PEGylation Improves the Pharmacokinetic Properties and Ability of Interferon Gamma to Inhibit Growth of a Human Tumor Xenograft in Athymic Mice

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Interferon gamma (IFN- γ) is a 28 kDa homodimeric cytokine that exhibits potent immunomodulatory, anti-proliferative, and antiviral properties. The protein is used to treat chronic granulomatous disease and malignant osteopetrosis, and it is under investigation as a treatment for a variety of cancer, fungal and viral diseases. IFN- γ has a short circulating half life *in vivo*, which necessitates frequent administration to patients. An unusual feature of IFN- γ is that the protein contains no native cysteines. To create a longer-acting and potentially more effective form of the protein, we introduced a cysteine residue into the IFN- γ coding sequence at amino acid position 103, which is located in a surface-exposed, non-helical region of the protein. The added cysteine residue served as the site for targeted modification of the protein with a cysteine-reactive polyethylene glycol (PEG) reagent. The recombinant protein was expressed in bacteria, purified and modified with 10, 20, and 40 kDa maleimide PEGs. The purified, PEGylated proteins had *in vitro* bioactivities comparable to IFN- γ , as measured using an *in vitro* cell growth inhibition assay. The PEGylated proteins displayed 20- to 32-fold longer half lives than IFN- γ in rats, and they were significantly more effective than IFN- γ at inhibiting growth of a human tumor xenograft in athymic mice.

Introduction

INTERFERON GAMMA (IFN- γ) belongs to a family of structurally related proteins that also includes interferon-alpha (IFN- α) and interferon-beta (IFN- β) (Wheelock 1965). IFN- γ activates cells through a different receptor than IFN- α and IFN- β , which accounts for the different physiological properties of the proteins (Farrar and Schreiber 1993; Pestka and others 1997). Production of IFN- γ is largely restricted to activated CD4⁺ T_H1 T cells, CD8⁺ T cells, and natural killer cells (Farrar and Schreiber 1993). One of the most important consequences of IFN- γ secretion is the activation of macrophages [reviewed in Schroder and others (2004)]. This is achieved through the induction of reactive oxygen intermediates and nitrogen monoxide, which activate a variety of anti-bacterial, anti-tumor, and anti-viral responses. IFN- γ plays a central role in inflammatory responses by activating endothelial cells, promoting T_H1 cell development and cellular immune responses, and up-regulation of major histocompatibility complex protein expression on antigen-presenting cells (Farrar and Schreiber 1993; Schroder and others 2004).

Native human IFN- γ is a homodimeric protein with 143 amino acids per monomer, no cysteine residues, and an

isoelectric point of ~ 8.7 (Gray and others 1982; Rinderknecht and others 1984). IFN- γ is enriched in basic amino acids and contains 40 lysines and 16 arginines per dimer. There are 2 potential N-linked glycosylation sites at asparagine-25 and asparagine-97, both of which can be glycosylated *in vivo* (Rinderknecht and others 1984). The absence of carbohydrate moieties in *Escherichia coli*-derived recombinant IFN- γ does not affect its biological activity (Arakawa and others 1985). The X-ray crystal structure of recombinant human IFN- γ has been determined for both the protein itself (Ealick and others 1991) and in complex with the IFN- γ receptor alpha subunit (Walter and others 1995). IFN- γ contains 6 alpha helical regions (helices A–F) that are joined by short unstructured sequences or loops. The homodimer is stabilized by the intertwining of the helices across the subunit interface with multiple intersubunit interactions. The first 4 helices (A–D) from 1 subunit form a cleft that accommodates helix F of the other subunit. The IFN- γ dimer binds 2 IFN- γ receptor alpha receptors and forms a 1:2 complex. Residues of IFN- γ in the binding interface are located on 2 discontinuous polypeptide segments (Lundell and others 1994; Walter and others 1995). The first segment includes helix A, a portion of the AB loop, and helix B. The second segment contains helix F and the

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C-terminal amino acids. The carboxy-terminal region of IFN- γ may interact with a second receptor subunit (IFNGR2) (Griggs and others 1992; Lunn and others 1992; Pestka 1997; Pestka and others 1997; Schroder and others 2004).

