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(54) Title: INTERFERON AND IMMUNOGLOBULIN FC FRAGMENT HYBRID

(57) Abstract: The present invention provides interferon-immunoglobulin Fc fusion proteins ("IFN-Fc hybrids") that are useful for removing or regressing tumors or delaying tumor development. The IFN-Fc hybrids preferably include peptide linkers between the IFN and the Fc fragment. These linkers are T cell inert sequences or any non-immunogenic sequence, preferably Gly-Ser repeat units. The preferred Fc fragment is a human immunoglobulin Fc fragment, preferably the γ4 chain.

INTERFERON AND IMMUNOGLOBULIN FC FRAGMENT HYBRID

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to protein hybrids having an interferon protein covalently linked to an immunoglobulin Fc fragment, including hybrids having peptide linkers linking the interferon protein and immunoglobulin Fc fragment.

Description of the Prior Art

Interferons, including interferon-α ("IFNα") and interferon-β ("IFNβ"), were among the first cytokines to be produced by recombinant DNA technology. IFNα has been shown to have therapeutic value in conditions such as hairy cell leukemia and inflammatory and viral diseases, including hepatitis B. IFNβ has been approved for use in treatment of multiple sclerosis.

Most cytokines, including IFNα, have relatively short circulation half-lives because they are produced *in vivo* to act locally and transiently. To use IFNα as an effective systemic therapeutic, relatively large doses and frequent administrations are needed. Such frequent administrations are inconvenient and painful. Further, the toxic side effects associated with IFNα administration are so severe that some cancer patients cannot tolerate the treatment. These side effects are probably associated with administration of a high dosage.

To overcome these disadvantages, the molecule can be modified to increase its circulation half-life or the molecule's formulation can be modified to extend its release time. The dosage and administration frequency can then be reduced to increase the efficacy. Efforts have been made to create a recombinant IFNα-gelatin hybrid with an extended retention time (Tabata, Y. et al., *Cancer Res.* 51:5532-8, 1991). A lipid-based encapsulated IFNα formulation has also been tested in animals and achieved an extended release of the protein in the peritoneum (Bonetti, A. and Kim, S. *Cancer Chemother Pharmacol.* 33:258-261, 1993).

IgG and IgM immunoglobulins are among the most abundant proteins in the human blood. They circulate with half-lives ranging from several days to 21 days. IgG has been found to increase the half-lives of several ligand binding proteins (receptors) when used to form recombinant hybrids, including the soluble CD4 molecule, LHR, and the IFN-γ receptor (Mordenti J. *et al., Nature*, 337:525-31, 1989; Capon, D.J. and Lasky, L.A., U.S. Patent number 5,116,964; Kurschner, C. *et al., J. Immunol.* 149:4096-4100, 1992).

Various linkers have proven useful for producing protein hybrids. US Patent No. 5,723,125 issued to Chang, et al. on March 3, 1998 entitled "Hybrid with interferon-alpha and an immunoglobulin Fc linked through a non-immunogenic peptide" and US Patent No.

5,908,626 issued to Chang, et al. on June 1, 1999 entitled "Hybrid with interferon-.beta. and an immunoglobulin Fc joined by a peptide linker" disclose hybrid recombinant proteins consisting of human interferon and a human immunoglobulin Fc fragment joined by a peptide linker comprising the sequence Gly Gly Ser Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser.

Given the problems with the use of interferons to treat patients, there is a need for improved compositions and methods for administering interferons to patients while lengthening the *in vivo* half-life and decreasing the adverse side effects.

SUMMARY OF THE INVENTION

It is, therefore, an object of the invention to provide interferon hybrids that have a longer *in vivo* half-life than interferon.

It is another object of the invention to provide interferon hybrids that can be administered less frequently that interferon.

It is a further object of the invention to provide interferon hybrids useful for removing or regressing tumors or delaying tumor development.

These and other objects are achieved using a protein hybrid comprising an interferon protein covalently linked at its C-terminal end to the N-terminal end of an immunoglobulin Fc fragment or a protein hybrid comprising an interferon protein covalently linked at its C-terminal end to the N-terminal end of a peptide linker that is covalently linked at its C-terminal end to the N-terminal end of an immunoglobulin Fc fragment. The preferred linker is an immunologically inert peptide consisting of Gly Ser repeat unit having from about 2 to about 40 amino acids. The linkers may also be T cell inert amino acid sequences or any non-immunogenic amino acid sequence. The hybrids of the present invention are useful for treating patients with tumors to remove tumors, regress tumors, or delay tumor development.

Other and further objects, features and advantages of the present invention will be readily apparent to those skilled in the art.

BRIEF DESCRIPTION OF THE SEQUENCE LISTING

SEQ ID NO:1 is the nucleotide and amino acid sequence of an IFN-α-Fc hybrid.

SEQ ID NO:2 is the amino acid sequence of an IFN-α-Fc hybrid shown in SEQ ID NO:1.

SEQ ID NOS:3-9 are the amino acid sequences of the various length peptide linkers used to conjugate the N-terminal end(s) of a heavy chain γ4 Fc fragment to the C-terminal end of an IFN-β protein.

