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Modulating pharmacokinetics of an anti-interleukin-8 $F(ab')_2$ by amine-specific PEGylation with preserved bioactivity

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Abstract

By covalently attaching biocompatible polyethylene-glycol (PEG) groups to ε-amino groups of the $F(ab')_2$ form of a humanized anti-interleukin-8 (anti-IL-8) antibody, we sought to decrease the in vivo clearance rate to give a potentially more clinically acceptable therapeutic. The in vivo clearance was modulated by changing the hydrodynamic size of the PEGylated antibody fragments. To achieve significant increases in the hydrodynamic size with minimal loss in bioactivity, high molecular weight linear or branched PEG molecules were used. Modification involved *N*-hydroxy-succinamide reaction of the PEGs with primary amines (lysines and/or the N-terminus) of the anti-IL-8 $F(ab')_2$. The process of adding up to four linear 20 kDa PEG, or up to two branched 40 kDa PEG, gave reproducible distribution of products. The components with uniform size (as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) were purified by a single-step ion-exchange high-performance liquid chromatography and showed no significant loss of biological activity in ligand binding and cell-based assays. Addition of a single branched 40 kDa PEG to a $F(ab')_2$ (molecular weight (MW) = 1.6 million Da) or up to two 40 kDa branched PEG (MW = 1.9 million Da) increased the serum half-life to 48 h as compared with the unPEGylated $F(ab')_2$ with a half-life of 8.5 h. This study shows that by attaching high molecular weight PEGs at a one or two sites, bioactive antibody fragments can be made reproducibly with sizes tailored to achieve the desired pharmacokinetics.

Keywords: Pharmacokinetics; Polyethylene-glycolylation; Bioactivity; Anti-interleukin-8 antibody

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1. Introduction

The rate of in-vivo clearance of human therapeutic proteins can be modulated by the chemical coupling of polyethylene glycol (PEG) moieties to the protein to increase the molecular weight (MW) beyond the 70 kDa cutoff of the kidney. For example, PEGylation of three amino-residues in interleukin (IL)-2 with 7 kDa PEG gave an average MW of 160 kDa (Knauff et al., 1988) and decreased the clearance by 17-fold, from 44 to 748 min. This molecule was active in vitro and in vivo by inducing systemic immunity in a tumor model with less side-effects compared with unPEGylated IL-2 (Balemans et al., 1995). Similar relationships of PEGylation and clearance have been reported for granulocyte-macrophage colony-stimulating factor (Carl et al., 1996), antibodies (Chamow et al., 1994; Delgado et al., 1996) and other proteins (Francis et al., 1996; Tsutsumi et al., 1996; Sakane and Pardridge, 1997). PEGylation has also been shown to increase stability against proteolytic digestion, reduce immunogenicity (e.g. in L-asparaginase) (Ashihara et al., 1978), and alter the biodistribution (e.g. in TNF-α) (Delgado et al., 1996; Francis et al., 1996). The most commonly used reaction is the nucleophilic attack of an amino-group of a protein on an electrophilic group of a PEG. There are limitations to this approach, such as generation of a distribution of products, based on the sites of modification on the protein and the heterogeneity of the PEG reagent. In addition, dramatic losses in protein activity have been noted in numerous studies due to labeling at multiple sites with small MW PEGs (Beauchamp et al., 1983). For developing a clinical product, reproducibility of the sites of attachment and of the degree of PEGylation are requirements, and minimal loss of activity is highly desirable. Site-directed modification involving selective mutagenesis at regions away from the domain required for bioactivity has been explored (Gaertner and Offord, 1996). This is a labor-intensive process and carries the risk of increasing the immunogenicity of the molecule. Described in this paper is the approach we followed for modulating the pharmacokinetics of a $F(ab')_2$ by minimal modification of lysines with high MW linear or branched PEGs.

The $F(ab')_2$ described in this study is a neutralizing antibody against IL-8, a cytokine implicated in the pathogenesis of adult respiratory distress syndrome (ARDS) a disease characterized by acute lung injury and pulmonary edema as well as multiple organ failure that results in 50% mortality (Kiehl et al., 1998). Blockade of IL-8 by antibodies decreases neutrophil-mediated tissue injury as well as neutrophil infiltration (Mukaida et al., 1998), both being associated with ARDS.

Initial efficacy studies in rabbit ARDS models showed that a dose of 5 mg/kg/day of a humanized full-length immunoglobulin IgG and about eight times higher for $F(ab')_2$ fragment were required for effective blockade of IL-8 (unpublished results). The requirement for a higher dose of $F(ab')_2$ was attributed to its faster clearance (about 15 times faster than IgG). The use of full-length IgG in the clinic poses some disadvantages: first, the half-life of several days to weeks is longer than might be desirable for an Intensive Care Unit setting, where earlier cessation of treatment might become necessary; second, the IgG has to be manufactured in mammalian cell lines that have lower expression yields and longer expression times than bacterial cell line-produced $F(ab')_2$ or Fab′ fragments. Thus, efforts were initiated to generate $F(ab')_2$ in *Escherichia coli* and to modify it with PEGylation to give clearance rates intermediate between the Fab′ and the full-length IgG. In addition, this approach has the advantage of avoiding any Fc receptor-mediated effects (Colcher et al., 1998).

2. Materials and methods

2.1. Source of PEG and $F(ab')_2$

All PEG reagents were purchased (some were custom ordered) from Shearwater Polymers (Huntsville, AL) and stored at −70°C in a desiccator. Branched *N*-hydroxysuccinamide-PEG (abbreviated as br-40k) had a 20 kDa PEG on

each of its two branches. Methoxy-succinimidyl-propionic acid-PEG (abbreviated as L-(1)-20k) was a linear PEG, 20 kDa. Recombinant $F(ab')_2$ was produced in *E. Coli* and purified to homogeneity at Genentech Inc. by several chromatographic steps.

Product nomenclature summarizes the modification: for example, linear-(1)-20 KDa-(N)-$F(ab')_2$ denotes PEG type (linear or branched)-(number of PEG molecules attached)-MW of PEG attached-site of linkage (N for amino and S for thio)-antibody fragment used.