Recombinant, *E. coli*-derived IFN- γ (Actimmune®; Vidara Therapeutics International Ltd., Alpharetta, GA) is clinically used to reduce the frequency and severity of serious infections that are associated with chronic granulomatous disease and for delaying time to disease progression in patients with severe malignant osteopetrosis (The International Chronic Granulomatous Disease Cooperative Study Group 1991; Bemiller and others 1995; Key and others 1995). IFN- γ has also proved efficacious in clinical studies for treating atopic dermatitis (Hanifin and others 1993) and fungal infections in immunocompromised patients (Pappas and others 2004; Armstrong-James and others 2010). IFN- γ inhibits growth of many types of cancer cells both *in vitro* and *in vivo* (Burke and others 1999; Wall and others 2003; Merchant and others 2004; Lissat and others 2007; Reid and others 2009). IFN- γ has a short circulating half life in humans and is typically administered to patients by thrice weekly subcutaneous (sc) injections (Kurzrock and others 1985; Wagstaff and others 1987; Bemiller and others 1995). This dosing regimen is inconvenient for patients and may not optimize therapeutic properties of the protein. The finding that continuous administration of IFN- γ using an osmotic pump is superior to an intermittent intraperitoneal (ip) administration of the drug for treating parasitic infections (Murray 1990) suggested that longer-acting forms of IFN- γ with improved pharmacokinetic properties might prove more efficacious *in vivo*. One method that has proved useful for improving the pharmacokinetic properties of proteins, including murine IFN- γ , is to covalently modify the proteins with polymers such as polyethylene glycol (PEG) (Harris and others 2001; Harris and Chess 2003; Bansal and others 2011). The most commonly used method to PEGylate proteins utilizes amine-reactive PEGs, which attach to lysine residues and the N-terminal amino acid. Since most proteins contain multiple lysine residues, amine-PEGylation typically yields a heterogeneous mixture of PEGylated protein species with varying specific activities and pharmacokinetic properties due to differences in the number and locations of the attached PEG residues. Indeed, amine-PEGylation of murine IFN- γ yielded a mixture of PEGylated species with multiple attached PEGs per subunit and reduced specific activities (Bansal and others 2011). Here, we describe a longer-acting form of human IFN- γ created using a targeted, site-specific PEGylation technology. We took advantage of the absence of native cysteines in human IFN- γ to create an IFN- γ analog containing an added cysteine residue, which was subsequently modified with a cysteine-reactive PEG reagent. We find that the site-specific PEGylated IFN- γ analog has similar *in vitro* bioactivity as IFN- γ , but superior pharmacokinetic properties compared with IFN- γ and is significantly more effective than IFN- γ at inhibiting growth of a human tumor xenograft in athymic mice.

Materials and Methods

A cDNA encoding human IFN- γ was amplified by the reverse transcriptase-polymerase chain reaction method (RT-PCR) (Kawasaki 1990) from total RNA isolated from

the human Jurkat T cell line (American Type Culture Collection, Manassas, VA). The Jurkat cells were activated *in vitro* for 6 h with 1 μ g/mL phytohemagglutinin-L (Sigma-Aldrich, Inc., St. Louis, MO) and 50 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich, Inc.) to induce IFN- γ expression before RNA isolation (Wislocki and others 1985) using an RNeasy Mini RNA isolation kit (Qiagen, Inc., Valencia, CA). The forward and reverse PCR primers contained *Hind*III and *Bam*HI sites, respectively, for cloning purposes. The PCR product was digested with *Hind*III and *Bam*HI, gel purified, and cloned into similarly digested pCDNA3.1(+) vector (Invitrogen Corporation, Carlsbad, CA). A clone with the correct DNA sequence (Gray and others 1982) was designated pBBT192. This IFN- γ gene was modified for expression as a 2 cistronic gene (Schoner and others 1984) in *E. coli* by PCR mutagenesis, which was performed in 2 steps. In the first step, forward primer BB1049 (5'CAG GAC CCA TAC GTA AAA GAA GCA GAA AAC CTT AAG) and reverse primer BB1050 (5'CCG GAATTC TTA CTG GGA TGC ACG TCG ACC TTG AAA CAG) were used to delete DNA encoding the IFN- γ signal sequence from the 5' end of the gene and to incorporate a TAA termination codon followed by an *Eco*RI recognition site (GAATTC) for cloning purposes at the 3' end of the IFN- γ gene. The product of this PCR was amplified in a second PCR step using forward primer BB1048 (5'CGC GGA TCC ATC TTG GAG GAT GAT TAA ATG CAG GAC CCA TAC GTA AAA G) and reverse primer BB1050 to add a *Bam*HI recognition site (GGATCC) preceding the 5' end of the IFN- γ gene for cloning into plasmid pET21a(+), a T7 promoter expression vector (Novagen, Birmingham, United Kingdom). The *Bam*HI site is in frame with the *Bam*HI site in the T7-tag gene in pET21a(+) and creates a first small cistron encoding a 19 amino-acid peptide with the sequence MASMTGGQQMGRGSILED (1-letter amino-acid code). The first cistron ends with a TAA termination codon, which is immediately followed by DNA encoding an initiator methionine and the native IFN- γ gene sequence (QDPYVK...). The N-terminal methionine is unlikely to be cleaved from the expressed IFN- γ protein based on studies in *E. coli* with other proteins containing an initiator methionine followed by a glutamine (Hirel and others 1989), although this was not confirmed. The PCR-modified IFN- γ gene was cloned as a *Bam*HI+*Eco*RI fragment into plasmid pUC19 (Sigma-Aldrich, Inc.), and its DNA sequenced was confirmed. The gene was then excised as a *Bam*HI+*Eco*RI fragment and cloned into *Bam*HI+*Eco*RI digested pET21a(+), creating pBBT939. The IFN- γ (L103C) mutein (cysteine substituted for leucine-103) was created by PCR mutagenesis (Higuchi 1990; Scharf 1990) of plasmid pBBT939 and cloned as a *Bam*HI+*Eco*RI fragment into plasmid pUC18. After sequence verification, the L103C gene was excised from pUC18 and cloned into *Bam*HI+*Eco*RI-digested pET21a(+). For expression experiments, the pET21a(+) plasmids encoding wild type WT IFN- γ (referred to as BBT IFN- γ) and the L103C mutein were introduced into *E. coli* strain BL21 (DE3) (Invitrogen Corporation).