SEQ ID NO:10 is the amino acid sequence of a linker used to conjugate the N-terminal end of a heavy chain γ1 Fc fragment to the C-terminal end of an IFN-α protein as used in an assay as described below.

SEQ ID NO:11 is the amino acid sequence of a linker used to conjugate the N-terminal end of a heavy chain γ4 Fc fragment to the C-terminal end of an IFN-α protein which molecule was then used in an *in vitro* cytopathic effect assay as described below.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows a virus cytopathic effect inhibition assay for various linkers in an IFN-β-Fc hybrid.

Fig. 2 shows a virus cytopathic effect inhibition assay for two different linkers in an IFN-α-Fc hybrid.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a protein hybrid comprising an interferon protein covalently linked at its C-terminal end to the N-terminal end of an immunoglobulin Fc fragment.

The interferon protein can be any Type I interferon including interferon-α, interferon-β, interferon-ω, interferon-δ, and interferon-τ or Type II interferon including interferon-γ. Preferably, the interferon protein in an interferon-α or interferon-β, most preferably an interferon-α2a or interferon-α2b.

The immunoglobulin Fc fragment can be a fragment from any class of immunoglobin including IgA, IgD, IgE, IgG, or IgM. Preferably, the Fc fragment is a human immunoglobulin Fc fragment, most preferably an IgG Fc fragment of the IgG4(γ4) subclass. The γ4 chain is preferred over the γ1 chain because the former demonstrates little or no antibody-dependant cell-mediated cytotoxicity (ADCC) or complement activating ability and is stable in human circulation.

The present invention also provides a protein hybrid homodimer comprising two interferon proteins covalently linked at their C-terminal end to each of the two N-terminal ends of an immunoglobulin Fc fragment, preferably a γ4 Fc fragment.

The present invention also provides a protein hybrid comprising an interferon protein covalently linked at its C-terminal end to the N-terminal end of a peptide linker that is covalently linked at its C-terminal end to the N-terminal end of an immunoglobulin Fc fragment.

The interferon protein and Fc fragments are linked through a T cell immunologically inert peptide, preferably peptides with Gly Ser repeat units. Because these peptides are immunologically inactive, their insertion at the fusion point eliminates any neoantigenicity that may develop when directly joining of the interferon and Fc fragment.

The peptide linker is preferably an immunologically inert peptide consisting of Gly Ser repeat unit having from about 2 to about 40 amino acids. Typical amino acid sequences for the peptide linker are shown in SEQ ID NOS 3, 4, 5, 6, 7, 8, and 9. These linkers have the unique ability to connect interferons and Fc fragments without significantly adversely affecting the biological activity of the interferon. While all linker lengths function in the present invention, some linkers are somewhat more effective that others, i.e., linkers with 12 or more amino acids are somewhat more active than the shorter lengths. Preferably, the peptide linker has from 12 to 15 amino acids or from about 17 to 40 amino acids.

The complete nucleotide sequence of an IFNα-Fc hybrid with no linker is shown in SEQ ID NO: 1 and the amino acid sequence is shown in SEQ ID NO:2. The linker, if present, is located between amino acid residue numbers 188 (Glu) and 189 (Glu). The sequences of a number of suitable linkers which were all shown to have about the same cytopathic effect *in vitro,* are shown in SEQ ID NOS 3, 4, 5, 6, 7, 8, and 9.

The preferred embodiment of the present invention is a protein hybrid comprising an interferon protein covalently linked at its C-terminal end to the N-terminal end of an immunoglobulin Fc fragment. However, the present invention also includes a protein hybrid comprising an immunoglobulin Fc fragment covalently linked at its C-terminal end to the N-terminal end of an interferon protein. Such proteins can be linked directly or through a peptide linker as described herein.

The present invention also provides a protein hybrid homodimer comprising two interferon proteins covalently linked at their C-terminal end to each of the two N-terminal ends of an immunoglobulin Fc fragment, preferably a γ4 Fc fragment, or a protein hybrid homodimer comprising two interferon proteins covalently linked at their C-terminal end to the N-terminal end of a peptide linker that is covalently linked at their C-terminal end to each of the two N-terminal ends of an immunoglobulin Fc fragment. The linkers can contain the same number of amino acids or a different number of amino acids.

If the Fc fragment selected is another chain, such as the μ chain, then, because the Fc fragments form pentamers with ten possible binding sites, the hybrid can comprise interferon and from 1 to 10 cytokines, including multiple interferons. Suitable cytokines that may

function in the present invention include, but are not limited to, interleukins, Colony Stimulating Factor, and Tumor Necrosis Factor.

In another embodiment, an interferon protein is linked, directly or through a linker, to one of the two N-terminal ends of an immunoglobulin Fc fragment and another cytokine is linked, directly or through a linker, to the other of the two N-terminal ends of an immunoglobulin Fc fragment.

One significant advantage of the hybrids of the present invention over the native cytokine is that the hybrids have been shown to ablate tumors in an animal model. IFNα is approved for use in treating certain tumors and hepatitis B. The hybrids may be more effective than IFNα in treating infectious diseases and a broad range of tumors.

