Poly(ethylene glycol)

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Chapter 11

Conjugation of High-Molecular Weight Poly(ethylene glycol) to Cytokines: Granulocyte–Macrophage Colony-Stimulating Factors as Model Substrates

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The ability of the small receptor-binding protein, recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF), to increase the abundance of certain blood cell types in mice was enhanced markedly by covalent attachment of a single long strand of PEG (30-40 kDa). Potency was not increased further by coupling a second strand. Such conjugates can be synthesized efficiently by reaction of protein amino groups with PEG propionaldehydes in the presence of $NaBH_3CN$ or with PEG p-nitrophenyl carbonates. Both methods have been used to prepare recombinant human GM-CSF conjugates of pre-determined composition, e.g. PEG_1GM-CSF and PEG_2GM-CSF, in high yield. These compounds, or analogous derivatives of other cyto-kines, purified by ion-exchange and size-exclusion chromatography, may be suitable candidates for pharmaceutical development.

Many therapeutic proteins have been coupled to poly(ethylene glycol) (PEG)* in order to prolong their circulating life-times and increase their potencies *in vivo* (reviewed in *1-3*). The premise underlying the present research is that the activities of small proteins that function by interacting with receptors on cell membranes may not be enhanced optimally by modification with 5-kDa PEG under the usual conditions. These proteins have molecular weights (M_r) in the range of 10-30 kDa, based on their amino acid sequences, and are generally glycosylated *in vivo*. The nature, extent, and pattern of glycosylation of their recombinant homologues depend on the organism in which they are expressed. Cytokines, which function as intercellular signals among white blood cells and their precursors, represent a physiologically important group of such receptor-binding (glyco)proteins (reviewed in *4, 5*). The steric and chemical requirements for selective, high-affinity interactions of these small proteins with their receptors are not compatible with covalent modification by PEG (PEGylation) at many sites. Thus, the technique of attaching a large number of strands of 5-kDa or 6-kDa PEG, which has been applied successfully to dozens of larger proteins, particularly enzymes that act on small substrates, is unlikely to enhance, or even to preserve, the efficacy of most cytokines.

Effects of the Number and Size of Attached PEG Strands. In several successful efforts to enhance the activities of enzymes, of which L-asparaginase (*6*), adenosine deaminase (*7*), and Cu,Zn-superoxide dismutase (SOD; *8*) are well-known examples, the increased plasma half-life resulting from the attachment of many strands of 5-kDa PEG more than compensated for the partial loss of catalytic activity observed *in vitro* (reviewed in *1-3*). The use of fewer strands of larger PEG has been explored by only a few research groups (*9-15*). For example, studies of PEG conjugates of both bovine and recombinant human SOD showed that two to five strands of PEG with $M_r > 30$ kDa were more effective than seven or 15 strands of 5-kDa PEG in preserving the biological activity, increasing the plasma persistence, and reducing the antigenicity and immunogenicity of these enzymes (*13*).

Attempts to engineer more potent forms of cytokines and other small receptor-binding proteins by PEGylation have met with mixed success (*1, 9-11, 14-20*). Studies of PEG conjugates of interleukin-2 (IL-2) (*11*), granulocyte colony-stimulating factor (G-CSF) (*14*), tumor necrosis factor α (*18*), and human growth hormone (hGH) (*19*) have demonstrated that the coupling of several strands of PEG prolonged their plasma half-lives substantially. For example, Niven *et al.* (*15*) reported that coupling the amino terminus of recombinant human G-CSF (rhG-CSF) to one strand of 6-kDa or 12-kDa PEG extended its plasma half-life in rats from one hour to two or four hours, respectively. On the other hand, an inverse relationship has been observed between the number of attached PEG strands and the receptor-binding affinities of several small proteins, measured *in vitro* (*18, 19*). In contrast with the results obtained with several enzymes, the most extensively PEGylated preparations of cytokines and peptide hormones have not proven to be the most active *in vivo* (*19, 20*). For example, Satake-Ishikawa *et al.* (*14*) found that G-CSF conjugates containing an average of two to three strands of 10-kDa PEG displayed longer duration of activity and higher potency *in vivo* than conjugates containing an average of five strands of 5-kDa PEG, despite the larger size of the latter forms, inferred from their electrophoretic mobilities in polyacrylamide gels. In other words, bigger PEG-cytokine conjugates are not necessarily better!

Objective of This Study. The research reported here was undertaken to test the proposal that the potency of certain cytokines could be optimized by their covalent linkage to a small number of PEG strands of high molecular weight (at least 18 kDa). This size was selected as the minimum because, as shown below, the molecular radius of 18-kDa PEG exceeds that of serum albumin, which is effectively retained by the kidneys (*21, 22*). While the rates of clearance of proteins from the plasma by mammalian kidneys depend on their molecular weights, with a fairly sharp cut-off near 70 kDa (about the size of serum albumin), the filtration of PEGs into the urine has a shallower dependence on M_r (*23*). In addition, the clearance of PEGs by the liver has a molecular weight dependence resulting in minimal accumulation of PEGs with M_r in the range of 20-50 kDa (*23*). For these reasons, the present research has focused on conjugates prepared from monomethoxy, monohydroxy PEGs with M_r of 19-42 kDa.

We have explored three alternative chemical approaches for coupling one or a few long strands of PEG to several cytokines, starting with recombinant murine granulocyte-macrophage colony-stimulating factor (rmuGM-CSF). The discovery of GM-CSF was based on its profound stimulation of the proliferation of white blood cells of the monocytic lineage in cell culture (reviewed in *4*). Because of the transient half-life of the unmodified cytokine *in vivo*, demonstration of its effects on peripheral blood cell counts in laboratory animals required repeated administration of high doses. This chapter includes results obtained using two conjugation chemistries to attach PEGs with M_r of 19-42 kDa to recombinant human GM-CSF (rhGM-CSF), and contrasts them with results obtained with 5-kDa PEG. Data are also presented to demonstrate that the conjugates formed by coupling a single strand of 36-kDa PEG to rmuGM-CSF promote dramatic increases in the abundance of several types of white blood cells in the peripheral circulation of mice.

