml to 3 mg/ml at room temperature leading to the formation of dimers (7%) but no higher multimers. These data suggest that *in vivo* expression and native purification of DI fusion proteins are essential for obtaining highly multimerized protein.

3.5.2. Heteromultimers

Since previous data not only suggest the formation of multimers but also that these multimers are active (Jensen et al., 2002), the possible heteromultimeric assembly of co-expressed R5 and D4 antibody fragments both fused to DI of protein III was investigated. R5 DI fusion from pKBJ3 was subcloned into a similar vector carrying a different antibiotic resistance marker and co-expression was performed from double transfected *E. coli* with plasmids encoding D4 and R5 DI fusion proteins. After IMAC purification of the expressed protein it was analyzed by gel filtration analysis and sandwich ELISA. The gel filtration analysis showed clear formation of multimer (Fig. 5A). Moreover, sandwich ELISA demonstrated that the co-expressed protein preparation contained active R5 and D4 scFvs heteromultimer binding immobilized and soluble antigen (Fig. 5B). This supports the formation of active heteromultimer upon co-expression of DI fused scFvs.

4. Discussion

Although the use of filamentous bacteriophage particles as adjuvant with the antigen displayed on either protein III (de la Cruz et al., 1988) or protein VIII (Greenwood et al., 1991; Felici et al., 1993; Minenkova et al., 1993; Willis et al., 1993; Yip et al., 2001) has been reported, this is to our knowledge the first time that fusion proteins with N-terminal fragments of protein III have been applied for this purpose. Previous studies also assessed that protein III is indeed immunogenic (Tsunetsugu-Yokota et al., 1991; Minenkova et al., 1993); in this work we demonstrate that DI of protein III is sufficient to confer multimerization and immunogenicity.

The mechanism for the generation of higher order multimers during cellular expression of protein III DI fusion scFvs in *E. coli* remains to be elucidated. Possible explanations might be higher intracellular protein concentrations, structural regions of domain I and domain II, which have been revealed by structural studies to interact (Holliger et al., 1999; Lubkowski et al., 1999) or intermolecular interactions between natively non-exposed regions of the polypeptide chain initiated before or during the folding process.

Generally multimerization leads to longer *in vivo* half-life due to the reciprocal proportional relation between the size of the molecule and its half life in blood (Willuda et al., 1999; Powers et al., 2001). Theoretically this should result in prolonged contact between the antigen containing multimeric compound and the immune system. This fact is used by a series of other vaccine studies primarily focusing on multivalent 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). The few fusion protein systems which have been investigated, such as the pentameric cholera toxin B subunit and related structures (Schodel et al., 1990; Liljeqvist et al., 1997), have only been demonstrated to evoke potent immune response in mixture with CFA or IFA. In contrast, the scFv-DI fusion proteins with their potential to multimerize do not need Freund's adjuvants in order to induce IgG immune responses. Furthermore, the introduction of heteromultimerization potentially enhances the immunological properties of the FuncFAb system by enabling (i) co-administration of both CTL and T-helper epitopes, when they are not both present in the monomeric multimer (De Berardinis et al., 2000) and (ii) dual display of two different scFvs to target an epitope of interest to a specific cell or tissue type, e.g. APCs within lymph nodes or peripheral tissues (Singh and O'Hagan, 1999; Schoonjans et al., 2000).

Our experiments document adjuvant property of the N-terminal fragment of protein III of the filamentous bacteriophage, since immunizations with otherwise non-immunogenic scFvs fused to the N-terminal domain(s) of protein III induce a significantly elevated T cell-dependent class-switched humoral immune response (IgG response) towards the scFv. A few mice seem to be able to generate a low antibody titer against the scFvs alone without the use of adjuvant. Interestingly the same does not seem to be the case when scFv is injected along with soluble non-fused DI (Fig. 3). One could speculate that such lack of response is due to DI working as a non-specific antibody decoy, but most importantly this underlines the importance of the covalent fusion between DI and the antigen. We also determined the Th1/Th2 profile via comparison of the antibody isotype responses (IgG1 vs. IgG2a) in mice showing that i.p. immunizations using IFA or DI fusion as adjuvant both give IgG1 antibody responses, suggesting that the Ig class switching is helped by Th2 cells. Administration route and vaccination format are of great importance in regard to the Th1/Th2 polarization (McCluskie et al., 2000; Serezani et al., 2002; Herrick et al., 2003), and thus we have previously reported that i.m. administration of the L36 domain I fusion protein as a DNA vaccine gives a response primarily aided by Th1 type $CD4^+$ T cells (Cuesta et al., 2006) compared to the Th2 shifted profile for i.p. immunization with L36-DI protein reported in the present study in concordance with published studies (Fyfe et al., 1991).

Our data show that immunization with scFv-DI fusion molecules induces *anti*-idiotypic antibodies. These antibodies are mixtures of Ab2β and Ab2γ antibodies, which are valuable for characterizing *anti*-idiotypic mimicry antibodies. R5 and PACA17 are selected for binding to the idiotype of tumor specific carbohydrate binding immunoglobulins in a manner competitive to the carbohydrate. To verify if R5 and PACA17 are true structural mimics of there corresponding carbohydrate structure it is ultimately necessary to perform mouse immunization studies. Here we show that scFv-DI fusions can be utilized for immunization purposes without the need of traditional adjuvants, and earlier studies showed that several phage display selected antibodies benefit from the fusion by improved folding properties; in extreme cases rendering non-active scFvs active (Jensen et al., 2002). Our results show that R5 and PACA17 are not structural mimics, and suggest that finding a structural mimic (Ab2β) of an antigen among all the *anti*-idiotypic antibodies capable of competing with the

antigen for the antigen binding site (Ab2β and Ab2γ) necessitates a labor intensive screening process.

Based on the above studies in mice, we suggest that the FuncFAb system shows great promise as an adjuvant for vaccine purposes, especially for *anti*-idiotypic antibody vaccines. The FuncFab system is especially valuable for scFvs, which are difficult to obtain in an active form (Jensen et al., 2002). We speculate that the potent effect of the FuncFab expression system is due to the multimerization and increased active folding of the antibody fragments. Therefore the FuncFab system is likely to be not only suitable for antibody fragments but also for other antigenic structures to be expressed in *E. coli*.

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