The IFNα cDNA can be obtained by reverse transcription and PCR using RNA extracted from cells that express IFNα and following the extraction with reverse transcription and expression in a standard expression system. There are several ways to express the recombinant protein *in vitro*, including in *E. coli,* baculovirus, yeast, mammalian cells or other expression systems. The prokaryotic system, *E. coli*, is not able to do post-translational modification, such as glycosylation. This could be a problem in these systems, and mammalian expression could be preferred for this reason, and because it offers other advantages in terms of simplifying purification.

There are several assay methods available for the measuring of the IFNα bioactivity, including an antiviral assay. The hybrids of the invention have a longer half-life *in vivo* than native IFNα based on *in vitro* experimental results. Even though the specific activity is lower, the hybrids are preferred to the native IFNα for clinical use. This is because, as a result of the longer half-life, the Cxt (the area under the concentration vs. time curve) is much greater based on *in vitro* results than for the native IFNα. This means that at the equivalent molar dosage of the native IFNα and the hybrid, the latter would provide a several hundred fold increased exposure to IFNα resulting in vastly increased efficacy at the same dosage and less frequent administration.

Immunoglobin Fc fragments useful in the present invention can be obtained by conventional methods well known to skilled artisans.

Peptide linkers useful in the present invention can be obtained by conventional methods well known to skilled artisans, including chemical synthesis and recombinant expression of the linker with the interferon and Fc fragment.

The IFNα-Fc hybrids of the present invention have a much longer half-life *in vivo* than the native IFNα. Cytokines are generally small proteins with relatively short half-lives. They usually dissipate rapidly among various tissues, including at undesired sites. It is believed that small quantities of some cytokines can cross the blood-brain barrier and enter the central nervous system and cause severe neurological toxicity. The IFN-Fc hybrids of the present invention are especially suitable for treating tumors, including lymphomas and leukemias, because these products will have a long retention time in the vasculature and will not penetrate undesired sites.

The hybrids of the present invention are therefore useful for treating patients with tumors to remove tumors, regress tumors, or delay tumor development. The hybrids are administered in amounts effective to treat the patient with tumors. The determination of the dosage is within the skill of the art based upon the patient size, tumor size, tumor type, and similar parameters.

The IFN-Fc hybrids can be administered in a pharmaceutical formulation including suitable excipients and additives. The dosage for human use can be readily determined by extrapolation from animal data, with compensation for differences in size, and routine experimentation in clinical trials.

The invention will now be described with reference to examples and experimental results, although it will be understood that these examples are included merely for purposes of illustration and are not intended to limit the scope of the invention unless otherwise specifically indicated..

Example 1

IFNα-Fc Hybrid Demonstrates a Large Increase in Half-Life over the Native IFNα

The disclosures of U.S. Patent No. 5,723,125, incorporated by reference, describes making an IFNα-Fc(γ1) hybrid with a linker having the sequence: Gly Gly Ser Gly Gly Ser (SEQ ID NO: 10). The specific activity of this hybrid was 7.7×10^8 units/μmole in an *in vitro* assay in Daudi cells, compared with 15.4×10^8 units/μmole for the unmodified interferon-α in the same assay. In a later cytopathic effect inhibition assay, the hybrid showed an antiviral specific activity of 2.2×10^8 IU/μmole, which is lower than the 3.8×10^9 IU/μmole of the unmodified interferon-α. In attempting to increase the specific activity of the hybrid, the linker was extended, to increase the flexibility and decrease steric hindrance. A linker having the sequence: Gly Gly Ser Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser (SEQ ID NO: 11) was used. Another difference in the new hybrid was that the Fc portion was γ4 Fc, rather than γ1Fc.

The results of a virus cytopathic effect inhibition assay, *in vitro*, showed that the new hybrid had an antiviral specific activity of 1.1-2.2x10^9 IU/μmole, a 5-10 fold increase over the old one. Nevertheless, it is still 2-3 fold less than that of the unmodified interferon-α, which had a specific activity of 3.8 x 10^9 IU/μmole in this same assay. However, in an *in vivo* pharmacokinetic study in primates, the serum half-life of the claimed new hybrid was about 40 fold longer than the unmodified interferon. Also, the clearance half-life after subcutaneous (s.c.) administration of the hybrid was almost 120 fold longer. The hybrid, when administered s.c., was also well absorbed. The large increase in the AUC (area under curve) for the new hybrid means that it likely would be more efficacious than native interferon-α, notwithstanding its lower specific activity.

Experiments described below were then conducted to determine the effect of using linkers of different lengths on cytopathic activity.