Granulocyte-Macrophage Colony-Stimulating Factors as Model Substrates for PEG Conjugation. During the decades since the first descriptions of the actions *in vitro* of the cell lineage-selective colony-stimulating factors (CSFs) found in conditioned cell culture media (reviewed in *4*), knowledge regarding these proteins has increased exponentially. This has included the elucidation of their amino acid sequences, patterns of glycosylation, structures, receptors, signal transduction pathways, and physiological actions, particularly in cell culture and experimental animals (reviewed in *4, 24-27*). Despite the potential utility of these factors in the management of a wide variety of diseases, progress in implementing their therapeutic use has been impeded, in most cases, by the high cost of producing recombinant human CSFs, their rapid clearance following administration by various routes, and several serious side effects (*5, 28-32*).

   Many academic laboratories and pharmaceutical companies have sought to overcome these problems by PEGylation of cytokines. The selection of rmuGM-CSF as the first model compound for our studies of cytokines coupled to high molecular weight PEG was based, in part, on the availability of a well-characterized laboratory model in which to test the potency of the conjugates *in vivo* (*33*). Another attractive feature of this cytokine is that, for a protein of its size, it has a relatively large number of potential sites of PEGylation: the amino-terminal alanine and 11 lysine residues (*34*). Our initial results with rmuGM-CSF included the demonstration that the covalent attachment of one or two strands of 36-kDa PEG via a urethane linkage increased its potency *in vivo* by more than an order of magnitude compared with the unmodified protein (*9, 10*). The resultant conjugates induced dose-dependent increases in the peripheral blood cell counts of all types of cells that had been shown previously to respond to GM-CSF *in vitro* (*9, 10*). Such effects on peripheral cell counts have not been reported in previous studies with conjugates of rhGM-CSF prepared by another PEGylation technique (*1, 16, 17*). Our successful preliminary studies with murine GM-CSF led us to extend this approach to recombinant human GM-CSF, which differs importantly in having only six lysine residues (*35*), and to recombinant murine stem cell factor.

Methods

Materials. Recombinant human GM-CSF (rhGM-CSF), expressed in yeast, was a gift from Immunex Corporation (Seattle, WA). Recombinant murine GM-CSF (rmuGM-CSF) and recombinant murine stem cell factor, both expressed in *Escherichia coli*, were from PeproTech (Rocky Hill, NJ). Monomethoxy PEGs of various molecular weights (19, 36, and 42 kDa) were from Polymer Laboratories, Ltd. (Church Stretton, Shropshire, UK). These preparations were characterized by very narrow polydispersities, *i.e.* the ratios of weight average to number average molecular weights (M_w/M_n) were less than 1.06. Their contamination with dihydroxy PEG (PEG diol) did not exceed 2%, which is the lower limit of quantitation by our current methods. Monopropionaldehyde derivatives of monomethoxy PEG (PEG aldehydes) were from Shearwater Polymers, Inc. (Huntsville, AL) or Polymer Laboratories. PEG standards for column calibration were from Polymer Laboratories and had the following values of M_r: 1.56, 4.82, 10.0, 18.3, 32.6, 50.1, 73.4, 120, 288, 448, 646, and 1,390 kDa.

   The following proteins were used for calibration of size-exclusion chromatography columns (values for M_r and corresponding references are indicated): equine cytochrome *c*, 12,360 (*36*); equine myoglobin, 17,641 (*37*); bovine carbonic anhydrase, 29,000 (*38*); bovine SOD, 31,200 (*39*); bovine serum albumin (BSA), 66,430, and BSA dimer, 132,860 (*40*); equine liver alcohol dehydrogenase, 79,700 (*41*); human immunoglobulin G, 150,000 (*42*); equine ferritin, 476,000, and ferritin dimer, 952,000 (*43*), and bovine thyroglobulin, 620,000 (*44*). These proteins were from Sigma (St. Louis, MO) except for BSA, which was from Calbiochem-

Novabiochem (San Diego, CA), and SOD, which was from Oxis International, Inc. (Portland, OR).   Sodium cyanoborohydride, p-nitrophenyl chloroformate, and most other reagents were from Aldrich Chemical Company (Milwaukee, WI).

Preparation and Coupling of PEG Derivatives. The attributes of various "activated" (*i.e.* electrophilic) derivatives of PEG, including three that we have coupled to GM-CSF, have been discussed in several recent reviews (*1-3, 45-47*).   In the case of cytokines, the use of highly efficient coupling techniques is mandated by the high cost of the proteins as well as the cost of the purest available PEGs and PEG derivatives.   Succinimidyl carbonate derivatives of monomethoxy PEG (SC-PEGs) were prepared and coupled to cytokines by modifications of published methods (*48, 49*).   These modifications were necessitated by the small amounts of cytokines available for conjugation (*e.g.* <1 mg of stem cell factor).   For the coupling of SC-PEG to rmuGM-CSF and stem cell factor, the input molar ratio of SC-PEG to protein was 2.5:1.   Methods for cytokine PEGylation by reductive alkylation using PEG aldehydes and $NaBH_3CN$ were adapted from those of Friedman *et al.* (*50*). Related methods have been described by Chamow *et al.* (*51*) and have been applied to G-CSF (*15*) and to brain-derived neurotrophic factor (*52*), among other small receptor-binding proteins.

The use of p-nitrophenyl chloroformate to activate PEG is based on methods previously applied to the synthesis of affinity chromatography matrices (*53*). p-Nitrophenyl carbonate derivatives of PEG (pNPC-PEGs) have been used previously in the synthesis of PEG conjugates of various proteins including SOD (*54*), horseradish peroxidase (*55*), and a chimeric toxin composed of *Pseudomonas* exotoxin and transforming growth factor α (*56*).   Various reaction conditions and input molar ratios of pNPC-PEG to GM-CSF (from 1.5:1 to 12:1) were tested in this study.