Protein expression and purification

E. coli cells were grown in shake flasks at 37°C in Luria Broth containing 100 μ g/mL ampicillin to an optical density

of 0.5–0.6 at 600 nm, at which time expression of the proteins was induced by adding 0.5 mM isopropyl β -D-1-thiogalactopyranoside to the cultures. The cells were harvested by centrifugation 3 h post induction and frozen at -20°C . The cell pellet from a 400 mL culture was thawed and treated with 10 mL of B-PERTM (Thermo Fisher Scientific, Inc., Rockford, IL). The insoluble material, which contained the bulk of the IFN- γ proteins, was recovered by centrifugation and resuspended in an additional 10 mL of B-PER. This mixture was treated with lysozyme (200 $\mu\text{g}/\text{mL}$) for 10 min, adjusted to 10 mM MgCl_2 , and treated with protease-free DNase (2 $\mu\text{g}/\text{mL}$). Insoluble proteins were collected by centrifugation and washed with water. The insoluble pellets were dissolved in 20 mL of 6 M guanidine, 10 mM *tris*(2-carboxyethylphosphine) hydrochloride (TCEP) in 20 mM Tris, pH 8.0. No reducing agent (TCEP) was necessary to dissolve wild type IFN- γ . TCEP also was omitted from the refolding solution and column purification buffers described later for purifying wild type IFN- γ . The solubilization mixture was stirred for 2 h at room temperature, centrifuged, and diluted into a renaturation solution consisting of 0.3 M guanidine, 1 mM TCEP, 1 mM EDTA, 20 mM Tris, pH 8.0. After overnight incubation at 4°C , each refold solution was clarified by centrifugation and diluted with 20 mM Tris, 1 mM TCEP, pH 8.0 (buffer A). The mixture was applied to a 10 mL S-Sepharose column (GE Healthcare, Piscataway, NJ) that was equilibrated in buffer A. The column was eluted with a linear salt gradient from 25% to 75% buffer B (buffer A + 1 M NaCl). S-Sepharose fractions were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and fractions that were enriched in the IFN- γ proteins were pooled. The purified L103C protein was modified with linear 10 and 20 kDa maleimide-PEGs, and a branched 40 kDa maleimide PEG (NOF Corporation, Irvine, CA) in a reaction mixture containing 100 mM Tris, pH 8, and a 10-fold molar excess of PEG. After 2 h at room temperature, the reaction mixture was diluted with 20 mM Tris, pH 8 and loaded onto a 1 mL S-Sepharose column that was equilibrated in 20 mM Tris, pH 8. The PEGylated protein was eluted using a 20%–60% gradient of buffer B (20 mM Tris, 1 M NaCl, pH 8.0). Fractions were analyzed by SDS-PAGE, and fractions enriched for the PEGylated proteins were pooled and stored at -70°C . Protein concentrations were measured using a Bradford dye-binding assay (Bio-Rad Laboratories, Hercules, CA). The mass of the PEGylated proteins used in *in vitro* bioassays and *in vivo* xenograft studies is stated as the mass of the protein portion only and does not include the mass contributed by the attached PEG moiety.

In vitro cell growth inhibition assay

The human ovarian NIH:OVCAR-3 cell line, which is sensitive to the growth inhibitory properties of IFN- γ (Burke and others 1999), was obtained from the American Type Culture Collection. The NIH:OVCAR-3 cell growth inhibition assay was performed as described (Bell and others 2008), except that the plates were incubated for 4 or 7 days rather than 3 days at 37°C . A commercial recombinant human IFN- γ standard (*E. coli*-expressed; catalog number RIFNG100; specific activity of 2×10^7 units/mg) was obtained from Pierce Endogen, a subsidiary of Thermo Fisher Scientific, Inc., and analyzed in parallel as a control. A

World Health Organization (WHO) International recombinant human IFN- γ standard was obtained from BEI Resources (Manassas, VA; catalogue number NR-3086, lot number Gxg01-902-535). The concentration of the WHO standard is 80,000 units/mL; we assumed a specific activity of 2×10^7 units/mg when using this standard in the bioassay. Protein dilutions were assayed in triplicate wells and averaged. Proteins were assayed at least 3 times. Concentrations of the PEGylated proteins refer only to the protein portions of the proteins, and they do not include mass contributions of the PEG moiety.

Pharmacokinetic experiments

Male Sprague–Dawley rats ($n=3/\text{group}$; weighing ~ 350 g) were obtained from Charles River Laboratories (Wilmington, MA). Rats received a single sc or ip injection (100 μg protein/kg) of IFN- γ prepared by us (BBT IFN- γ) or the L103C protein modified with a 10, 20, or 40 kDa PEG (only the protein mass was used to calculate doses of the PEG proteins for all experiments). Blood samples were drawn at various times post-injection. Plasma levels of the proteins were quantitated using human IFN- γ ELISA kits (R&D Systems, Minneapolis, MN). Titration experiments with the purified proteins indicated that the ELISA sensitivity for detecting BBT IFN- γ and the 10, 20, and 40 kDa PEG-L103C proteins were 158%, 25%, 20%, and 9%, respectively. Plasma levels of the proteins shown in the figures were adjusted based on these numbers. Differences in pharmacokinetic parameters between test groups were compared using a Student's 2-tailed *t*-test, with significance set at $P \leq 0.05$.