Example 2

Study of the Effect of Various Linkers on IFN-Fc Cytopathic Activity

Comparison of IFN-α(16)Fc and IFN-α-Ala-Fc

The effect of peptide linkers was tested by comparing IFN-α(16)Fc and IFNα-Ala-Fc. IFN-α(16)Fc contains IFNα linked to the hinge region of the human IgG4 Fc fragment through the sixteen amino acid peptide linker shown in SEQ ID NO: 11, an amino acid sequence as follows: GlyGlySerGlyGlySerGlyGlyGlyGlySerGlyGlyGlyGlySer. The IFN-α-Ala-Fc construct contains IFN-α linked to the hinge region of the human IgG1 Fc with one amino acid (Ala) between the two domains. DNA fragments encoding IFN-α(16)Fc and IFN-α-Ala-Fc were inserted, respectively, at the polycloning site of the pcDNA3 expression plasmid. Purified plasmid DNA was then used to transfect NS0 cells by electroporation. Stably-transformed cell lines were selected in the presence of G418. Cell lines expressing these linker variants were then grown in spinner culture flasks. Spent culture supernatant was collected and purified proteins were prepared using the protein A affinity column. Purified proteins were used in the same virus cytopathic effect inhibition assays as described in Example 1. Both IFN-α-Ala-Fc and IFN-α(16)Fc were shown to have equivalent activities (Figure 1).

Constructs for IFNβ-Fc Linker Variants

A number of different constructs of interferon-β linked to an Fc ("IFNβ-Fc") were made, to determine the effect of linker length on the activity of the IFNβ-Fc hybrid. The amino acid sequences of these constructs are listed in the following Table 1.

Table 1
Translated Amino Acid Sequences of Various IFNβ-Fc.

Linker Variants	Linker Sequence between IFNβ and the hinge of IgG4(Fc)
IFNβ-(2)Fc	GlySer (SEQ ID NO:3)
IFNβ-(8)Fc	GlyGlyGlySerGlyGlyGlySer (SEQ ID NO:4)
IFNβ-(12)Fc	GlySerGlyGlyGlyGlySerGlyGlyGlyGlySer (SEQ ID NO:5)
IFNβ-(18)Fc	GlyGlyGlySerGlyGlyGlySerGlyGlyGlyGlySerGlyGlyGlyGlySer (SEQ ID NO:6)
IFNβ-(23)Fc	GlyGlyGlySerGlyGlyGlySerGlyGlyGlyGlySerGlyGlyGlyGlySerGly GlyGlyGlySer (SEQ ID NO:7)
IFNβ-(30)Fc	GlyGlyGlySerGlyGlyGlySerGlyGlyGlyGlySerGlyGlyGlyGlyGly SerGlyGlyGlyGlySerGlyGlyGlyGlySer (SEQ ID NO:8)
IFNβ-(40)Fc	GlyGlyGlySerGlyGlyGlySerGlyGlyGlyGlySerGlyGlyGlyGlyGly SerGlyGlyGlyGlySerGlyGlyGlyGlySerGlyGlyGlyGlySerGlyGlyGly GlySer (SEQ ID NO:9)

Expression of IFNβ-Fc Linker Variants

DNA sequences containing different IFNβ-Fc linker variants were inserted, respectively, at the polycloning site of the pcDNA3 expression plasmid. Purified DNA was then used to transfect NS0 cells by electroporation. Stably-transformed cell lines were initially selected in the presence of G418. Cell lines expressing these linker variants were then grown in the absence of G418. Spent culture supernatant was collected and filtered through a 0.22 μm membrane. The concentration of IFNβ-Fc was estimated by PCFIA using purified IFNβ-Fc protein as the standard. Concentrations of culture supernatant were estimated to be 5.4, 22.5, 15.9, 5.7, 10.2, 5.5, and 4.5 μg/ml for the IFNβ-Fc variants containing peptide linkers of 2, 8, 12, 18, 23, 30 and 40 amino acids, respectively. These supernatants were used in the following *in vitro* cytopathic effect experiments.

In vitro Cytopathic Effect Assays using the IFNβ-Fc Variants.

In 96-well plates, human lung carcinoma A549 cells were plated at 100μl/well containing $5x10^4$ cells using DMEM containing 5% FBS. Plates were incubated at 37°C for 24 hrs in the 5% CO_2 incubator. Culture supernatants containing the IFNβ-Fc linker variants were diluted. These solutions were then used to make 2-fold serial dilutions in a 96-well plate using DMEM containing 5% FBS. One hundred μl of the diluted samples were added to each well and the plates were incubated at 37°C for an additional 24 hours in the incubator. Culture supernatant was removed and encephalomyocarditis (EMC) virus was added at 100 μl/well (the virus is diluted 1:200 in D15 containing 5% FBS from virus stock). The plates

were then incubated at 37°C for 48hrs in the 5% CO_2 incubator. Culture supernatant was removed and the wells were washed 2 times with PBS. Cells were then fixed with paraformaldehyde; and stained with the giemsa dye, then left for 5 minutes at room temperature. Thereafter, the plates were rinsed gently with tap water several times. Methanol was added to each well and the wells were read at 630nm using the Dynatech MR5000 ELISA reader.

The results of several experiments with IFNβ-Fc hybrids, as shown in Figure 2, and the results for the two different IFNα-Fc hybrids shown in Example 2, show that the cytopathic effect did not change significantly no matter which linker was used. Further *in vivo* experiments on one of the IFNα-(16)Fc hybrids were conducted as described below.