2.2. Ion exchange high-performance liquid chromatography method

A J.T. Baker wide-pore carboxysulfone, 5 μm, 7.75×100 mm high-performance liquid chromatography (HPLC) column was used for fractionation of the different PEGylated products, taking advantage of the difference in charge of the lysine-modified species. The column was maintained at 40°C, and a combination of salt and pH gradient elution was used: Buffer A was 25 mM sodium borate, 25 mM sodium phosphate (pH 6.0), Buffer B was 1 M ammonium sulfate, and Buffer C was 50 mM sodium acetate (pH 5.0). The column had up to 10 mg capacity for protein loading and a maximum of 5 mg was loaded at each time, to minimize interference by unreacted PEG. The samples were filtered through 0.2 μm PVDF filter units prior to loading.

2.3. Size exclusion HPLC-UV detection

The hydrodynamic size of each species was estimated by size exclusion (SEC) using a Pharmacia Superose-6 HR 10/30 column (10×300 mm). The mobile phase was 200 mM NaCl, 50 mM sodium phosphate (pH 6.0). The flow rate was 0.5 ml/min and the column was kept at ambient temperature. Absorbance at 280 nm was monitored where PEG had minimal signal contribution. MW standards (Biorad, Richmond, CA) containing cyanocobalamin, myoglobin, ovalbumin, IgG and thyroglobulin were used to generate a standard curve, from which the hydrodynamic size of the PEGylated species was estimated.

2.4. Size exclusion-light scattering detection

For determination of the actual molecular weight, the SEC column was connected to an on-line static light-scattering detector (Wyatt Minidawn) equipped with three detection angles of 50, 90 and 135°. A refractive index detector (Wyatt) and a UV detector (HP1090 HPLC) were also placed on-line, both of which were used to provide independent measure of MW. All buffers were filtered with Millipore 0.01 μm filters; in addition, a 0.02 μm Whatman Anodisc 47 was placed in-line prior to the column.

The intensity of scattered light is directly proportional to the molecular weight (M) of the scattering species, independent of shape, according to:

$$M = R_0/Kc$$

where R_0 is the Rayleigh ratio, K is an optical constant related to the refractive index of the solvent, the wavelength of the incident light, and the differential refractive index (dn/dc) between the solvent and the solute with respect to the change in solute concentration, c. The system was calibrated with toluene ($R_0 = 1.406 \times 10^{-5}$ at 632.8 nm); $dn/dc = 0.16$ and an extinction coefficient of 1.34 was used. The system parameters were confirmed using DNase (produced at Genentech) with an extinction coefficient of 1.6 and 32 kDa molecular weight ($dn/dc = 0.18$). The system had a mass accuracy of ~ 3–5%.

2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Tris–Glycine Novex minigels (4–12%) were used along with the Novex supplied Tris–Glycine running buffers. Between 10 and 20 mg protein was applied in each well, and the gels were run in a cold box at 150 mV/gel for 45 min. Gels were stained with colloidal Coomassie Blue (Novex), washed with water for a few hours; and then preserved and dried in drying buffer (Novex). For rapid staining of gels in less than 5 min for pooling of IEX fractions, Fast-Stain (Pierce) was used.

2.6. Preparation of a linear (1)-20 kDa-(N)-F(ab′)$_2$

A 4 mg/ml solution of anti-IL-8 F(ab′)$_2$ was dialyzed overnight against 50 mM sodium phosphate buffer (pH 8.0). Five milliliters of the protein solution was mixed with the L-20 kDa-PEG reagent:protein at a molar ratio of 3:1. The reaction was carried out in a 15 ml polypropylene tube (Falcon), and the PEG was added while vortexing the sample at low speed. The sample was then incubated at room temperature while shaking on a nutator for 30 min. The extent of modification was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The complete reaction mixture was injected on ion exchange (IEX) for removal of any unreacted PEG and purification of singly or doubly PEGylated species. This reaction generated a mixture of 50% singly-labeled and 50% unlabeled anti-IL-8. The 50% unreacted anti-IL-8 was recycled through the PEGylation/purification steps. The pooled PEGylated product was dialyzed against formulation buffer (as already described) for in vitro assays and pharmacokinetic studies in animals. Endotoxin levels were measured and found to be below 0.5 EU/ml. The fractions were also analyzed on SDS-PAGE to confirm size homogeneity. The concentration of the final product was assessed by absorbance at 280 nm using an extinction coefficient of 1.34, as well as by amino acid analysis.

2.7. Preparation of branched (1)-40 kDa-(N)-F(ab′)$_2$

A 4 mg/ml solution of anti-IL-8 F(ab′)$_2$ was dialyzed overnight against 50 mM phosphate buffer (pH 8.0). Solid Br-40kDa-PEG powder was added to 5 ml protein solution in two aliquots to give a final PEG:protein molar ratio of 6:1. Each solid PEG aliquot was added to the protein in a 15 ml polypropylene Falcon tube while vortexing at low speed for 5 s, and then placing the sample on a nutator for 15 min. The extent of modification was evaluated by SDS-PAGE. The 5 ml PEG-protein mixture was injected on the ion exchange column for removal of any unreacted PEG. This reaction generated a mixture of unreacted (37%), singly-labeled (45%), doubly- and triply-labeled (18%) species. These were the optimal conditions for obtaining the greatest recovery of the protein with only one PEG per antibody rather than the higher molecular weight adducts. The unmodified anti-IL-8 was recycled. The PEGylated products were separated and fractionated in Falcon tubes, and then dialyzed against formulation buffer for assays and animal PK studies. Endotoxin levels were below 0.5 E.U./ml. The fractions were also run on SDS-PAGE to confirm homogeneity. The concentration of the final product was assessed by absorbance at 280 nm using an extinction coefficient of 1.34, as well as by amino acid analysis.