Fractionation and Characterization of PEGylated GM-CSF. Mixtures of proteins PEGylated to different extents can be fractionated chromatographically on the basis of size, hydrophobicity and/or ion exchange properties (*12, 57-59*).   Each strand of high molecular weight PEG provides sufficient shielding of charges on the protein that the relative retention times on an ion exchange column of components in a PEGylated preparation of GM-CSF are: free $PEG < PEG_2GM\text{-}CSF < PEG_1GM\text{-}CSF <$ GM-CSF.   This sequential elution was used in the fractionation of PEG conjugates of GM-CSF on analytical or preparative Mono Q columns (Pharmacia,   Piscataway, NJ). Other preparations were fractionated on DEAE Sepharose CL-6B columns (Pharmacia).   Aliquots of PEGylation reaction mixtures or fractions from the Mono Q or Sepharose columns were characterized by size-exclusion high performance liquid chromatography (HPLC) on a TSK 5000 PW_{XL} column or on TSK 4000 PW_{XL} and TSK 2500 PW_{XL} columns in series (TosoHaas, Montgomeryville, PA) at room temperature in 100 mM sodium phosphate buffer, pH 6.8, containing 100 mM NaCl. Proteins and PEGs were monitored by ultraviolet absorbance and/or refractive index detectors.   The elution profiles were analyzed using the program PeakFit (Jandel Scientific, San Rafael, CA) and the results were interpreted with reference to standard curves for both protein and PEG molecular weights.

The fractions of PEG molecules linked to the amino-terminal alanine and to lysine residues in preparations of rhGM-CSF that were PEGylated by reductive alkylation were inferred from the results of amino acid analyses performed by Analytical Biotechnology Services (Boston, MA).

Evaluation of the Potency of PEGylated Murine GM-CSF in Mice. Methods used to assess the hematologic effects of PEGylated rmuGM-CSF in normal mice were adapted from Metcalf *et al.* (*33*).   Briefly, groups of 10 female BALB/c mice were injected *i.p.* twice daily for six days with vehicle or with unmodified murine GM-CSF or PEG conjugates containing 100, 200 or 400 ng of GM-CSF protein.   On

the seventh and eighth days, retroorbital blood samples (*c.* 200 μl) were drawn and on the eighth day the mice were sacrificed and their spleens were weighed. Numbers of platelets and the following types of blood cells were determined by Maryland MetPath (Baltimore, MD): red cells; total white blood cells; total, normal and "activated" lymphocytes (the latter characterized by enlarged nuclei); polymorphonuclear (PMN) neutrophils and bands (immature neutrophils); eosinophils; basophils, and monocytes.

Results

Size-exclusion Chromatography of PEGs and Proteins. The size-exclusion HPLC columns were calibrated using both PEG and protein standards, and the data for log M_r as a function of retention time were fit to cubic equations. For 12 PEG standards with M_r of 1.6-1390 kDa, the fit was virtually perfect, with a correlation coefficient (r^2) of 1.000 (Figure 1). Analogous data for 11 protein standards with M_r of approximately 12-952 kDa fit less well to a cubic equation ($r^2 = 0.983$), since the molecular radii of proteins are influenced by factors other than M_r, particularly the shape and degree of hydration (*60*). The two standard curves were not parallel and diverged as the molecular weight increased. For example, the retention time of the pre-
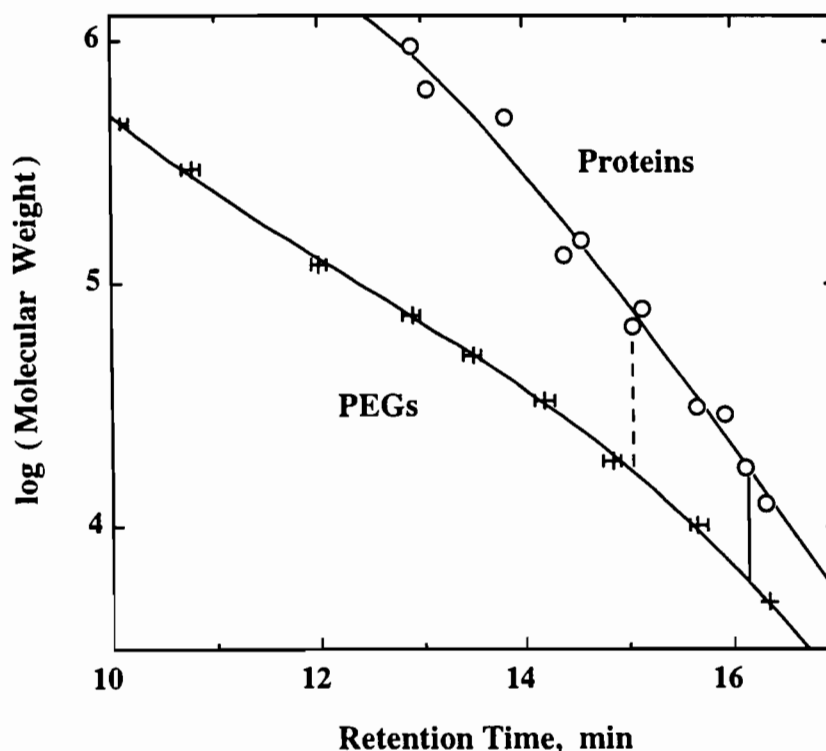

Figure 1. Calibration curves for PEG and protein standards on a size-exclusion HPLC column. Mean retention times ± s.d. are shown for nine PEG standards ($M_r = 4.8$-448 kDa). The 11 protein standards (O; $M_r = 12$-952 kDa) were cytochrome *c*, myoglobin, carbonic anhydrase, Cu, Zn-superoxide dismutase, bovine serum albumin (BSA), liver alcohol dehydrogenase, BSA dimer, immunoglobulin G, ferritin, thyroglobulin, and ferritin dimer. The retention time of BSA ($M_r = 66.4$ kDa) corresponds to an apparent M_r of 17 kDa on the PEG calibration curve (*dashed line*). The retention time of the peak in recombinant human GM-CSF corresponds to 17 kDa on the protein calibration curve and an apparent M_r of 6 kDa on the PEG calibration curve (*solid vertical line*).

dominant form of rhGM-CSF is nearly identical to that of myoglobin ($M_r = 17,641$; *37*), but corresponds to a molecular weight of only 6 kDa on the PEG calibration curve.   In other words, these small proteins would coelute with PEGs that have molecular weights about one-third as large as theirs. For a very large protein, *e.g.* thyroglobulin, the discrepancy between its molecular weight and that of coeluting PEG species would be even more pronounced, corresponding to a ratio of approximately 9:1. The major virtue of this analytical method is that the range of molecular sizes it can resolve extends from those of the smallest unmodified cytokines to those of the multiply PEGylated conjugates, even when very large PEGs are coupled.