Example 3
Animal Tumor Model.

Tumor Initiation in Mice

Female CB17/scid mice (Charles River Laboratories; seven and half weeks old) were inoculated subcutaneously (s.c.) with Daudi Burkitt lymphoma cells at the lower right flank at a total volume of 100 μl. There were four different cell densities tested in five animals in each group (Table 2). The injection site was monitored one day after inoculation and then daily three weeks after inoculation.

Palpable tumors were measured by caliper. Tumor volume was determined and calculated using the formula, V=4 xyz/3, where 2x, 2y and 2z are the three perpendicular diameters of the tumor and the average of two measurements.

For inoculation, cells were grown *in vitro* in D15 media with 10% fetal calf serum in100 ml spinners to a density of 0.6X106/ml with 94% viability. Cells were harvested by centrifugation at 300g for 10 minutes, washed twice in cold PBS, and resuspended to the desired density in PBS. Cell counting and Tryptan Blue staining confirmed the cell density and viability.

Table 2
Cell Density and Route of Inoculation

Cell Density	No. of Animals	Route of Administration
PBS	5	s.c.
$0.5X10^6$/100ul PBS	5	s.c.
$2.5X10^6$/100ul PBS	5	s.c.
$1.25X10^7$/100ul PBS	5	s.c.

Human tumor xenografts became detectable in the$1.25X10^7$ group at the site of injection four weeks after inoculation. One week later, the tumor take rate reached 80% and

was maintained at this level throughout the entire pilot study period. It took about three weeks (2.5-3.5 wks) for a palpable tumor to grow up to 10-15% of the animal's body weight. In the $2.5X10^6$ and $0.5X10^6$ groups, the take rate reached 60% by the end of the nine and half weeks. The tumors did not kill the mice and there was no sign of metastases.

Thus, it is concluded that a subcutaneous inoculation of $1.25X10^7$ Daudi Burkitt lymphoma cells will yield about 80% tumor takes in about four weeks.

In vivo Antiproliferation Study
Experiment with Daily Dosing

Thirty-two mice inoculated with $12.5X10^6$ Daudi Burkitt lymphoma cells were randomly assigned to one of four treatment groups as shown in Table 3. Roferon A (IFN-α-2a, Hoffmann La Roche, Nutley, NJ) and IFN-α(16)-2a-Fc (having the linker shown in SEQ ID NO:11) treatment began the day after tumor inoculation. All the animals were dosed daily subcutaneously over the scruff and the treatment continued for eight consecutive weeks. During the treatment period, animals were monitored every 3-4 days for tumor development, and tumor size was measured as above. After the treatment period, weekly observations were continued for additional six months for animals that were tumor free by the time when treatment stopped.

Blood was collected retro-orbitally 24 hours post the last dosing day, one, two and four weeks after termination of the treatment for IFN-α-2a-Fc and one, two and three weeks after termination of Roferon A treatment. Serum Interferon level was determined by ELISA.

Table 3
Dose, Route and Schedule

Group	Dose	Route of Administration	Schedule
Control	Diluent	s.c.	daily
Roferon A	$1X10^6$ IU/100μl	s.c.	daily
IFN-α-Fc	$1X10^6$ IU/100μl	s.c.	daily
IFN-α-Fc	$1X10^5$ IU/100μl	s.c.	daily

Effect of IFN-α on Tumor Take Rate and Tumor Progression

Tumor development in different treatment groups is shown in Table 4. In control animals, the first tumor was detected 24 days after inoculation and within 6 days thereafter 7/8 (87.5%) of the animals had developed tumors. The average time of tumor detection was 25.1± 2.3 days (The mouse that developed a tumor at day 75 was not included.). In Roferon A treated animals, the first tumor became detectable 32 days after the inoculation. After

another two weeks, 87.5% had developed tumors. The average tumor detection time was 39.6±4.7 days ($t > t_{0.05\ (12)}$, $P<0.05$). Roferon A delayed tumor development for about two weeks. IFN-α-2a-Fc treatment at both doses completely prevented the Daudi lymphoma from developing throughout the entire dosing period. At the lower dose, two mice developed detectable tumors at 2 and 19 days after cessation of the treatment. While all mice in $1x10^6$ IU/day group and the remaining six mice in $1X10^5$ IU/daily still remained tumor free six months post treatment. (Table 4). This experiment was repeated once with similar results, as shown in Table 4.

Table 4

Tumor Development in CB17/scid Mice (Exp.1.)