Tumor xenograft study

Female athymic nude mice were obtained from Charles River Laboratories and weighed 20–25 g at study initiation. The mice were injected with 5×10^6 NIH:OVCAR-3 tumor cells in Matrigel (BD Biosciences, Bedford, MA) in the dermis overlying the axillae on day 0 and were randomly assigned to test groups, consisting of 10 mice each. Beginning on day 3, the different test groups received sc injections in the abdominal region of vehicle (Dulbecco's phosphate-buffered saline) or 15 μg protein/injection of IFN- γ prepared by us (BBT IFN- γ) or the PEG-L103C proteins using a 3 $\times$ /week dosing regimen (Monday, Wednesday, Friday). Only the protein mass was used to calculate doses of the PEG proteins for this experiment. The proteins were formulated in vehicle solution. Tumor width and length were measured weekly using calipers. Tumor volumes were calculated using the formula: volume = [(width $\times$ width) $\times$ length/2]. On day 70, the animals were sacrificed and their tumors were excised and weighed. Differences in tumor volumes and tumor weights between test groups were compared using a Student's 2-tailed *t*-test, with significance set at $P \leq 0.05$.

Results

We expressed wild type IFN- γ (referred to as BBT IFN- γ) and the IFN- γ (L103C) mutein as intracellular proteins in *E. coli*. Both proteins were largely insoluble and were recovered in the pellet fraction after centrifugation of cell lysates. The insoluble proteins were solubilized using a denaturing agent, refolded, and purified by ion-exchange column

chromatography. Solutions used for solubilization, refolding, and purification of L103C contained a reducing agent (TCEP) to maintain the protein in a reduced state. The purified proteins co-migrated by reducing and non-reducing SDS-PAGE (Fig. 1). The proteins migrate as monomers with apparent molecular weights of ~ 17 kDa by SDS-PAGE, because SDS disrupts the hydrophobic interactions that hold IFN- γ dimers together. Under non-reducing conditions, purified L103C preparations contain a minor species with an apparent molecular weight of about 35 kDa. This minor species appears to be disulfide-linked L103C dimers, as it is not observed when the protein is analyzed using reducing SDS-PAGE conditions. Between 20 and 30 mgs of purified protein were typically obtained from a 400 mL *E. coli* culture.

Preparation of PEGylated L103 proteins

The purified L103C protein was modified with linear 10 and 20 kDa maleimide PEGs, and a branched 40 kDa maleimide PEG as described in the "Materials and Methods" section. PEGylated protein was separated from unPEGylated protein and unreacted PEG by S-Sepharose column chromatography. Using optimized PEGylation conditions, the L103C protein was converted near quantitatively to diPEGylated dimers (homodimers in which both subunits are PEGylated), resulting in a single large PEG-protein peak eluting off the S-Sepharose column (Fig. 2A). An example of the products of an optimized PEG reaction is shown in Figure 2B (lane Rx). Column fractions across the main PEG-protein peak showed only a single predominant PEG-protein species by SDS-PAGE, which is consistent with only a single PEG target site per subunit (Fig. 2B). The main PEG-protein peak fractions contained only trace amounts of protein that co-migrated with unPEGylated L103C, indicating that essentially all of the L103C monomers were PEGylated. The high PEGylation efficiency suggested that attachment of PEG to one L103C monomer did not inhibit attachment of a second PEG to the other subunit of the

FIG. 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of purified wild-type BBT IFN- γ , L103C, and PEG-L103C proteins. Proteins were analyzed under non-reducing (left panel) and reducing (right panel) conditions. Lane 1, wild type BBT IFN- γ ; lane 2, L103C; lane 3, 10 kDa PEG L103C; lane 4, 20 kDa PEG L103C; lane 5, 40 kDa PEG L103C; lane 6, molecular weight markers.

FIG. 2. Column fractionation of PEG-L103C from unPEGylated L103C protein. L103C was reacted with a 20 kDa PEG, and the PEGylation reaction mixture was fractionated by S-Sepharose column chromatography. The column chromatogram (A) comprises a single, large eluting peak, beginning at about fraction 33 and ending at about fraction 45. Non-reducing SDS-PAGE analysis of the PEGylation reaction (lane Rx) and fractions 33–45 of the main S-Sepharose column peak are shown in (B). Fractions 33–45 contain predominantly PEG-L103C protein. Lane MW, molecular weight markers; Lane Rx, the PEGylation reaction before column fractionation; Lanes 33–45, fractions across the main protein peak of the S-Sepharose column (A). The position of PEG-L103C and unmodified L103C protein are indicated on the right.

L103C homodimer. Column fractions from the main column peak were pooled and used for further studies of the PEG-L103C proteins. The purified PEGylated proteins migrated predominantly as single species by SDS-PAGE (Fig. 1), indicating that they do not contain significant quantities of unPEGylated subunits. The apparent molecular weights of the 10, 20 and 40 kDa PEG L103C proteins were 37, 53, and 97 kDa, respectively, by non-reducing SDS-PAGE.