Kinetics of *p*-Nitrophenyl Carbonate-PEG Conjugation to GM-CSF. During 24 hours of incubation of rhGM-CSF with 19-kDa pNPC-PEG at 0 - 4 °C, the size of the predominant conjugates gradually increased as an increasing fraction of the rhGM-CSF was PEGylated (Figure 2). After two hours of incubation with an initial molar ratio of PEG to protein of 2.5:1, nearly half of the protein had been converted to mono-PEGylated GM-CSF (PEG_1GM-CSF). After 6.5 hours, PEG_1GM-CSF still constituted approximately half of the total protein, but more PEG_2GM-CSF had formed and the quantity of unmodified protein had decreased. After a further addition of pNPC-PEG to increase the total molar ratio of PEG to protein to 4:1, and incubation for a total of 24 hours, nearly all of the GM-CSF had been PEGylated and the predominant
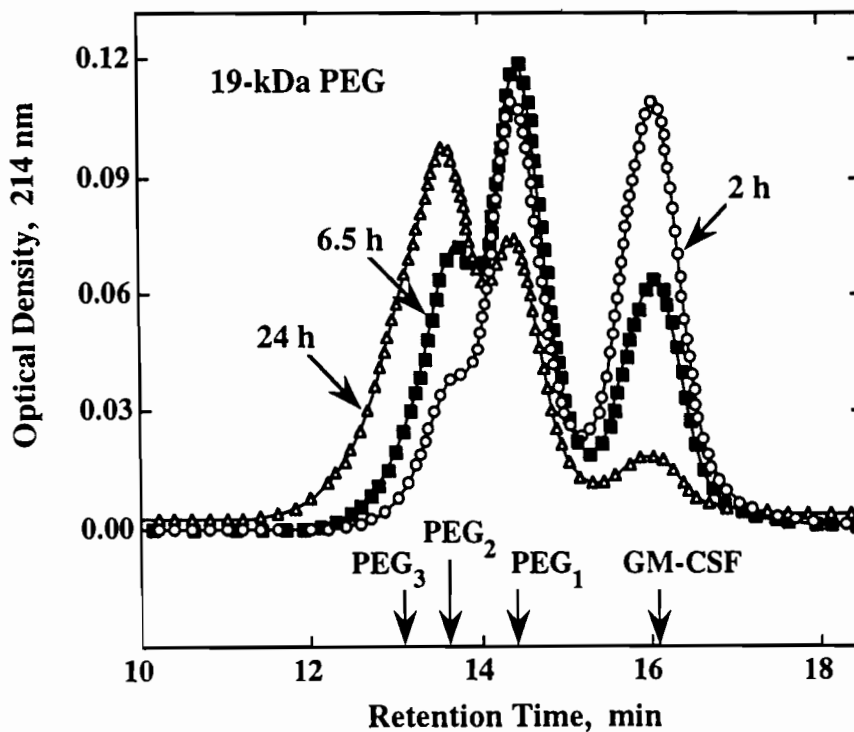

Figure 2. Sequential formation of rhGM-CSF conjugates containing one, two or three strands of PEG (*PEG₁*-, *PEG₂*- or *PEG₃GM-CSF*) by reaction with a *p*-nitrophenyl carbonate derivative of 19-kDa PEG (pNPC-PEG). Portions of a reaction mixture containing pNPC-PEG and rhGM-CSF at an initial molar ratio of 2.5:1 were analyzed by size-exclusion HPLC after incubation at 0-4 °C for 2 h (O) or 6.5 h (■). After the addition of pNPC-PEG to give a total molar ratio of 4:1, incubation was continued for a total of 24 h (Δ).

product was PEG$_2$GM-CSF (Figure 2). Analysis of these elution patterns using the program PeakFit revealed that a small amount of PEG$_3$GM-CSF had also formed in 24 hours under these conditions.

Effect of the Ratio of PEG Aldehyde to GM-CSF on Adduct Formation. Incubation of rhGM-CSF with aldehyde derivatives of PEG produced stable conjugates containing small numbers of strands of PEG only in the presence of the reducing agent, sodium cyanoborohydride (NaBH$_3$CN) (*10*). The number of strands of PEG coupled per molecule of rhGM-CSF, evaluated by size-exclusion HPLC, increased with the input molar ratio of PEG aldehyde to protein, which ranged from 1.5:1 to 6:1 in the studies shown in Figure 3. The observed retention times of the mono-PEGylated and di-PEGylated conjugates (PEG$_1$GM-CSF and PEG$_2$GM-CSF) formed with 42-kDa PEG aldehyde were within two seconds of the retention times calculated from the standard curves shown in Figure 1.

During incubation of either 5-kDa or 42-kDa PEG aldehyde with rhGM-CSF and NaBH$_3$CN for two weeks at 0-4°C at a 6:1 molar ratio of PEG to protein, virtually all of the GM-CSF was PEGylated and the predominant conjugates contained two strands of PEG per molecule of protein. The relatively poor resolution among the conjugates formed with 5-kDa PEG precluded reliable quantitation of the extent of PEG modification, as shown in Figure 3 (top). In contrast, conjugates formed with 42-kDa PEG were clearly resolved from unmodified GM-CSF and partially resolved from each other by chrotography on the TSK 5000 PW$_{XL}$ column (Figure 3, bottom). PeakFit analysis of these patterns indicated that approximately 25% and 50% of the GM-CSF molecules were present as PEG$_1$GM-CSF and PEG$_2$GM-CSF, respectively. At lower input ratios of PEG to protein, *e.g.* 1.5:1 or 3:1, there was some residual unmodified protein and the predominant conjugates contained only one strand of PEG. With an input molar ratio of 1.5:1, approximately 80% of the PEGylated species consisted of PEG$_1$GM-CSF. Qualitatively similar results were obtained when rmuGM-CSF was coupled to 36-kDa SC-PEG (see Figure 4).