2.8. Preparation of branched (2)-40 kDa-(N)F(ab′)$_2$

The Br-(2)-40kDa-(N)-F(ab′)$_2$ molecule was most efficiently made by adding three times in 15 min intervals a 3:1 molar ratio of PEG:protein to the already modified Br-(1)-40kDa-(N)-F(ab′)$_2$. The molecule was purified on IEX. The reaction yielded 50% Br-(2)-40kDa-(N)-F(ab′)$_2$. The unmodified molecule was recycled until ~ 20 mg protein was isolated for animal PK studies. The product was characterized by SDS-PAGE and SEC with on-line dual light scattering and UV detection.

The procedures described were highly reproducible (gave the same banding pattern on SDS-PAGE and on IEX) and the recoveries from IEX were $> 90\%$.

2.9. In-vitro bioassays

In order to determine whether the PEGylated F(ab′)$_2$ molecules were capable of binding to and inhibiting the biological activity of IL-8 similar to unPEGylated-F(ab′)$_2$, the PEG-F(ab′)$_2$ molecules and their appropriate controls were analyzed on three different bioassays using freshly isolated neutrophils. These bioassays included: first, an IL-8-binding assay in which the ability of the antibody fragments to inhibit binding of [^{125}I]-IL-8 to the neutrophil cell surface receptors was

assessed (Leong et al., 1997); second, a neutrophil chemotactic assay was used to examine the ability of the antibodies to block neutrophil chemotaxis in response to IL-8 (Leong et al., 1994); third, β-glucoronidase release assay was tested to assess the ability of the PEG-F(ab′)$_2$ molecules to inhibit IL-8-mediated degranulation (Lowman et al., 1996). Protein concentration for all samples tested were determined by amino acid analysis, and activities were corrected for concentration.

2.9.1. IL-8 binding ELISA

Recombinant human IL-8 (produced in *E. coli* at Genentech) was diluted to 1 μg/ml in 0.05 M carbonate buffer (pH 9.6), and 100 μl aliquots were placed in the wells of enzyme-linked immunosorbent assay (ELISA) plates (Nunc Immunoplate Maxisorb, Neptune, NJ). After an overnight incubation at 4°C, the plates were washed three times with 400 μl/well wash buffer (phosphate-buffered saline (PBS)/0.05% Tween-20) and blocked for 1 h at room temperature by adding 200 μl/well assay diluent (PBS/0.5% bovine serum albumen (BSA)/0.05% Tween-20/0.01% thimerosal). This and all subsequent incubations were performed at room temperature on an orbital plate shaker. The PEG-anti-IL-8 corresponding to the samples to be analyzed was diluted in assay diluent to a range of 100–1.56 ng/ml for use as a standard curve; samples were diluted to fall within this standard curve range. For analysis of rabbit serum samples from pharmacokinetic studies, dilutions were made in assay diluent containing control rabbit serum such that the concentration of serum was uniform for all samples and standards. After completion of the blocking step, the plates were washed. The standards and samples (100 μl/well) were then added, the plates were incubated for 2 h, and again washed. Horseradish peroxidase (HRP)-conjugated goat anti-human Fab′ (ICN Immunobiologicals/Cappel, Costa Mesa, CA) diluted 1/10 000 in assay diluent was added (100 μl/well), and the plates were incubated for an additional 2 h. The plates were then washed, and HRP substrate (TMB; Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added. After adequate color was achieved (10–25 min depending on the PEG-F(ab′)$_2$ variant), the reaction was stopped by adding 100 μl/well 1 N phosphoric acid. The plates were read in a microplate reader at a wavelength of 450 nm with a 620 nm reference. The concentration of PEG-F(ab′)$_2$ in the samples was extrapolated from a four-parameter fit of each PEG-F(ab′)$_2$ standard curve. In general, the F(ab′)$_2$ molecules with the larger molecular weight PEG variants tended to be less reactive in the assay; for this reason, it was critical that standard curves of each variant be run in order to accurately determine the unknown concentration of the same variant in samples.

2.9.2. Inhibition of IL-8-mediated neutrophil chemotaxis by F(ab′)$_2$ and PEG-F(ab′)$_2$

Fresh human neutrophils were isolated, counted and resuspended at 5×10^6 cells/ml in Hank's balanced salt solution (HBSS; without calcium and magnesium) containing 0.1% bovine serum albumin. The neutrophils were labeled by adding calcein AM (Molecular Probes, Eugene, OR) at a final concentration of 2.0 μM. Following a 30 min incubation at 37°C, cells were washed twice with HBSS-BSA and resuspended at 5×10^6 cells/ml.

Chemotaxis experiments were carried out in a Neuro Probe (Cabin John, MD) 96-well chamber, model MBB96. Experimental samples (buffer only control, IL-8 alone or IL-8 + antibody) were loaded in a polyfiltronics 96-well View plate (Neuro Probe Inc.) placed in the lower chamber. One hundred microliters of the calcein AM-labeled neutrophils were added to the upper chambers and allowed to migrate through a 5 μm porosity PVP free polycarbonate framed filter (NeuroProbe Inc.) towards the bottom chamber sample. The chemotaxis apparatus was then incubated for 40–60 min at 37°C with 5% CO_2. At the end of the incubation, neutrophils remaining in the upper chamber were aspirated and the upper chambers were washed three times with PBS. Then the polycarbonate filter was removed, non-migrating cells were wiped off with a squeegee wetted with PBS, and then the filter was air dried for 15 min. The relative number of neutrophils that migrate through the filter (neutrophil migration index) was determined by measuring fluores-

cence intensity of the contents of the lower chamber and adding the two values together. Fluorescence intensity was measured with a CytoFluor 2300 fluorescent plate reader (Millipore Corp., Bedford, MA) configured to read a Corning 96-well plate using the 485–20 nm excitation filter and 530–25 nm emission filter, with the sensitivity set at 3.