Wild type IFN- γ [both a commercial standard (Pierce Endogen) and BBT IFN- γ prepared by us], L103C, and the PEG-L103C proteins inhibited *in vitro* growth of the human NIH:OVCA-3 ovarian cancer cell line to similar extents and had similar mean IC_{50} values (protein concentration required for half-maximal inhibition of cell growth) in this

assay (Fig. 3), indicating essentially no loss of *in vitro* bioactivity due to the added cysteine residue or to PEGylation. IC₅₀ values for the proteins ranged from about 5 to 150 ng/mL in assays performed on different days and with different lots of NIH:OVCAR-3 cells; thus, it was important to assay IFN- γ and the PEG-L103C proteins together on the same day for comparative purposes. In initial experiments, the IC₅₀ for the commercial IFN- γ standard was 81 ± 53 ng/mL, as compared with an IC₅₀ of 35 ± 7 ng/mL for BBT IFN- γ and an IC₅₀ of 45 ± 21 ng/mL for L103C. In experiments performed at a later date with a new lot of NIH:OVCAR-3 cells, BBT IFN- γ and the PEG-L103C proteins had similar IC₅₀ values in the 5–13 ng/mL range (Table 1 and Fig. 3). We also tested a WHO recombinant human IFN- γ standard in the assay and estimated an IC₅₀ of 14 ± 2.5 ng/mL for this protein. BBT IFN- γ and the

TABLE 1. *IN VITRO* BIOACTIVITIES OF IFN- γ AND PEG-L103C PROTEINS FOR INHIBITING PROLIFERATION OF HUMAN NIH:OVCAR-3 CELLS

Protein	IC ₅₀ (ng/mL) ^a
BBT IFN- γ ^b	8.4 ± 2.8
10 kDa PEG L103C	8.0 ± 2.4
20 kDa PEG L103C	5.2 ± 2.4
40 kDa PEG L103C	12.5 ± 3.5

Serial dilutions of each protein were incubated with human NIH:OVCAR-3 cells in 96-well tissue culture plates at 37°C in a humidified tissue culture incubator. After 4 days, CellTiter 96® Aqueous One Solution was added to each well and the absorbance, which is proportional to cell number, was measured. Absorbance values were used to calculate an IC₅₀ value for each protein. Data are means $\pm$ SD for at least 3 assays for each protein.

^aMeans $\pm$ SD for at least 3 assays for each protein. IC₅₀ values for PEG-L103C proteins were calculated using only the protein component of the molecules.

^bIFN- γ , lab synthesized.

IFN- γ , interferon gamma.

FIG. 3. Representative dose-response curves for BBT IFN- γ and 20 kDa PEG-L103C (A) and BBT IFN- γ and 10 kDa and 40 kDa PEG-L103C (B) proteins for inhibiting growth of human NIH:OVCAR-3 cells *in vitro*. Serial dilutions of the protein samples were mixed with NIH:OVCAR-3 cells and incubated for 4 days at 37°C in a tissue culture incubator. On day 4, cell number was quantified by adding CellTiter 96® Aqueous One Solution to the wells, and reading the absorbance of the wells in a plate reader 1–4 h later. Absorbance on the Y-axis is proportional to cell number, which was confirmed by visual inspection of the assay wells. Data are means $\pm$ SD for triplicate wells for each protein dilution. PEG-L103C protein concentrations were calculated using only the protein component of the molecules.

commercial IFN- γ standard had IC₅₀s of 33 ± 2.4 and 35 ± 7 ng/mL, respectively, when assayed on the same days as the WHO standard using a third lot of NIH:OVCAR-3 cells. The IC₅₀ determined for the WHO standard could only be estimated, because the standard has a low IFN- γ protein concentration (4 μ g/mL) that prevented accurate growth inhibition data from being obtained when the protein was used in the assay at concentrations greater than ~ 100 ng/mL. Above this concentration, the volume of the WHO standard that needed to be added to the assay caused non-specific cell growth inhibition, which was mimicked by adding an equal volume of buffer without IFN- γ (data not shown).

Rat pharmacokinetic experiments with BBT IFN- γ and PEG-L103C proteins

The circulating half lives of BBT IFN- γ and the PEG-L103C proteins were compared after sc and ip administration to rats, which are the routes of administration expected to be most commonly used for human clinical studies. Plasma levels of the proteins were measured by ELISA. Selected pharmacokinetic parameters for the proteins after sc and ip injection are presented in Tables 2 and 3, respectively. Nearly all of the pharmacokinetic parameters measured for BBT IFN- γ were significantly different than the corresponding parameters measured for the PEG-L103C proteins ($P \leq 0.05$; Tables 2 and 3). After sc administration (Fig. 4A), BBT IFN- γ reached peak plasma levels 2 h post-administration and was cleared rapidly from the rats. The protein could not be detected at 24 h post-administration. The terminal half life of BBT IFN- γ was 1 h. In contrast, the PEG-L103C proteins did not reach peak plasma levels until 24 h post-administration and could be easily detected even at 144 h post-administration. Peak plasma levels of the PEG-L103C proteins were 44- to 84-fold higher than peak plasma levels of BBT IFN- γ . Circulating levels of the 20 and 40 kDa PEG proteins were similar to each other and higher than circulating levels of the 10 kDa PEG protein. Terminal half lives for the 10, 20, and 40 kDa PEG-L103C proteins were similar, being 26, 28.6, and 27.4 h, respectively. Systemic exposure, as measured by area under the concentration curve

TABLE 2. SELECTED PHARMACOKINETIC PARAMETERS FOR BBT IFN- γ AND 10, 20, AND 40 kDa PEG L103C PROTEINS AFTER SC ADMINISTRATION OF 100 μ g PROTEIN/KG OF THE PROTEINS TO RATS