Hematologic Effects of PEGylated rmuGM-CSF in Mice. Recombinant murine GM-CSF was PEGylated by incubation for 2 hours at room temperature with 36-kDa SC-PEG at an input molar ratio of 2.5:1. Fractions obtained by chromatography on DEAE-Sepharose in isotonic buffer were analyzed by size-exclusion HPLC and combined into pools containing predominantly mono-PEGylated GM-CSF (PEG$_1$ Pool) or predominantly di-PEGylated GM-CSF (PEG$_2$ Pool), as shown in Figure 4.

Solutions of unmodified or PEGylated murine GM-CSF containing 200 ng of GM-CSF protein were injected into mice according to the protocol of Metcalf *et al.* (*33*). Under these conditions, unmodified GM-CSF caused no statistically significant increases in peripheral blood counts of any cell type, including PMN neutrophils, eosinophils or lymphocytes (hatched bars in Figure 5), or in the numbers of monocytes (*10*) or platelets (unpublished results). As observed previously (*33*), the unmodified cytokine had a dramatic effect on spleen weight, causing a 70% increase over the mean value in vehicle-treated mice (open bars in Figure 5). PEG$_1$GM-CSF (light gray bars) and PEG$_2$GM-CSF (dark gray bars) were similarly effective to each other in increasing the cell counts and spleen weight, and each was far more effective than unmodified GM-CSF. In mice treated with either PEG$_1$GM-CSF or PEG$_2$GM-CSF, the increases in the numbers of circulating lymphocytes, PMN neutrophils, and eosinophils were approximately 3-fold, 7-fold, and 