2.9.3. Inhibition of IL-8-stimulated β-glucuronidase release

The ability of control and PEGylated anti-IL-8 $F(ab')_2$ molecules to inhibit the IL-8-stimulated release of β-glucuronidase from neutrophils was assessed as previously described. Briefly, varying concentrations of the PEG-$F(ab')_2$ molecules were pre-incubated with 10 nM IL-8 in polypropylene 96-well plates. Freshly isolated human neutrophils at a concentration of 1×10^7/ml were stimulated for 15 min with 5 μg/ml cytochalasin B (Sigma Chemical Co., St. Louis, MO) and were then added (100 μl/well) to the wells containing the PEG-$F(ab')_2$/IL-8 mixtures. After a 3 h incubation at room temperature, the plates were centrifuged to pellet the neutrophils, and the supernatants were harvested. β-Glucuronidase activity was detected by incubating the supernatants with a substrate (10 mM 4-methylumbelliferyl-β-D-glucuronide; Calbiochem, La Jolla, CA) and reading the plates in a microplate fluorometer at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The extent of enzyme release in each sample is expressed as a percentage of the total cellular β-glucuronidase content, determined by analyzing a sample of neutrophil lysate.

2.10. Pharmacokinetic studies

Male New Zealand White (NZW) rabbits ($n = 2$ or 3) were dosed with an equivalent amount of humanized anti-IL-8 protein ($F(ab')_2$ fragment) at 2 mg/kg, either PEGylated or unmodified, via a single intravenous bolus dose in a marginal ear vein. Amino acid composition analysis and absorbance at 280 nm were utilized to determine the protein concentration. Whole blood samples were collected via a cannulated ear artery (contra-lateral to dosing ear). Animals which received unmodified anti-IL-8 $F(ab')_2$ were sampled at the following post-dose times: 0, 5, 30 min, and 1, 2, 4, 6, 8, 10, 12, 24, 28, 32, 48, 52, 56 and 336 h. Animals which received Br-(1)-40kDa-(N)-$F(ab')_2$ were sampled at the following post-dose times: 0, 5, 30 min, and 1, 2, 4, 6, 8, 10, 12, 24, 28, 32, 48, 72, 96, 168, 216, 264, 336 and 360 h. Animals which received Br-(2)-40kDa-(N)-$F(ab')_2$ were sampled at the following post-dose times: 0, 5, 30 min, 1, 2, 4, 6, 8, 10, 12, 24, 28, 32, 48, 72, 144, 192, 240 h, and days 13, 16, 20, 23. Samples were harvested for serum and assayed for anti-IL-8 $F(ab')_2$ concentrations using an IL-8 binding ELISA (see method already described). Beginning on day 14, serum samples were also analyzed for antibodies against anti-IL-8. Noncompartmental pharmacokinetic analyses were conducted on concentration–time data up to and including 168 h using the WINNONLIN pro-

Fig. 1. The reaction scheme for PEG attachment to the amine groups on $F(ab')_2$. A nucleophilic attack onto the carbonyl of the PEG succinimidyl propionate (SPA) is followed by leaving of the *N*-hydroxysuccinamide moiety and the formation of a covalent amide bond between the lysine on $F(ab')_2$ and the PEG chain.

Fig. 2. Monitoring $F(ab')_2$ PEGylation reaction process by SDS-PAGE: 20 kDa PEG was reacted with $F(ab')_2$ at ratios of 3:1 (top) or 9:1 (bottom). Due to the relatively long half-life of the SPA-PEG in solution ($t_{1/2} = 25$ min at pH 8, 25°C), reaction time was used to modulate the extent of PEGylation. Duplicate preparations at 10, 20 and 30 min were analyzed on SDS-PAGE (Coomasie stain) in reducing (right half of gel) or nonreducing (left half of gel) conditions. The light and heavy chains with similar MW (21 and 23 Da) were not well resolved in the reduced portion of the gel; upon PEGylation, broad bands corresponding to one, two, or more PEG additions are seen in the reduced samples. In the nonreduced samples, a series of doublet bands appear, suggesting PEGylation at different sites. At 3:1 PEG:protein, the reduced samples showed addition of only one PEG, whereas the nonreduced sample showed a series of doublet bands; this is consistent with increasing total number of PEGs per $F(ab')_2$, but only one PEG adduct per light or heavy chain.

Fig. 3. IEX chromatograms of Br-(1)-40kDa-(N)-$F(ab')_2$ (prepared at 3:1 PEG:protein, 10 min, room temperature, pH 8.5; top), and L-(1)-20kDa-(N)-$F(ab')_2$ (prepared at 3:1 PEG:protein, room temperature, pH 8.5, 50 min; bottom). A cluster of at least three ion-exchange peaks (bar lines) corresponded to a similar SDS-PAGE band pattern, suggesting the same number of PEG additions to three different sites on the protein within each cluster. Each cluster was pooled for PK analysis.

gram. The following pharmacokinetic parameters were considered: central volume of distribution (V_c), steady-state volume of distribution (V_{ss}), peak concentrations (C_{max}), time to C_{max} (T_{max}), clearance (CL), area under the curve from time = 0 to infinity and mean residence times (MRT).