Mouse I.D.	Date of Inoculation	Date of Tumor Detection	Tumor Development Time (days)	Mean±S.D.
C* 116	5/27/98	6/20/98	24	
117	5/27/98	6/20/98	24	
125	5/27/98	6/20/98	24	
134	5/27/98	6/20/98	24	
114	5/27/98	6/20/98	24	
101	5/27/98	6/22/98	26	
119	5/27/98	6/26/98	30	25.1±2.3
R* 133	5/27/98	6/28/98	32	
104	5/27/98	7/1/98	35	
103	5/27/98	7/6/98	40	
115	5/27/98	7/6/98	40	
110	5/27/98	7/7/98	41	
113	5/27/98	7/9/98	43	
128	5/27/98	7/12/98	48	39.6± 4.7

*C indicates a control

*R indicates that Roferon A was administered at $1X10^{6\ IU/day}$

Effect of IFN-α on tumor growth rate

Once the tumor grew to about 1% of the mouse's body weight, tumor growth rate in control and Roferon A treated animals were very close. In control animals, average tumor volume increased 10 times in two weeks, while Roferon A treated mice showed a 9-fold increase.

Table 5
Tumor Take Rate in Different Treatments

Group	Treatment	Tumor Take Rate (%) (N=8)
Control	Diluent	100 (8/8)
Roferon A	$1X10^6$ IU /100ul	87.5 (7/8)
IFN α-2a-Fc	$1X10^6$ IU /100ul	0
IFN α-2a-Fc	$1X10^5$ IU /100ul	25.0 (2/8)

Quantization of Serum IFN-α Level

Serum concentration of IFN-α and IFN-α-2a-Fc was determined by ELISA procedures. In Roferon A treated mice, IFN-α -2a was undetectable 24 hours after the last dose. In IFN-α-2a-Fc treated mice, serum IFN-α-2a-Fc concentration was 3.5 ug/ml for the$1x10^6$ IU/day group and 0.7 ug/ml for the $1x10^5$ IU/day group 22 days after termination of the treatment (Table 6). There was a decrease in serum concentration between 1 and 22 days after the end of the treatment. The data indicate that IFN-α-2a-Fc has a half-life of about one week in mice after being administered subcutaneously $1X10^6$ IU/day or $1X10^5$ IU/day for 8 weeks.

Table 6
Serum IFN-α-2a Level (μg/ml)

Treatment	Days Post Treatment Termination		
	1	8	22
IFN-α-2a-Fc $1X10^6$ IU	25.370±6.885	12.080±3.477	3.477±0.525
IFN-α-2a-Fc $1X10^5$ IU	2.766±1.138	1.549±0.536	0.691±0.141
Roferon A	Undetectable	Undetectable	Undetectable

Experiment with an Increased-Dosing-Interval

In this experiment, Roferon A $1X10^6$ IU was given every 3 days and $1X10^6$ IU IFN-α-2a-Fc was dosed every three days and weekly. The results are shown in Table 7. Roferon A $1X10^6$ IU for 3 days failed to show any protection against tumor formation as compared to the control animals in tumor volume and average time for tumor development, while $1X10^6$ IU IFN-α-Fc administered every three days and weekly effectively inhibited the tumor formation during the eight week treatment period. This inhibition extended to seven weeks after the treatment period.

Table 7
Tumor Development in animals with an increased dosing intervals

Treatment	Tumor Take Rate (%) (N=8)	Average Time for Tumor Development (days)
Control	100 (8/8)	21.1±1.1
Roferon A 10^6 IU/ 3 days	100 (8/8)	22.0±1.9
IFN-FC 10^6 IU/ 3 days	N/A	N/A
IFN-FC 10^6 IU/ weekly	N/A	N/A

Preliminary study with established Daudi Burkitt Lymphomas

Two mice with well established 5-week-old Daudi Burkitt lymphomas were treated with IFN-α-Fc at10^6 IU/daily. After ten days, complete regression was observed in both of the animals (Table 8). Two other mice with established 6.5-week-old Daudi lymphomas were treated with 10^6 IU Roferon A every three days for eight weeks. In the latter mice, tumor volume decreased rapidly, declining from 2.7 cm^3 and 4.6 cm^3 to 0.3 cm^3, a reduction of 89% to 94% in the first two weeks. Complete regression was not achieved.

Table 8
Tumor Regression in Control Mice

Mouse I.D.	Date	Tumor Volume (cm^3)
416	11/20/98	0.195 (7 mm X7.6 mm)
	11/24/98	0.161 (6.4 mm X7.6 mm)
	11/25/98	palpable
	11/26/98	palpable
	11/27/98	complete regression
453	11/20/98	0.858 (10 mm X 16 mm)
	11/24/98	0.393 (6.4 mm X 7.6 mm, 7.6 mm X7.6mm)
	11/25/98	palpable
	11/26/98	palpable
	11/27/98	palpable
	11/28/98	barely palpable
	11/29/98	complete regression

Summary and Conclusions

A murine human tumor xenograft model has been established by inoculating subcutaneously female CB17/scid mice (six and half weeks old) with 1.25X10^7 Daudi Burkitt lymphoma cells in the lower right flank at a total volume of 100 µl.

Roferon A 1x10^6 IU/day treatment delayed the Daudi B cell lymphoma development by two weeks ($t > t_{0.05\,(12)}$, $P<0.05$). IFN-α-2a-Fc 1X10^6 IU/day completely inhibited the tumor formation throughout the entire dosing period and this inhibition has been extended to six

months after termination of the treatment. Partial to full inhibition was also shown in the $1X10^5$ IU /day IFN-α-2a-Fc treated mice.