10-fold, respectively, relative to the corresponding cell counts in vehicle-treated mice. The numbers of circulating monocytes, platelets, and activated lymphocytes were also increased significantly after treatment with PEG$_1$GM-CSF or PEG$_2$GM-CSF (unpublished results). In addition, administration of PEG$_1$GM-CSF or PEG$_2$GM-CSF increased the spleen weight approximately 60% above the elevated weight observed after treatment with unmodified GM-CSF.
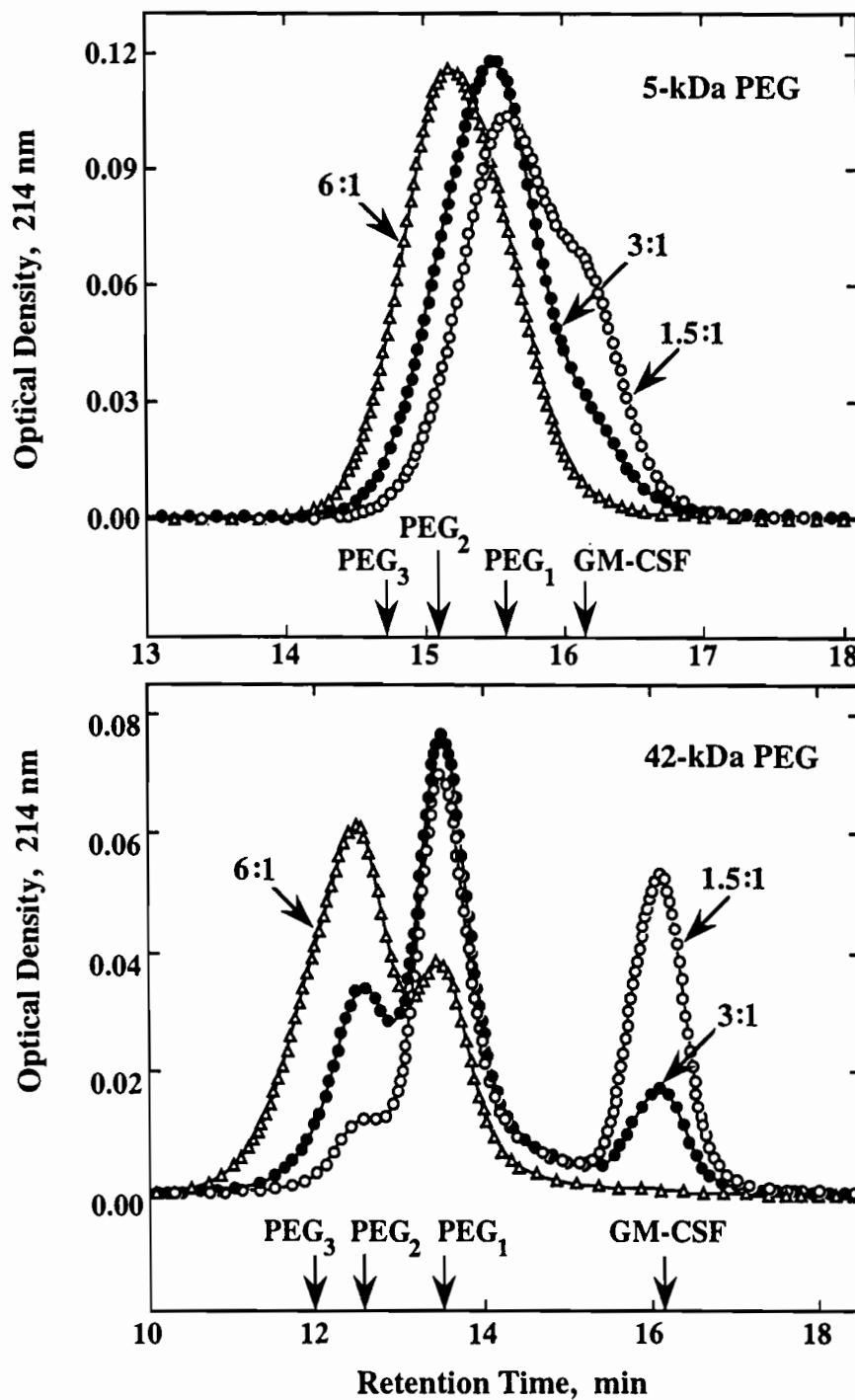
Figure 3. Effects of PEG size and input molar ratio of PEG to rhGM-CSF on the conjugates formed and resolution among them. Products of 2-week incubations of rhGM-CSF, NaBH$_3$CN, and either 5-kDa PEG aldehyde (*top*) or 42-kDa PEG aldehyde (*bottom*) at PEG:protein molar ratios of 1.5:1 (O), 3:1 (●) or 6:1 (Δ) were analyzed by size-exclusion HPLC. Vertical arrows indicate the mean retention time of unmodified rhGM-CSF and the calculated retention times of conjugates containing one, two or three strands of PEG (*PEG$_1$*-, *PEG$_2$*- or *PEG$_3$GM-CSF*).
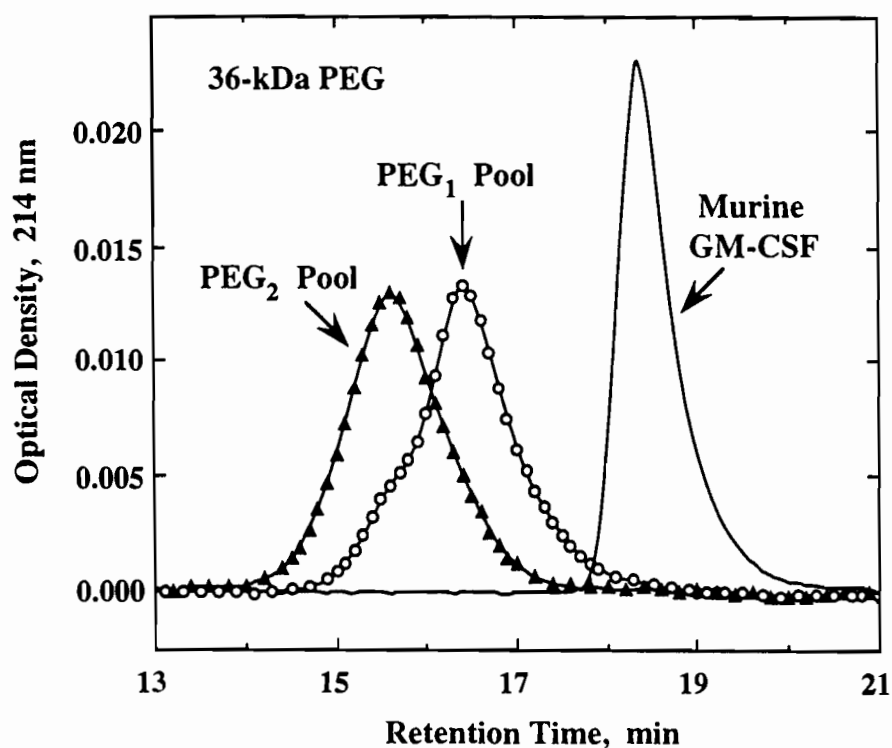
Figure 4. Size-exclusion HPLC analysis of the PEGylated rmuGM-CSF preparations tested in mice (see Figure 5). Conjugates were prepared by incubation of GM-CSF with 36-kDa SC-PEG and fractionated by chromatography on DEAE-Sepharose. Optical density (OD) data for unmodified rmuGM-CSF (——) and for pools containing predominantly PEG_1GM-CSF (O) or PEG_2GM-CSF (▲) were normalized to give equal areas under the OD-time curves.