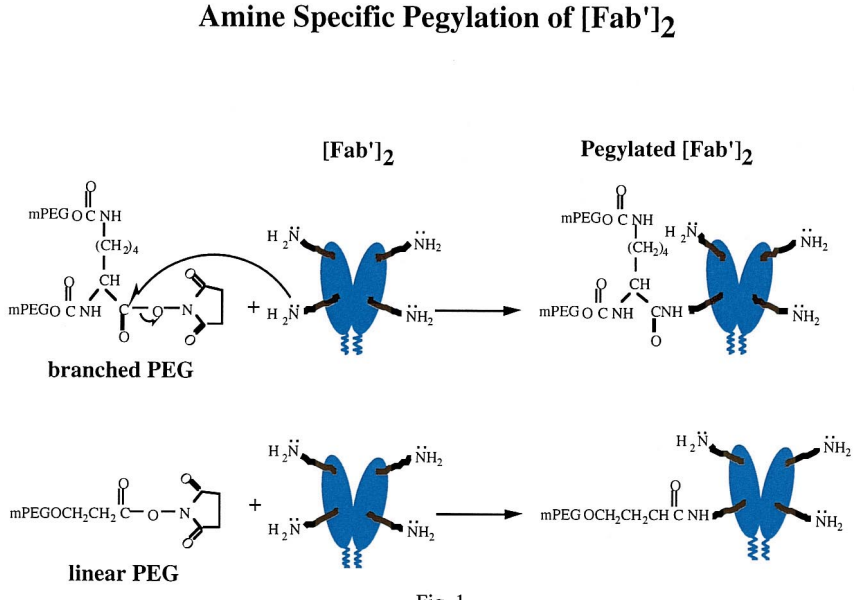


Fig. 1

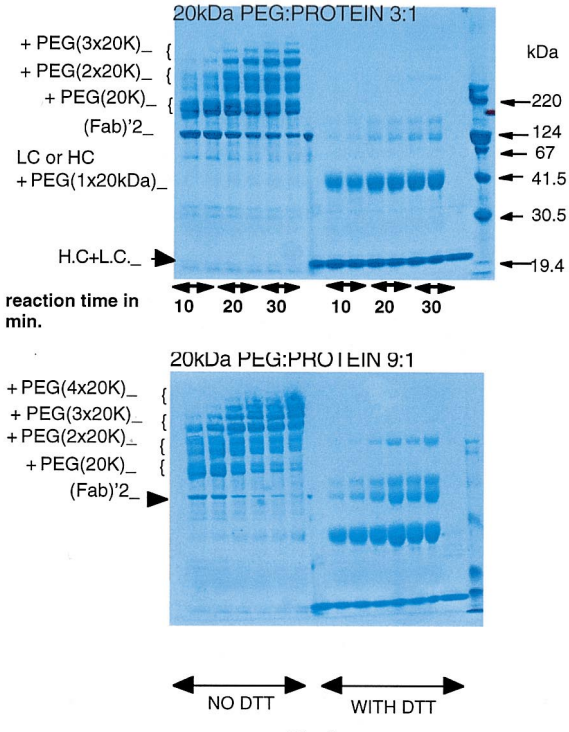


Fig. 2

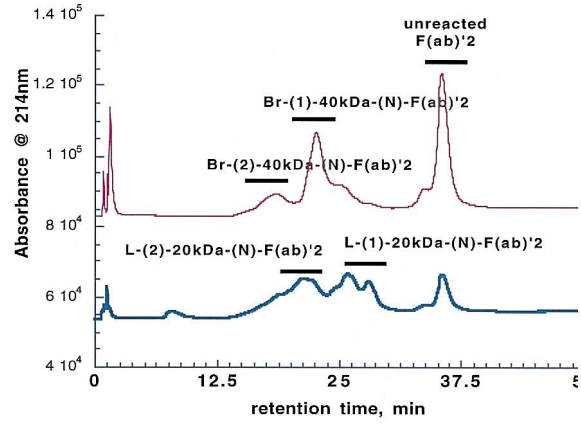


Fig. 3

3. Results and discussion

3.1. Preparation of PEG-anti-IL-8

Attaching PEG to the amino groups of proteins occurs through a nucleophilic attack of the electron pair on the nitrogen of the NH_2 moiety onto an electrophilic group; in this case, the carbonyl group connected to succinimido-propionate-PEG (Fig. 1). To decrease the probability of modifying the N-terminus, which is in close proximity to the CDR region, the reaction was performed at pH 8. At pH 8.0, both the α-amino group ($pK_a \sim 7$) of the N-terminus as well as the ε-NH_2 group of lysines ($pK_a = 10.3$) will be available to react (reactive species is uncharged NH_2). Nevertheless, there are multiple lysine sites available to react compared with the single N-terminus, which increases the chances of the lysines to PEGylate rather than the N-terminus. For the linear PEGs, a methoxy-succinimidyl-propionate derivative of an NHS-PEG (SPA-PEG) was used with significantly longer half-life in solution (17 min at 25°C at pH 8.0) compared with the NHS esters of PEGs (S-PEG) (which have 5–7 min half-life under the presented conditions). By using a PEG reagent that is less prone to hydrolysis, a greater extent of modification was achieved with lower ratios of PEG:protein. Branched 40 kDa PEG (20 kDa in each arm) was used to achieve a large increase in size while minimally modifying the molecule due to steric hindrance and size of PEG.

Factors affecting the degree of PEGylation included pH and temperature, initial PEG:protein molar ratio, and protein concentration. As already indicated, the pH of the reaction was maintained at 8.0 to maximize the ε-amino labeling. Although the reaction was faster at higher pH values, protein instability and reactivity with sites other than lysines required pH at or below 8. For the same PEG:protein molar ratio, the extent of PEGylation was not affected by protein concentration in the range 1–7 mg/ml. For convenience in later purifications, the protein concentration was maintained at 5 mg/ml. The reaction temperature was kept at ambient temperature (20–25°C) because of rapid reaction and lack of adverse effect on protein stability. The effect of reaction time and molar ratio of PEG:protein as monitored by SDS-PAGE is illustrated in Fig. 2. Since SDS-PAGE mobility depends on steric effects as well as on MW, patterns of doublets might indicate conformational variances depending on the actual amino acid modified by the PEG. Alternatively, they might indicate PEGylation on both the light and heavy chains. The SDS-PAGE of the reduced sample shows modification of the light or heavy chain; the gel resolution is not high enough to determine whether one or both chains were labeled. For the linear SPA-PEG, which has a 16 min half-life in solution at room temperature, reaction time was a significant factor for optimizing the degree of PEGylation for the desired product. This was not an option with the branched S-PEG which has a 2 min half-life in solution. Also, the ratio of PEG to protein affected PEGylation with either the SPA or the S-PEG. From these studies, a ratio of 6:1 and 3×3:1 PEG:protein gave optimal yield of one- and two-branched 40 kDa PEGylated species, respectively.