Parameter	10kDa PEG L103C	20kDa PEG L103C	40kDa PEG L103C	BBT IFN- γ
T _{max} (h)	24	24	24	2 ^a
C _{max} (pg/mL)	15,625	29,528	23,474	353 ^a
AUC _{0→∞} (h·pg/mL)	1,126,546	2,307,378	2,124,462	947 ^a
CL/F (mL/h/kg)	88.8	43.3	47.1	105,602 ^a
T1/2 (h)	26.0	28.6	27.4	1.0 ^a
MRT (h)	55.1	61.4	68.2	1.9 ^a

Rats ($n=3$ males/group) received a single sc injection of 100 μ g protein/kg. Only the protein component of the PEG-L103C proteins was used for calculating doses and pharmacokinetic parameters. Blood samples were obtained pre-dose and from 1 to 144 h post-injection, and plasma levels of the proteins were measured by ELISA. Plasma levels were averaged for the 3 rats in each group and used to calculate pharmacokinetic parameters using WinNonlin software.

^a $P \leq 0.05$ versus each PEG-L103C protein.

AUC_{0→∞}, area under the concentration curve from time 0 to infinity; CL/F, apparent clearance after sc administration; C_{max}, maximal blood concentration; MRT, mean residence time extrapolated to infinity; sc, subcutaneous; T1/2, terminal half-life; T_{max}, time to maximal blood concentration.

over time (AUC_{0→∞}), was 1,189- to 2,436-fold greater for the PEG-L103C proteins than for BBT IFN- γ . Qualitatively similar results were obtained after ip administration of the proteins to rats (Fig. 4B), although plasma levels, systemic exposure (AUC_{0→∞}), and terminal half lives of the PEG proteins showed a greater correlation with increasing PEG size by this route of administration. Terminal half lives for the proteins after ip administration were as follows: wild type BBT IFN- γ (0.9 h), 10 kDa PEG L103C (18.4 h), 20 kDa PEG L103C (22.7 h), and 40 kDa PEG L103C (29.1 h).

Inhibition of tumor xenograft growth in mice by PEG-L103C

We compared the ability of BBT IFN- γ and the different PEG-L103C proteins to inhibit growth of human NI-H:OVCAR-3 tumors in athymic nude mice. Human IFN- γ is not biologically active in mice; therefore, inhibition of human tumor xenograft growth in athymic mice by human IFN- γ occurs through a direct anti-proliferative effect on the human tumor cells. Mice were implanted with tumor cells in the dermis overlying the axillae on day 0, and tumor growth followed for the next 70 days. Mice received 3 $\times$ /week sc injections into the abdominal region of vehicle solution (phosphate-buffered saline) or 15 μ g protein/injection of BBT

IFN- γ or the PEG-L103C proteins. As shown in Figure 5, mean tumor volumes in mice receiving BBT IFN- γ were essentially the same size as mean tumor volumes in mice receiving vehicle solution throughout the course of the study. By contrast, mean tumor volumes in mice receiving any of the PEG-L103C proteins were significantly smaller than mean tumor volumes in mice receiving vehicle solution or IFN- γ . Differences in mean tumor volumes between vehicle or BBT IFN- γ test groups and the PEG-L103C test groups were statistically significant from day 35 to study termination on day 70 ($P < 0.05$). At study termination, mean tumor volumes in animals receiving the 10, 20, and 40 kDa PEG L103C proteins were 61%, 72%, and 76% smaller, respectively, than mean tumor volumes in animals receiving vehicle solution or BBT IFN- γ ($P < 0.005$ for the 20 and 40 kDa PEG L103C proteins and $P < 0.02$ for the 10 kDa PEG L103C protein compared with vehicle or BBT IFN- γ ; Table 4). Similarly, mean tumor weights measured at necropsy were 61%, 71%, and 73% smaller in mice receiving the 10, 20, and 40 kDa PEG-L103C proteins, respectively, than mean tumor weights in mice receiving vehicle solution or BBT IFN- γ ($P < 0.005$ for the 20 and 40 kDa PEG L103C proteins and $P < 0.02$ for the 10 kDa PEG L103C protein compared with vehicle or BBT IFN- γ ; Table 4). Day 70 mean tumor volumes and weights were smaller in mice receiving the 20 and

TABLE 3. SELECTED PHARMACOKINETIC PARAMETERS FOR BBT IFN- γ AND 10, 20, AND 40 kDa PEG L103C PROTEINS AFTER IP ADMINISTRATION OF 100 μ g PROTEIN/KG OF THE PROTEINS TO RATS

Parameter	10kDa PEG L103C	20kDa PEG L103C	40kDa PEG L103C	BBT IFN- γ
T _{max} (h)	4	4	10	2
C _{max} (pg/mL)	160,038	179,502	567,297	3,411 ^a
AUC _{0→∞} (h·pg/mL)	3,301,498	6,487,514	28,250,446	12,545 ^a
CL/F (mL/h/kg)	30.3	15.4	3.5	7,972 ^b
T1/2 (h)	18.4	22.7	29.1	0.9 ^a
MRT (h)	22.5	32.1	41.9	2.4 ^a

Rats ($n=3$ males/group) received a single ip injection of 100 μ g protein/kg. Only the protein component of the PEG-L103C proteins was used for calculating doses and pharmacokinetic parameters. Blood samples were obtained pre-dose and from 0.25 to 144 h post-injection, and plasma levels of the proteins were measured by ELISA. Plasma levels were averaged for the 3 rats in each group and used to calculate pharmacokinetic parameters using WinNonlin software.

