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Production, purification and biological characterization of mono-PEGylated anti-IL-17A antibody fragments

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ABSTRACT

The aim of this study was to maximize the yield of the production of mono-PEGylated anti-interleukin-17A (anti-IL-17A) antibody fragments using large (≥20 kDa) polyethylene glycol (PEG) chains. Particular attention was paid to selectively yield mono-PEGylated species to maintain the maximum possible functionality and to simplify the purification. Neutralization of IL-17A by antibody constructs might find application for the treatment of bronchial hyperreactivity. Amino-directed and sulfhydryl-directed PEGylation of the native antibody fragments were compared. The former was selected as it produced the most interesting construct in terms of yield and preservation of biological activity. In particular, the $F(ab')_2$-PEG conjugate with one 40 kDa branched PEG prepared in this study was produced at a 42% yield. The conjugate presented only a slight decrease in its binding activity and in its *in vitro* inhibitory potency offering interesting perspectives for *in vivo* studies.

1. Introduction

Monoclonal antibodies (mAbs) are of particular therapeutic interest due to their high specificity in binding to their target molecules. Numerous mAbs are currently used clinically or undergoing late-stage clinical trials (Chan and Carter, 2010).

Recombinant antibody fragments ($F(ab')_2$, Fab) and genetically engineered antibody constructs (Fab′ with a single hinge cysteine, scFv, single V-type domains antibodies, diabodies, triabodies, minibodies) can provide an effective alternative to full length antibodies (Holliger and Hudson, 2005). Among others, the advantages of antibody fragments are the lack of Fc-dependent inflammation, enhanced tumor and tissue penetration (King et al., 1994; Oh et al., 2004), penetration into inaccessible antigen sites (Stijlemans et al., 2004) as well as increased avidity and multispecific action (Casey et al., 2002; Griffiths et al., 2004; Weir et al., 2002). Furthermore, antibody fragments can be economically produced in bacterial fermentation in contrast to full length antibodies which require mammalian cultures with lower capacity (Chapman, 2002; Weisser and Hall, 2009). Yet, a main drawback of antibody fragments is the short residence time in intact form in the body. This is in part due to the fact that they have a molecular weight below the kidney filtration threshold (~60 kDa) but also due to the lack of recycling by the neonatal Fc receptor (FcRn)-mediated recycling pathway (Roopenian and Akilesh, 2007).

PEGylation of proteins is a common approach to increase serum half-life (Knight et al., 2004; Kontermann, 2009; Koumenis et al., 2000; Leong et al., 2001). The conjugation of PEG chains to proteins and antibody fragments may prolong the residence in the body due to reduced renal clearance, enhanced proteolytic resistance and reduced recognition by specific antibodies (Bailon and Won, 2009; Pasut et al., 2004; Veronese and Pasut, 2005).

Proteins attached to PEG chains ranging from 20 to 40 kDa are believed to be sufficiently large to overcome kidney ultrafiltration and to obtain a clinically useful circulation half-life (Jorgensen and Moller, 1979). Single and short-chain PEGs usually fail to yield sufficient steric protection of large proteins (Park et al., 2010). In the case of IgGs (~150 kDa) or large IgG fragments, the molecular

Abbreviations: PEG, polyethylene glycol; mAbs, monoclonal antibodies; AUC, area under the curve; BHR, bronchial hyperreactivity; HSFM, hybridoma serum free medium; LPS, lipopolysaccharide; PBS, phosphate buffered saline; PVDF, polyvinylidene fluoride; FPLC, fast protein liquid chromatography; GFC, gel filtration chromatography; CEC, cation exchange chromatography; 2-MEA, 2-mercaptoethylamine·HCl; GBS, glycine buffered saline; FCS, fetal calf serum; NHS, N-hydroxysuccinimide; GCSF, granulocyte colony-stimulating factor.

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weight is above the glomerular filtration limit (70 kDa for globular proteins), therefore, PEGylation might not offer retarded renal clearance (Bailon and Won, 2009). Large proteins with a molecular weight above 70 kDa are chiefly eliminated from the body *via* alternative routes such as liver uptake, degradation by proteolytic enzymes and clearance by the immune system (Caliceti and Veronese, 2003). Addition of a single branched 40 kDa PEG to a F(ab′)$_2$ anti-IL-8 antibody fragment led to approximately 5-fold increase in serum half-life and an 16-fold increase in area under the curve (AUC) after intravenous administration as compared to the unmodified protein. Furthermore, the attachment of two 40 kDa PEG molecules to the same antibody fragment led to an AUC which was about 18-fold higher than the unmodified protein (Koumenis et al., 2000). Even though the unconjugated constructs were exceeding the kidney ultrafiltration limit, PEGylation had an impact on the residence time in the bloodstream, indicating that it also affects the alternative routes of protein catabolism.

In this study, PEGylation methods for murine anti-IL-17A Fab′ and F(ab′)$_2$ antibody fragments are presented. Interleukins (IL)-17 contribute to bronchial hyperreactivity (BHR), mucus secretion and inflammation suggesting that they play a pivotal role in asthma pathogenesis (Barczyk et al., 2003; Nembrini et al., 2009). Therefore, the inhibition of IL-17 by anti-cytokine antibodies (anti-IL-17) may offer a new opportunity to target mechanisms of asthma. A single large PEG chain (≥20 kDa) was attached to Fab′ or F(ab′)$_2$ antibody fragments to produce conjugates retaining the maximum possible functionality and which can be easily purified. The generated products were purified, characterized and their biological activities were assessed *in vitro*.