3.2. Analytical characterization of purified PEG-anti-IL-8 molecules

Since amine specific PEGylation results in loss of positive charge, ion-exchange HPLC was used to purify the PEGylated species from the un-

Fig. 4. SEC pattern of PEGylated antibody fragments purified by IEX. The dramatic increase in hydrodynamic size with increasing PEG chains is evident. The inset shows the SDS-PAGE of the purified samples. From left: lane 1, $F(ab')_2$; lane 2, Br-(2)-40 kDa-(N)-$F(ab')_2$ showing ~90% purity as defined by PEG additions; lane 3, Br-(1)-40 kDa-(N)-$F(ab')_2$ showing >95% purity; lane 4, linear(3 + 4)-20 kDa-(N)-$F(ab')_2$ showing ~95% purity; lane 5, linear(1)-20 kDa-(N)-$F(ab')_2$; lane 6, MW markers (Gibco BRL).
Fig. 5. Comparison of the hydrodynamic size with actual MW of the IEX-purified PEGylated samples as determined by SEC with UV or on-line light scattering detections, respectively.
Fig. 6. Pharmacokinetics of PEGylated anti-IL-8 antibody fragments: Rabbits were dosed with 2 mg/kg of each species and the concentration of anti-IL-8 in serum was quantitated with an ELISA. The clearance decreases with increasing size/number of added PEGs

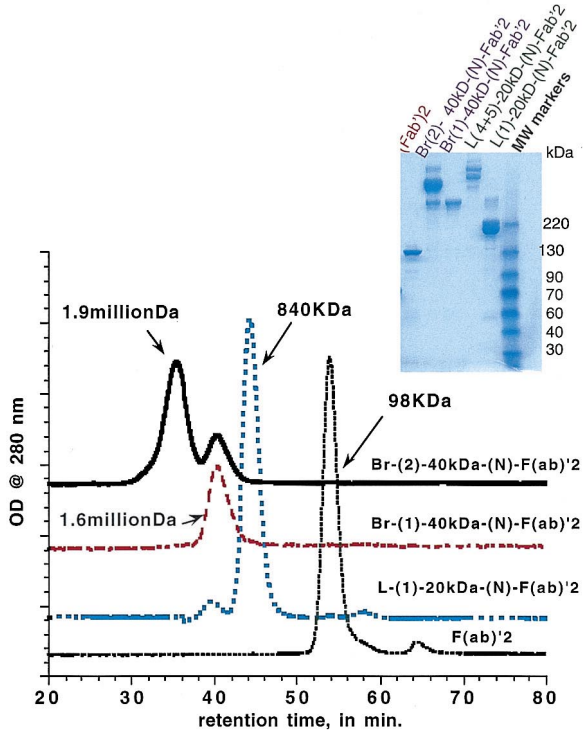


Fig. 4

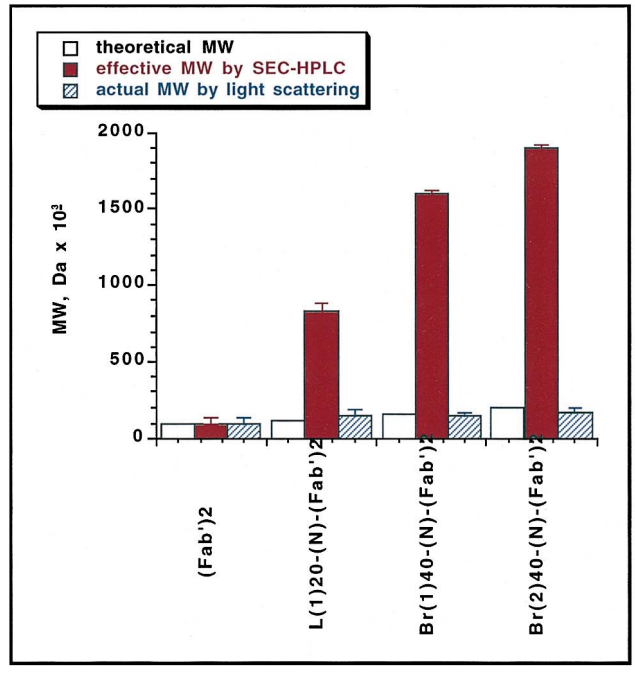


Fig. 5

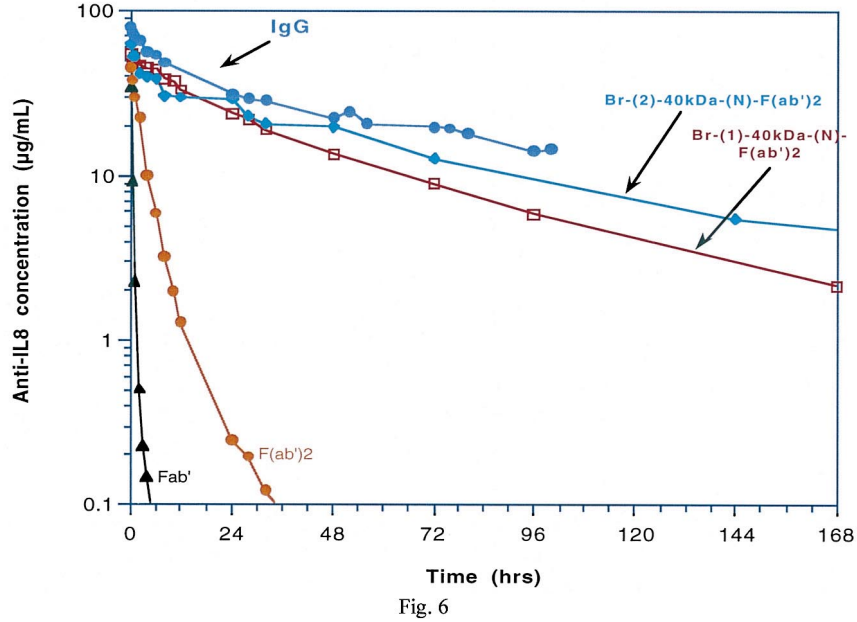


Fig. 6

modified $F(ab')_2$ as well as from any unreacted PEG. In Fig. 3, two representative IEX chromatograms are shown for the branched and the linear PEGylated anti-IL-8 mixtures at a 5 mg load. A repeated pattern of clusters of three peaks was resolved on IEX. The peaks within each cluster on IEX differed in charge distribution and were probably modified to the same extent, but at different lysine sites on the molecule. This is supported by SDS-PAGE (Fig. 2), where each three-peak IEX cluster corresponded to a single peak indicating a single modification with PEG. Based on that data, the peaks within each cluster were pooled as one product.