^a $P \leq 0.05$ versus each PEG-L103C protein.

^b $P \leq 0.05$ versus the 20 and 40 kDa PEG L103C proteins; $P=0.09$ versus the 10 kDa PEG-L103C protein.

CL/F, apparent clearance after ip administration; ip, intraperitoneal.

FIG. 4. Clearance of BBT IFN- γ and PEG-L103C proteins after subcutaneous (A) and intraperitoneal (B) administration to rats. Plasma samples were obtained at various times post-injection from the rats, and plasma levels of the proteins were quantified by ELISA. Data are means $\pm$ SD for 3 rats per group. Rats were dosed at 100 μ g protein/kg. Only the protein component of the PEG-L103C proteins was used for dose calculations.

40 kDa PEG L103C proteins compared with mice receiving the 10K-PEG L103C protein, although the differences were not statistically significant. Mean tumor volumes began increasing in mice treated with the PEG-L103C proteins beginning around day 42 and continued to increase until study termination on day 70. This increase reflected growth of tumors in 60%–80% of mice in these treatment groups. None of the mice showed any apparent drug-related toxicities during the course of the study.

Discussion

The data presented here demonstrate the utility of site-specific PEGylation for improving the pharmacokinetic properties and *in vivo* biological activity of human IFN- γ . The L103C mutain was chosen as a lead development candidate from a screen of about 20 IFN- γ cysteine mutains, because it expressed well, refolded and PEGylated efficiently, and possessed high *in vitro* bioactivity when PEGylated.

FIG. 5. Inhibition of human NIH:OVCAR-3 tumor growth in athymic mice by BBT IFN- γ and PEG-L103C proteins. Mice were implanted in the axillary area on day 0 with 5×10^6 NIH:OVCAR-3 cells. On day 3, the mice began receiving 3 $\times$ /week sc administrations of vehicle solution (phosphate-buffered saline), BBT IFN- γ , or 10, 20, or 40 kDa PEG L103C for 70 days. Mice were administered an injection in the abdomen, and they received 15 μ g of protein or an equivalent volume of vehicle solution per injection. Only the protein component of the PEG-L103C proteins was used for dose calculations. Tumor volumes were measured at weekly intervals. Data are means $\pm$ SE for 10 mice per group.

Amino acid position 103 was chosen as a promising site for PEG attachment, because it is a surface-exposed amino acid located in the EF loop of IFN- γ , which is removed from the known receptor binding sites of IFN- γ and is predicted to point away from the cell surface when IFN- γ is bound to its cellular receptor (Ealick and others 1991; Walter and others 1995). We hypothesized that attachment of PEG to the L103C amino acid would not interfere with receptor binding, and, indeed, modification of L103C with

TABLE 4. FINAL TUMOR VOLUMES AND TUMOR WEIGHTS ON DAY 70 IN MICE RECEIVING 3 $\times$ /WEEK SC INJECTIONS OF VEHICLE SOLUTION, BBT IFN- γ , AND 10, 20, AND 40 kDa PEG L103C PROTEINS

Group	Tumor volume (mm ³) ^a	Tumor weight (mg) ^a
Vehicle	1,521 $\pm$ 318	827 $\pm$ 151
BBT IFN- γ	1,525 $\pm$ 235	820 $\pm$ 131
10 kDa PEG L103C	594 $\pm$ 178 ^b	323 $\pm$ 95 ^b
20 kDa PEG L103C	420 $\pm$ 133 ^c	237 $\pm$ 72 ^c
40 kDa PEG L103C	367 $\pm$ 100 ^c	226 $\pm$ 64 ^c

Female athymic mice were implanted in the dermis overlying the axillae with NIH:OVCAR-3 cells on day 0. Starting on day 3, mice received 3 $\times$ /week sc injections of the test proteins or vehicle solution in the abdominal region. Tumor volume was measured at weekly intervals. At sacrifice on day 70, final tumor volumes and weights were measured.

^aData are means $\pm$ SE for 10 mice per group.

^b $P < 0.02$ versus vehicle and BBT IFN- γ groups.

^c $P < 0.005$ versus vehicle and BBT IFN- γ groups.

PEG did not interfere with *in vitro* bioactivity of the protein. This was the case even when the protein was modified with a large 40 kDa branched PEG, which, because of the dimeric structure of IFN- γ , represents 80 kDa of PEG attached to the homodimer. The high PEGylation efficiency of the L103C mutein (estimated to be >90% by SDS-PAGE) indicates that attachment of a PEG to the L103C amino acid of one subunit in the homodimer does not appreciably interfere with the attachment of a second PEG to the L103C amino acid in the other subunit of the homodimer. This feature may be amino-acid position dependent, because we observed lower PEGylation efficiencies (approaching 50%) with some of the other IFN- γ cysteine muteins in our preliminary screen (data not shown). Consistent with findings for many other proteins (Harris and others 2001; Harris and Chess 2003), PEGylation dramatically slows the clearance of IFN- γ after sc or ip administration to animals, enabling the PEGylated IFN- γ proteins to maintain higher circulating levels for longer periods of time *in vivo* compared with IFN- γ .