Discussion

Chromatographic Resolution and Characterization of PEG-Protein Conjugates. High performance size-exclusion chromatography has been found to be a reliable method for characterizing and quantitating PEG-protein conjugates containing small numbers of PEG strands, as long as each strand of PEG is sufficiently large to alter the radius of the conjugate significantly (*12*). The results reported here confirm and extend those findings. In contrast, when the hydrodynamic radii of the PEG strands are smaller than those of the proteins to which they are coupled, as is generally the case when 5-kDa PEG is used, more laborious methods are required to quantitate the various conjugates (*16, 17*). As a corollary to these analytical results, the resolving power of preparative-scale size-exclusion chromatography is far greater for conjugates of proteins with high molecular weight PEGs than for conjugates with 5-kDa PEG.

The identification of cytokine species PEGylated to different extents was facilitated by calibration of the columns with respect to M_r of both PEGs and proteins and the use of the program PeakFit. The elution patterns from these columns are determined primarily by the effective molecular radii of the components, but may be influenced by adsorption to the column matrix (*61*). Since the PEG standards are homologous molecules, data for the PEG standards fit a smooth curve more closely than data for this particular selection of proteins. Nevertheless, it is clear from the results in Figure 1 that the retention times of proteins in the molecular weight range studied (12-952 kDa) are similar to those of PEGs of much lower molecular weight.
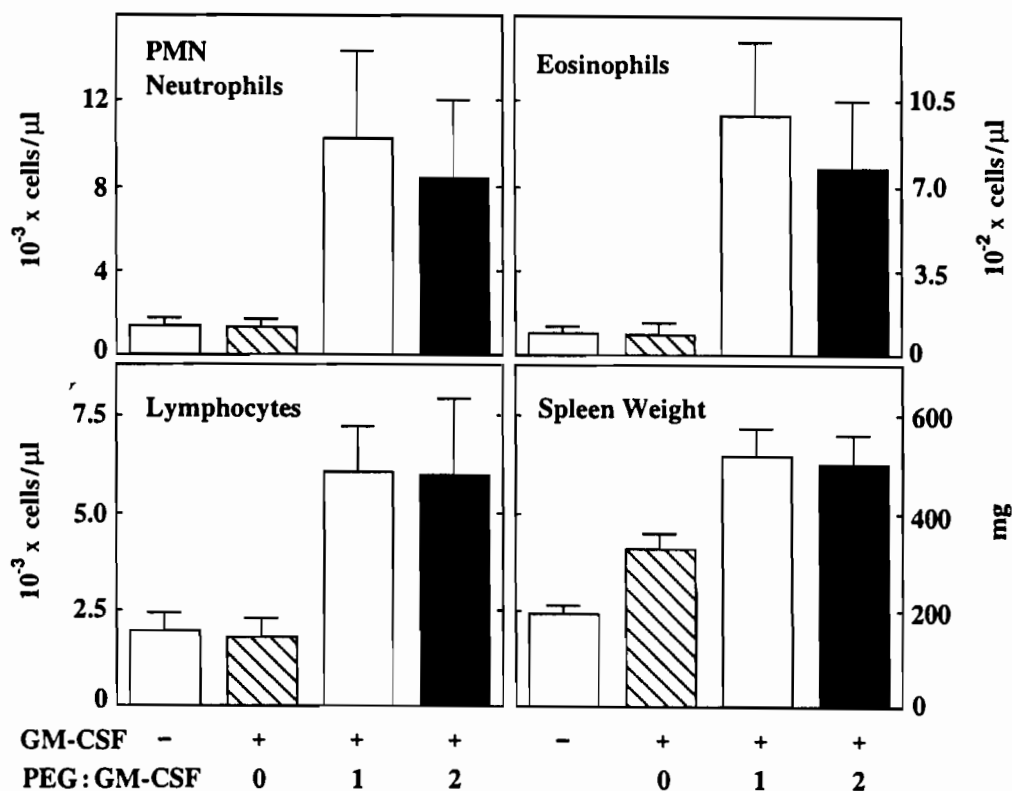
Figure 5. Dramatic effects of one or two strands of 36-kDa PEG on hematologic responses to rmuGM-CSF in mice. On Days 1-6, mice were injected twice daily with vehicle (*open bars*) or with 200 ng of GM-CSF that was unmodified (*hatched*), coupled to one strand of PEG (*light gray*) or coupled to two strands of PEG (*dark gray*). Mean values ± s.d. ($n = 10$) are shown for the numbers of three types of white cells per microliter of blood sampled on Day 7 and for spleen weight on Day 8.

The pharmacokinetics and tissue distributions of PEGs and proteins are profoundly affected by their sizes in aqueous solution. The disproportionate increase in the effective molecular radii of PEGs of high molecular weight, reflected in the diverging curves in Figure 1, makes it possible to produce conjugates of cytokines with very large radii by coupling a small number of strands of PEG of moderately high molecular weight (*e.g.* 18 kDa). While it is known that molecular radius is only one of several factors that determine the rate of renal clearance (*21*), the results in Figure 1, as well as the data of Yamaoka *et al.* (*23*), suggest that the addition of one or two strands of 18-kDa PEG would increase the molecular radius of a cytokine such as GM-CSF sufficiently that its clearance by the kidneys would be retarded substantially.

Comparison of Three Conjugation Chemistries. The rates of coupling of GM-CSF and other proteins to the three types of activated PEG used in this study decrease in the order: succinimidyl carbonate > p-nitrophenyl carbonate >> aldehyde. When both the protein and the activated PEG are relatively stable under the coupling conditions, the slowest method permits the closest monitoring, and hence the most precise regulation of the degree of modification. For example, gradual coupling facilitates the optimization of the formation of either the mono-PEGylated product

(PEG$_1$GM-CSF) or the di-PEGylated product (PEG$_2$GM-CSF). This situation is illustrated by the results in the lower panel of Figure 3, obtained by incubating 42-kDa PEG aldehyde with rhGM-CSF for two weeks at 0-4°C at an input molar ratio of 1.5:1, 3:1 or 6:1. Based on amino acid analyses and additional data, including peptide maps (unpublished results), the first molecule of PEG attached under these conditions was linked nearly exclusively to the amino-terminal alanine residue. Assessment of the activities of these products *in vitro* and *in vivo* is in progress.

The data in Figure 2 illustrate the more rapid attachment of pNPC-PEG to GM-CSF. Under the coupling conditions used, the activated PEG is hydrolyzed to release *p*-nitrophenol (detectable by its yellow color) during several hours at 0-4°C. If the desired product is PEG$_1$GM-CSF, then a 6- or 7-hour incubation of pNPC-PEG with GM-CSF at an input molar ratio of 2.5:1 is optimal. On the other hand, the formation of PEG$_2$GM-CSF is favored by incubation for 24 hours at a molar ratio of 4:1.

Practical Advantages of Coupling Long Strands of PEG. In contrast to the coupling of cytokines to 5-kDa PEG (*1;* Figure 3, top), an advantage of coupling cytokines and other small proteins to high molecular weight PEG is the possibility of producing well-characterized conjugates of predetermined composition in high yield (see Figures 2, 3 bottom, and 4). From the standpoints of the cost-effectiveness of their synthesis, purification, and analysis, as well as their potential regulatory acceptability, mono-PEGylated or di-PEGylated conjugates that are active and stable *in vivo* are preferable to the heterodisperse mixtures obtained by coupling smaller PEGs to multiple sites on the protein.

Factors that Modulate the Clearance Rates of Proteins and PEGs. The attachment of PEG may retard several processes involved in the removal of proteins from the circulation, including receptor binding, proteolysis, immune complex formation, and filtration through the renal glomeruli. While anionic proteins that are at least as large as serum albumin are not generally subject to glomerular filtration by healthy kidneys (*21*), smaller proteins such as G-CSF (*62*), ribonuclease, various protein hormones, and Bence-Jones proteins (immunoglobulin fragments) are cleared rapidly from the circulation by the kidneys (*22*). Similarly, short strands of PEG are cleared more rapidly than longer strands. For example, 6-kDa PEG has a terminal half-life in mice of <10 minutes (*63*), while 20-kDa PEG has a terminal half-life of about 3 hours (*23*). It can be inferred from previous studies of neutral, anionic, and cationic forms of dextrans and derivatives of a model protein (horseradish peroxidase) (*64-66*), that the glomerular filtration rates of free PEGs and PEG-protein conjugates may be influenced by their charges and deformability, as well as by their molecular radii. The deformability of PEG is particularly relevant to the ability of high molecular weight PEGs, PEGylated forms of small proteins, and their proteolytic cleavage products to be cleared slowly by the kidneys, despite the fact that their apparent molecular radii, as determined by size-exclusion chromatography, may be much larger than that of serum albumin.