The SEC profile of the IEX-purified PEGylated $F(ab')_2$ species with UV detection at 280 nm is shown in Fig. 4. Using a calibration curve from the standard MW markers, an estimate of the hydrodynamic size was made. The increase in the size of $F(ab')_2$ was about 7-fold by adding one 20 kDa PEG and about 11-fold by adding one branched 40 kDa. On-line light-scattering detection gave the exact molecular weight of the products and confirmed the extent of modification (Fig. 5). The homogeneity of the purified material was determined by SDS-PAGE (Fig. 4, inset); $F(ab')_2$ migrated as a 120 kDa species, the L-(1)-20kDa-(N)-$F(ab')_2$ migrated as a band at 220 kDa, the Br-(1)-40kDa-(N)-$F(ab')_2$ migrated as one major band at 400 kDa, and the Br-(2)-40 kDa-(N)-$F(ab')_2$ migrated as a major band at around 500 kDa with less than 10% singly- or triply-labeled species. The proteins appeared somewhat larger than their absolute MW on SDS-PAGE due to the steric effect of PEG.

3.3. Pharmacokinetics of PEG anti-IL-8 molecules

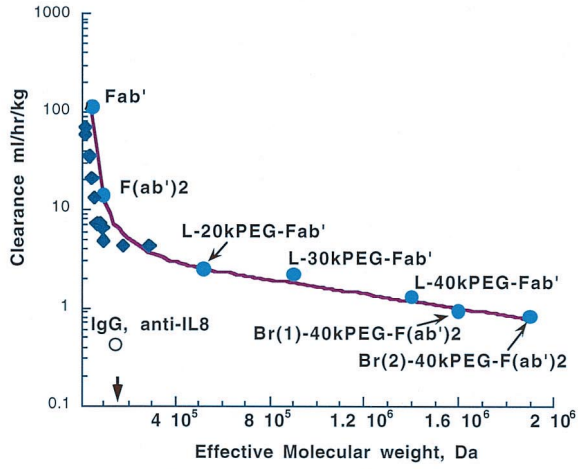
Evaluation of the pharmacokinetics of PEGylated antibodies in rabbit showed a progressive increase in the half-life with increasing size or number of added PEGs (Table 1 and Fig. 6). The PK data are included here to illustrate the dependence of protein clearance on the hydrodynamic size, in ranges well beyond that reported in the literature.

The pharmacokinetic data demonstrated that PEGylation can significantly decrease the clearance and increase the terminal half-life and MRT of the antibody fragment $F(ab')_2$ (Table 1 and Fig.

Table 1
Serum pharmacokinetic parameters (mean ± standard deviation) of anti-IL-8 $F(ab')_2$ antibody fragments (unmodified and PEGylated) following 2 mg/kg intravenous administration in New Zealand White rabbits

Molecule	Fab′	F(ab′)2		
PEG structure	None	None	Branched	Branched
Number of PEGs	None	None	1	2
Total PEG MW (kDa)	None	None	40	80
Apparent MW (kDa)	45	98	1600	1900
CL (ml/h/kg)[a]	110 ± 17	14 ± 0	0.92 ± 0.03	0.83
Terminal $t_{1/2}$ (h)[b]	3.0 ± 0.9	8.5 ± 2.1	45 ± 3	48
MRT (h)	0.6 ± 0.2	4.2 ± 0.3	55 ± 3	64
V_c (ml/kg)[c]	58 ± 3	45 ± 5	36 ± 1	32
V_{ss} (ml/kg)	68 ± 8	59 ± 4	50 ± 3	52
C_{max} (μg/ml)[d]	35 ± 1	45 ± 6	56 ± 2	62
T_{max} (min)[e]	5	5	5	5
AUC_0-∞ (h μg/ml)[f]	18 ± 3	140 ± 3	2200 ± 77	2500
Number of animals	3	3	3	2

[a] CL, weight-normalized serum clearance.
[b] Terminal $t_{1/2}$, half-life associated with the terminal phase of the concentration–time profile; MRT, mean residence time.
[c] V_c, Volume of distribution; V_{ss}, volume of distribution at steady state.
[d] C_{max}, Mean maximum concentration of compound.
[e] T_{max}, Time to reach C_{max}.
[f] AUC_0-∞, Area under the concentration versus time curve (extrapolated to infinity).



CL = 2071*exp(-6.5E^{-5}*MW) + 16*exp(-1E^{-5}*MW) + 3.7*exp(-0.08E^{-5}*MW);
R=0.99

Fig. 7

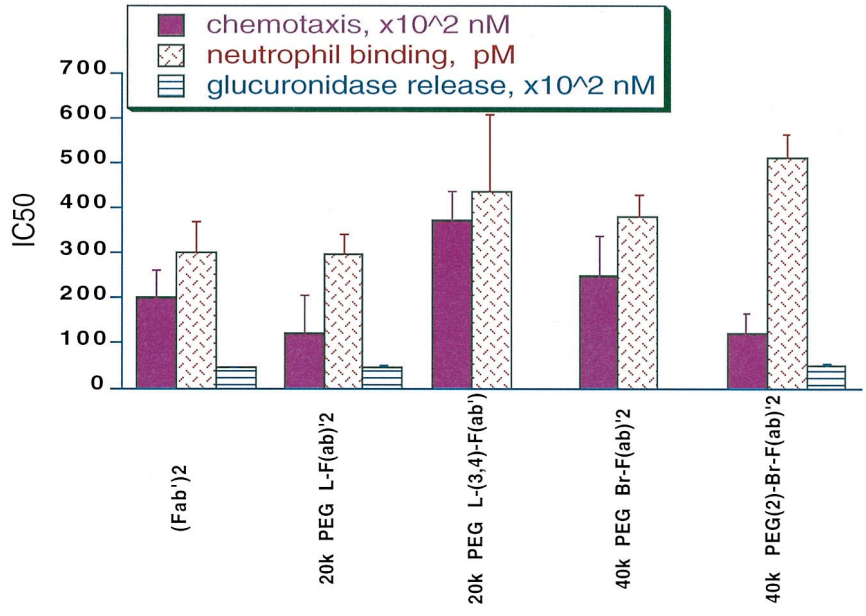


Fig. 8

Fig. 7. MW dependence of in vivo clearance of PEGylated and nonPEGylated antibody fragments in rabbits (●). Included in this graph are PK data from thiol-specific PEGylated Fab′ (S. Leong, G. Zapata, manuscript in preparation), as well as data from PEGylation of IL-2 (◆). These data show the broad applicability of the clearance model over a wide MW range and also with a molecule other than antibody; in this case, a cytokine. There is a significant decrease in the clearance after PEGylation, which has a dependence on the hydrodynamic size well beyond glomerular filtration cutoff of ~70 kDa (vertical arrow). The data fits the model described here, which can be used to predict the clearance of other PEGylated proteins when the apparent MW is known. The anti-IL-8 IgG (○) showed a much slower clearance.