Roferon A $1x10^6$ IU/ 3 days treatment failed to show any protection against the tumor development whereas Daudi Burkitt lymphoma has been completely inhibited by either IFN-α-Fc at $1x10^6$ IU/3 days or the IFN-α-2a-Fc $1x10^6$ IU/ weekly, and inhibition continued for at least seven weeks after cessation of the treatment.

Preliminary data demonstrated that established, 5-week-old Daudi Burkitt lymphomas are completely regressed when treated with IFN-α-2a-Fc 10^6 IU/daily for ten days. A 90% reduction of tumor volume in 2 weeks is also achieved in Daudi Burkitt lymphomas which were treated with 10^6 IU Roferon A/every 3 days for seven weeks before the IFN-α-2a-Fc treatment started.

IFN-α-2a-Fc has a half-life of about one week, when administered subcutaneously $1X10^6$ IU/day or $1X10^5$ IU/day for eight weeks.

It should be understood that the terms and expressions used herein are exemplary only and not limiting, and that the scope of the invention is defined only in the claims which follow, and includes all equivalents of the subject matter of those claims.

What is Claimed is:

1. A protein hybrid comprising an interferon protein covalently linked to an immunoglobulin Fc fragment.
2. The protein hybrid of claim 1 comprising an interferon protein covalently linked at its C-terminal end to the N-terminal end of an immunoglobulin Fc fragment.
3. The protein hybrid of claims 1 or 2 further comprising a peptide linker between the interferon protein and the immunoglobulin Fc fragment wherein the interferon protein is covalently linked at its C-terminal end to the N-terminal end of the peptide linker and the peptide linker is covalently linked at its C-terminal end to the N-terminal end of the immunoglobulin Fc fragment.
4. The protein hybrid of claim 3 wherein the linker comprises Gly Ser repeat units.
5. The protein hybrid of claim 4 wherein the Gly Ser repeat units comprise from 2 to 40 amino acids.
6. The protein hybrid of claim 4 wherein the Gly Ser repeat units comprise from 12 to 15 amino acids.
7. The protein hybrid of claim 4 wherein the Gly Ser repeat units comprise from 17 to 40 amino acids.
8. The protein hybrid of claim 3 wherein the linker comprises a T cell inert amino acid sequence or a non-immunogenic amino acid sequence.
9. The protein hybrid of claims 1, 2, or 3 wherein the interferon protein is selected from the group consisting of interferon-α and interferon-β.
10. The protein hybrid of claims 1, 2, or 3 wherein the interferon protein is interferon-α.
11. The protein hybrid of claim 9 wherein the interferon protein is selected from the group consisting of interferon-α2a or interferon-α2b.
12. The protein hybrid of claims 1, 2, or 3 wherein the Fc fragment is an IgG Fc fragment.
13. The protein hybrid of claims 1, 2, or 3 wherein the Fc fragment is an IgG γ4 chain Fc fragment.
14. The protein hybrid of claims 1, 2, or 3 further comprising another cytokine covalently linked at its C-terminal end to the N-terminal end of the other chain of the immunoglobulin Fc fragment, thereby forming a homodimer.
15. The protein hybrid of claim 14 wherein the cytokine is an interferon.
16. A protein hybrid comprising an interferon protein covalently linked at its C-terminal end to the N-terminal end of a peptide linker that is covalently linked at its C-terminal end to

the N-terminal end of an immunoglobulin Fc fragment, wherein the peptide linker comprises Gly Ser repeat units of having 2 to 15 and 17 to 40 amino acids.

17. A method of treating patients with tumors to remove tumors, regress tumors, or delay tumor development, comprising administering the protein hybrid of any of claims 1 to 16 to the patient in amounts effective to remove tumors, regress tumors, or delay tumor development.