IFN- γ inhibits growth of many tumor types *in vitro*, either alone or in combination with other drugs (Merchant and others 2004; Lissat and others 2007; Reid and others 2009). Human ovarian cancer cell lines are sensitive to the direct growth inhibiting effects of IFN- γ *in vitro* (Burke and others 1999 and our data). However, clinical trials of IFN- γ to treat human ovarian cancers have met with variable results (Windbichler and others 2000; Marth and others 2006; Alberts and others 2008). Burke and others (1999) reported that continuous exposure of human ovarian cancer cells to IFN- γ for at least 3 days was required for IFN- γ to kill the tumor cells. Maintaining optimal therapeutic levels of IFN- γ for 3 days *in vitro* is straightforward; however, maintaining these levels *in vivo* is difficult due to rapid clearance of IFN- γ from the circulation. Failure to maintain constant therapeutic levels of IFN- γ *in vivo* may contribute to the lack of clinical success with IFN- γ as a cancer treatment. Loss of IFN- γ receptor expression by ovarian tumor cells may also contribute to the variable responses reported for IFN- γ in ovarian cancer clinical trials (Duncan and others 2007; Alberts and others 2008). IFN- γ also has been shown to possess protumorigenic properties, depending on the tumor type and local cellular and microenvironmental factors (Zaidi and Merlino 2011).

The availability of a highly active PEG-IFN- γ protein enabled us to test whether continuous exposure of cancer cells to IFN- γ improves the ability of IFN- γ to inhibit tumor growth *in vivo*. We found that PEGylated IFN- γ proteins have 20- to 30-fold longer half lives than IFN- γ (BBT IFN- γ) in rats and maintain elevated levels for at least 6 days after a single injection. By contrast, IFN- γ could not be detected in the circulation of the rats at 10 h post-injection. Consistent with the short half life of the protein, IFN- γ failed to inhibit growth of human NIH:OVCAR-3 tumors in mice compared with a placebo using an every other day dosing regimen. By contrast, the same dose and dosing regimen of the longer-acting PEG-IFN- γ proteins strongly inhibited growth of NIH:OVCAR-3 tumors in mice. Thus, there was a strong correlation between circulating half lives and blood levels of the IFN- γ proteins and the abilities of the proteins to inhibit growth of NIH:OVCAR-3 tumor cells in mice. These data support the hypothesis that maintaining continuous exposure of tumor cells to optimal therapeutic levels of IFN- γ is important for tumor growth inhibition by

IFN- γ *in vivo*. We did not find statistically significant differences in the abilities of the different size PEG-IFN- γ proteins to inhibit tumor growth *in vivo*, which is consistent with their similar *in vitro* potencies and *in vivo* half lives after sc injection. Mean tumor volumes were slightly smaller in mice treated with the 20 and 40 kDa PEG-L103C proteins compared with mice treated with the 10 kDa PEG L103C protein, suggesting that the 20 and 40 kDa PEG proteins may be more efficacious than the 10 kDa PEG protein *in vivo*, but additional studies using larger numbers of mice, and a variety of doses and dosing regimens will be needed to properly answer this question. Factors such as biodistribution, immunogenicity, and manufacturing cost will also need to be evaluated for the different PEG-L103C proteins before deciding on a final clinical development candidate. Although mice treated with the PEG-L103C proteins had significantly smaller tumors compared with mice treated with vehicle solution or BBT IFN- γ , all of the PEG-L103C-treated mice still had detectable tumors on day 70, and mean tumor volumes had increased in size between day 45 and 70. This finding indicates that the PEG-L103C proteins had not killed all the tumor cells in the mice, which is in contrast to results reported for PEG-IFN- α (Bell and others 2008) and PEG-IFN- β -1b (Lee and others 2013) in the same tumor model. Whether tumor growth between days 45 and 70 in the PEG-L103C treatment groups represents selection and growth of IFN- γ -resistant tumor cells *in vivo* remains to be determined. Resistance to IFN- γ therapy could result from multiple mechanisms, including loss of IFN- γ receptor expression (Duncan and others 2007) and changes in IFN- γ intracellular signaling pathways.

Creation of long-acting murine IFN- γ proteins using amine PEGylation technology has been reported (Bansal and others 2011). In contrast to our results using site-specific PEGylation, amine-PEGylation of murine IFN- γ resulted in a heterogeneous mixture of PEGylated proteins containing an average of 2–3 PEGs per subunit. This result is consistent with the large number of lysine residues in IFN- γ proteins (40 lysines per homodimer in human IFN- γ , and 20 lysines per homodimer in murine IFN- γ). Amine-PEGylated murine IFN- γ proteins had reduced potencies in several *in vitro* assays compared with murine IFN- γ . Despite their lower *in vitro* potencies, amine-PEGylated murine IFN- γ proteins had superior pharmacokinetic properties compared with murine IFN- γ in mice, and they were more effective than murine IFN- γ at inhibiting fibrosis in a murine CCL4-induced acute liver fibrosis model (Bansal and others 2011).

Long-acting, PEGylated IFN- α proteins provide hepatitis patients with greater therapeutic benefits and dosing convenience compared with IFN- α , without significantly increasing toxicities (Zeuzem and others 2000). Similar beneficial clinical effects have been reported for other PEGylated protein therapeutics (Harris and others 2001; Harris and Chess 2003). The promising preclinical data reported here and elsewhere (Bansal and others 2011) suggest that long-acting, PEGylated IFN- γ proteins also may provide patients with superior therapeutic benefits compared with IFN- γ in a variety of disease states.

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