Fig. 8. The effect of PEGylation on the ability of the antibody fragments to bind IL-8, or to inhibit IL-8-mediated neutrophil chemotaxis and degranulation (see Section 2 for details). There is minimal effect on the activity for one or two PEG adducts.

6), in part presumably due to decreased renal filtration. Clearance was decreased up to 16-fold and the half-life was increased by approximately 5-fold. MRT was increased by 14-fold for the 40 kDa PEG branched di-substituted $F(ab')_2$. Note that V_{ss} changed very little with PEGylation since the $F(ab')_2$ is already distributed in a volume equal to the blood volume ($\sim$ 50 ml/kg).

Given that the 98 kDa $F(ab')_2$ is already at the limit of glomerular filtration with a molecular weight cutoff of $\sim$ 70 kDa, this dramatic increase in half-life is beyond what we expected. A combined analysis of the effect of PEGylation on the clearance of various antibody fragments (Fig. 7) shows the dependence of clearance on apparent molecular weight up to 1.9 million Da. Thiol-specific chemistry was also explored using Fab′ fragments (Leong et al., in preparation) and the data is included in this figure to show that in vivo clearance is affected similarly to amine specific chemistry. IgG has a much slower clearance (about 1 week), presumably due to both specific and nonspecific Fc interactions (Colcher et al., 1998). Knauff et al. (1988) have compared IL-2 modification with either PEG or polyoxyethylated glycerol and obtained molecules up to 140 kDa (IL-2, 21 kDa) with biological activity similar to the unmodified cytokine. Their study suggests that there is a linear relationship between apparent molecular weight and clearance, regardless of the nature of the modifier or the number and size of the modifiers attached, until the apparent MW reaches $\sim$ 200 kDa. Their data is plotted in Fig. 8 for comparison. In contrast to Knauff et al. who showed that above 200 kDa clearance was not apparently affected by any further increase in MW, our investigation shows continued decrease in clearance for molecules with an apparent MW of up to 1.9 million Da with minimal losses in bioactivity.

3.4. In-vitro biological activity of PEG-anti-IL-8 molecules

Neutrophil binding and functional assays were performed to test the effect of PEGylation on the neutralizing activity of the antibody; No significant differences were observed between the $F(ab')_2$ control, the L-(1)-20kDa-(N)-$F(ab')_2$ and the Br-(1)-40kDa-(N)-$F(ab')_2$ variants in neutrophil binding (Fig. 8). However, the variants containing higher numbers (three or more) of PEGs showed a slight reduction, less than 2-fold, in IC_{50} values compared with $F(ab')_2$ alone (data not shown), suggesting partial masking of the binding region by the attached PEG molecules. The neutrophil chemotaxis assay, also shown in Fig. 8, is consistent with the neutrophil binding data, where the singly modified linear or branched variants were able to block IL-8 similar to the control. The doubly modified branched variant had the same IC_{50} as the control, but the 20 kDa PEG variant showed an increased IC_{50} (about 2-fold). The ability of the antibody to inhibit IL-8-mediated degranulation was also unchanged for the variants tested, except for the Br-(2)-40kDa-(N)-$F(ab')_2$ molecule which had a 2-fold increase in IC_{50} (Fig. 8).

Although increased plasma half-life through lysine specific PEGylation has been reported for IL-2 (Knauff et al., 1988) and IL-15 (Pettit et al., 1997), those studies showed marked losses in bioactivity. In the case of IL-15, Pettit et al. (1997) report that although PEGylation through the lysines achieved a 50-fold increase in the half-life, the biological activity was decreased several fold. Sakane and Pardridge (1997) report the need to modify BDNF through the carboxy terminus using harsh conditions because multiple amino-terminal modification resulted in loss of bioactivity. In other studies, more than 50% loss in bioactivity has been described with modification of α_2-macroglobulin (Beauchamp et al., 1983), ribonuclease (Veronese et al., 1985), and tissue plasminogen activator (Pizzo, 1991), where multi-PEGylation with small MW PEGS was used. In our studies, mild conditions were used to produce large MW molecules and achieve increases in half-life greater than 15-fold with no significant decrease in bioactivity.

4. Conclusions

PEGylated anti-IL-8-$F(ab')_2$ molecules with one or two modified NH_2 groups, by either a linear 20 kDa PEG or branched 40 kDa PEG, could be made reproducibly without significant losses in

biological activity. With succinimidyl propionate PEG (SPA-PEG-NHS), which has a long solution half-life (> 15 min), the reaction products were optimized by changing the reaction times and the ratio of PEG:protein. With a succinamide PEG (S-PEG-NHS) that has a short half life (< 5 min), the most significant factor was the ratio of PEG to protein, and not the pH, nor the temperature or the reaction time. N-Terminal PEGylation was intentionally avoided because the N-terminus is in the CDR region, where protein–receptor interactions take place, and PEGylation at that site could potentially have a detrimental effect on bioactivity. SDS-PAGE and SEC were reliable assays for determining the extent of PEGylation, purity, actual MW and hydrodynamic size. Ion exchange was used for purification of large amounts of PEGylated species from unreacted PEG, as well as for separating singly from doubly PEG-modified products. This approach presents an excellent method for altering the pharmacokinetics of proteins that cannot be modified at the N-terminus because of compromises in bioactivity.

These data describe approaches to tailoring the pharmacokinetic parameters of antibody fragments to give half-lives over several days, possibly allowing decreased dose and/or lowering the frequency of administration.

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