Figure 1

Virus Cytopathic Effect Inhibition Assays Using Purified Proteins

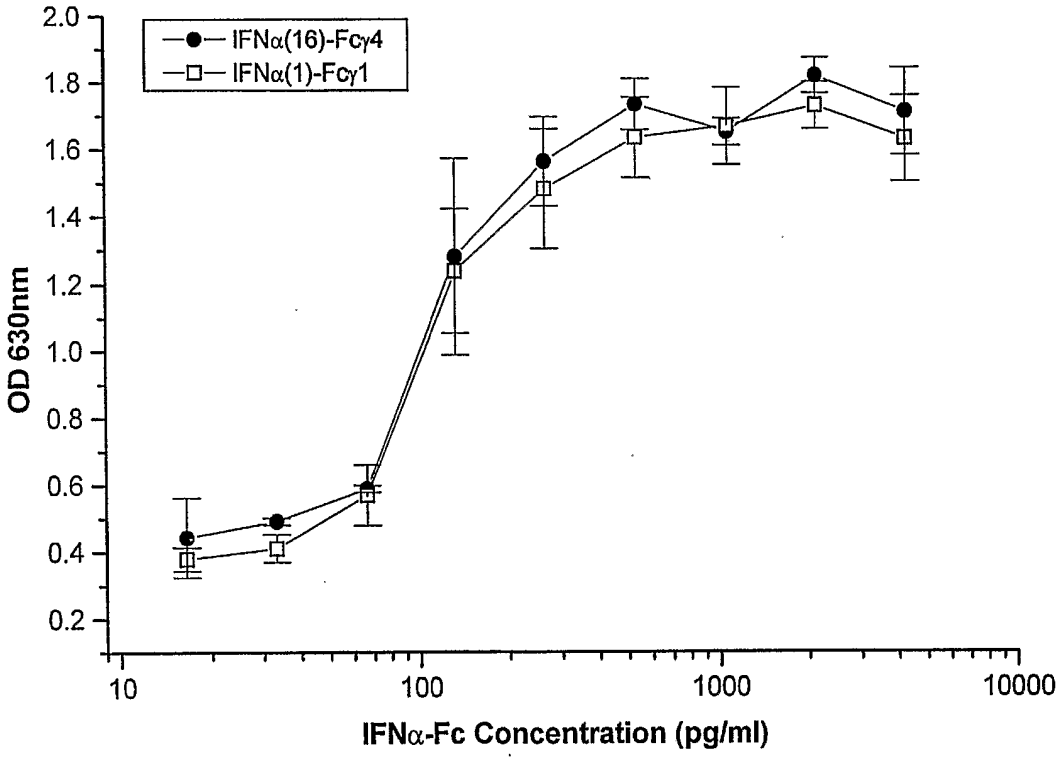
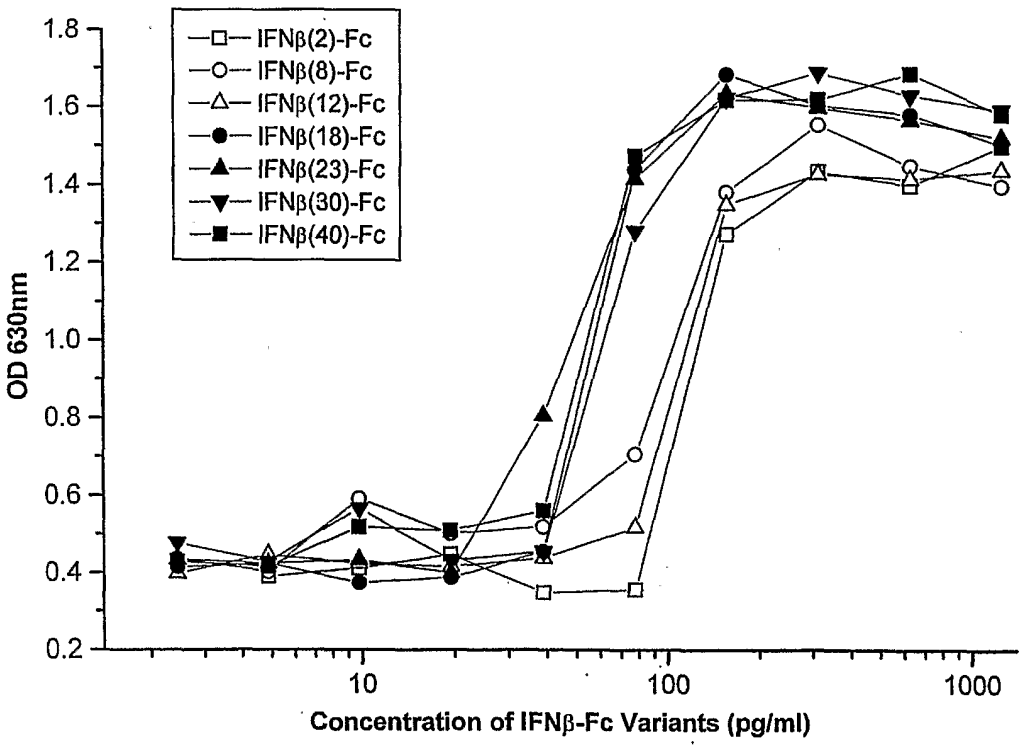


Figure 2

Virus Cytopathic Effect Inhibition Assays Using Culture Supernatants



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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/25154

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 38/21, 39/395, 39/00; C07K 1/00, 16/00

US CL : 424/85.4, 134.1, 185.1, 192.1; 435/69.7; 530/387.3

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/85.4, 134.1, 185.1, 192.1; 435/69.7; 530/387.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,723,125 A (CHANG et al) 03 March 1998 (3.03.1998), entire document	1-2,6-8 and 16
X	US 5,908,626 A (CHANG et al) 01 June 1999 (1.06.1999), entire document	1-2,6-8 and 16

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

20 September 2002 (20.09.2002)

Date of mailing of the international search report

05 MAR 2003

Name and mailing address of the ISA/US

Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703)305-3230

Authorized officer

Jegatheesan Seharaseyon

Telephone No. 703-308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/25154

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claim Nos.: 4-5,9-15 and 17
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Continuation of B. FIELDS SEARCHED Item 3:
WEST Search on USPAT
Search terms: interferon alpha, immunoglobulin Fc fragment