2. Materials and methods

2.1. Materials

Linear 5 and 20 kDa maleimide PEG (abbreviated as PEG5-mal and PEG20-mal, respectively) were obtained from Creative PEGworks (Winston Salem, NC, USA). Linear 20 kDa and branched 40 kDa N-hydroxysuccinimide-PEG (abbreviated as PEG20-NHS and PEG40-NHS, respectively) purchased from NOF Corporation (Tokyo, Japan). Unless otherwise stated, chemicals and reagents were from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Production and purification of anti-IL-17A

Anti-IL-17A hybridoma (MM17F3, IgG1-kappa) was derived from mice vaccinated with mouse IL-17A conjugated to ovalbumin (Uyttenhove and Van Snick, 2006). Hybridoma cells were cultured in hybridoma serum free medium (HSFM; Invitrogen, Carlsbad, CA, USA) supplemented with IL-6 (1 ng/ml). The antibody was purified by passage over a Protein G Sepharose™ 4 Fast Flow column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and eluted with 0.1 M glycine–HCl buffer pH 2.8. Eluted antibody was collected in tubes containing 1 M Tris–HCl buffer pH 8 for immediate neutralization. Lipopolysaccharide (LPS) traces were removed by passage over Sartobind® IEC MA 15 (Sartorius-stedium biotech GmbH, Goettingen, Germany). Purified antibody was concentrated and dialyzed against phosphate buffered saline (PBS) before use.

2.3. Fast protein liquid chromatography (FPLC)

Chromatography was performed on an ÄKTA™purifier 10 system (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Absorbance at 280 nm was monitored where PEG has minimal signal contribution. The samples were filtered through a polyvinylidene fluoride (PVDF) syringe-tip filter of 0.2 μm prior to loading. Data were recorded by UNICORN software (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Product yields were calculated by the comparison of the relative area under the peak of each molecular species.

A HiLoad 16/60 Superdex 200 pg column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was used for purification of the F(ab′)$_2$ and the Fab′ antibody fragments by gel filtration chromatography (GFC). The mobile phase was PBS and the elution was isocratic. The flow rate was 0.8 ml/min.

The diverse PEGylated products were purified by cation exchange chromatography (CEC) on a Resource S, 1 ml column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). A salt gradient elution was used. Buffer A was 20 mM CH_3COONa, 5 mM NaCl, pH 4.7 and buffer B was 20 mM CH_3COONa, 350 mM NaCl, pH 4.6. The column had a maximum capacity of 25 mg. To reduce interference of excess PEG with the column medium about 0.25 mg of protein was loaded each time.

2.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with PEG staining

SDS-PAGE was performed using a gradient 4–20% Mini-PROTEAN®TGX™ precast gel (Bio-Rad, Hercules, CA, USA) along with custom made buffer containing 0.025 M tris(hydroxymethyl)aminomethan, 0.192 M glycine and 0.1% sodium dodecyl sulfate, pH 8.7. Ten μg of protein was applied in each well, and the gels were run at 125 V, constant voltage, for 110 min. The protein bands were visualized by staining with GelCode® Blue Stain Reagent (Thermo Fisher Scientific, Rockford, IL, USA). PEG species were specifically stained with a barium iodide solution based on Kurfürst's method (Kurfürst, 1992). Briefly, the gel was soaked in 0.1 M $HClO_4$ for 30 min and then immersed in a 5% $BaCl_2$ in 1 M HCl solution for 10 min. Then, iodine solution (1.3% I_2 + 4% KI) was added and incubated for 5 min. Finally, the gel was left to destain in 0.05 M HCl for 30 min.

2.5. Production of F(ab′)$_2$

Anti-IL-17A (4 mg/ml) at a w/w [enzyme]:[antibody] ratio of 1:20 in 20 mM CH_3COONa buffer, pH 4 was incubated with pepsin at 37 °C for 39 h. The reaction was quenched by addition of 2 M Tris base, pH 7.5. The mixture was then analyzed by SDS-PAGE and the F(ab′)$_2$ fragment was purified by GFC. The eluted fractions were further analyzed by SDS-PAGE to confirm the size and the purity. The concentration of the final product was measured by absorbance at 280 nm on a NanoDrop spectrophotometer 2000 (Fisher Scientific, Waltham, MA, USA) using an extinction coefficient of 1.25. The procedure described was highly reproducible and generated F(ab′)$_2$ at a yield of 92%.

2.6. Preparation of PEG-Fab′ via sulfhydryl group-directed PEGylation

Prior to the conjugation reaction, the anti-IL-17A F(ab′)$_2$ fragment (1 mg/ml) was treated with 10 mM 2-mercaptoethylamine·HCl (2-MEA) in 100 mM sodium phosphate, 150 mM NaCl, 8 mM EDTA, pH 7.2 at 37 °C for 90 min to cleave the inter-heavy disulfide bonds. The reaction mixture was cooled to room temperature. To remove the 2-MEA excess, the solution was loaded to a PD-10 desalting column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), pre-equilibrated with 100 mM sodium phosphate buffer, 2 mM EDTA, pH 6.8. Elution was then performed using the same buffer and 0.5 ml fractions were collected. All fractions were measured at 280 nm in order to identify which ones contained the Fab′ construct. An Ellman's thiol assay (Thermo Fisher Scientific, Rockford, IL, USA) was then performed to all of the fractions according to the manufacturer's instructions to