While the persistence of cytokines or peptide hormones in plasma is prolónged to some extent by the attachment of one strand (*15*) or a few strands of 5-kDa PEG (*67*), the half-lives of the complexes increase further as more PEG strands are coupled (*11, 18, 19*), up to at least four strands for PEG-IL-2 (*20*), five strands for PEG-G-CSF (*14*), and at least seven strands for PEG-hGH (*19*). The further increments in plasma persistence when additional strands of PEG are coupled to conjugates that are already larger than albumin may be explained, in part, by the deformability of PEG.

Optimization of the Composition of PEG-Cytokine Conjugates. As discussed above, the attachment of one strand of 18-kDa PEG to a cytokine or peptide hormone is expected to decrease its rate of clearance from the plasma markedly. In contrast, the coupling of one or two strands of 5-kDa PEG to small proteins that have

molecular radii similar to that of 5-kDa PEG has been shown to produce conjugates that are smaller than serum albumin and are cleared from the plasma fairly rapidly (11, 15, 19). Since the attachment of PEG to many sites on these proteins may inhibit their ability to bind to receptors and to mediate the subsequent steps in signal transduction, it is difficult to achieve the ideal balance between the retention of intrinsic activity and the extension of plasma half-life by coupling many short strands of PEG.

Insights into the structure-function relationships of some cytokines may be provided by a comparison of the physiological activity of the amino-terminally PEGylated derivative, which is preferentially formed with PEG aldehyde at low pH, with that of a mixture of mono-PEGylated conjugates in which PEG is linked to either the amino terminal or one of the lysine residues. While the former is a unique species, it may be either more active or less active than a mixture of PEGylated isomers with the same average degree of modification. The relative activities of these products would depend on the proximity of the site(s) of PEG attachment to the domains of the cytokine that are critical for receptor binding and/or signal transduction.

Enhancement of Cytokine Potency by PEGylation. As shown in Figure 5, the covalent attachment of a single strand of 36-kDa PEG dramatically increased the activity of rmuGM-CSF in mice, and no further increase resulted from coupling a second strand. These observations and analogous results for several other hematologic parameters (unpublished results) imply that the attachment of each additional strand of PEG to a receptor-binding protein enhances its potency only if the resultant increase in half-life more than compensates for the decrease in its activity. This phenomenon has been observed with 5-kDa PEG conjugates of hGH (19) and IL-2 (20). Our results are consistent with the concept that coupling a few long strands of PEG to a cytokine interferes less with its function than coupling many short strands, making it possible to prolong its circulating life-time while preserving (more of) its intrinsic activity.

In addition to the results presented here, we have obtained encouraging preliminary data on high molecular weight PEG conjugates of recombinant murine stem cell factor (see 27), including evidence of enhanced radioprotective activity in mice (unpublished results). These observations indicate that the methods described here for coupling PEG to GM-CSF are effective for some other cytokines and may be useful for other types of small receptor-binding proteins such as chemokines and peptide hormones. For each protein, the conditions of PEGylation need to be optimized with respect to the number and size of the attached strands, as well as the coupling chemistry.

An Alternative Explanation for the Extraordinary Potency of PEGylated GM-CSF. Reports that high concentrations of unbound PEG potentiate the activation of murine lymphocytes *in vitro* (e.g. 68, 69) may provide some additional insights into the remarkable increase in the potency of PEGylated GM-CSF compared with the unmodified cytokine. It is possible that the cytokine may function, in part, as a targeting device for the delivery of PEG to the membranes of receptor-bearing cells, thereby increasing the local concentration of PEG. This process might account, in part, for the discrepancy of many orders of magnitude between the circulating concentrations of PEG in the mice following injections of less than one microgram of PEG_1- or PEG_2GM-CSF and the concentrations of unbound PEG that were found to be maximally stimulatory in the *in vitro* experiments (at least 10 mg/ml).

Conclusions

The data obtained by size-exclusion chromatography of conjugates of recombinant human or murine GM-CSF with one to three strands of 19- to 42-kDa PEG (Figures 1-4), the demonstration of the enhanced potency *in vivo* of recombinant murine GM-CSF coupled to 36-kDa PEG (Figure 5), and dose-response data for the

effects of PEGylated GM-CSF on several hematologic parameters published previously (*10*) lead to the following conclusions: 1) The covalent coupling of a single long strand of PEG to murine GM-CSF potentiates its ability to increase the numbers of several types of peripheral white blood cells in mice, either directly or indirectly, for example by stimulating the secretion of other cytokines (*70, 71*). 2) The potency of murine GM-CSF in mice is not increased further by coupling a second long strand of PEG. 3) The preceding results suggest that conjugates containing a small number of strands of high molecular weight PEG linked to human GM-CSF and other receptor-binding proteins, including other cytokines, chemokines, and hormones, may be suitable candidates for pharmaceutical development.

Abbreviations

The following abbreviations are used: BSA, bovine serum albumin; CSF, colony-stimulating factor; DEAE, diethylaminoethyl; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; hGH, human growth hormone; HPLC, high performance liquid chromatography; IL-2, inter-leukin-2; M_r, relative molecular weight; OD, optical density; PEG, poly(ethylene glycol); PEG_1-, PEG_2- or PEG_3GM-CSF, covalent complexes of GM-CSF with one, two or three molecules of PEG; PEGylation, covalent attachment of PEG; PMN, polymorphonuclear; pNPC-PEG, *p*-nitrophenyl carbonate derivative of PEG; rhG-CSF, recombinant human G-CSF; rhGM-CSF, recombinant human GM-CSF; rmuGM-CSF, recombinant murine GM-CSF; SC-PEG, succinimidyl carbonate derivative of PEG; SOD, Cu,Zn-superoxide dismutase.

Acknowledgments

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