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Recombinant Interferon-α Cross-linked with Thymosin α1 is Biologically Active

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Abstract: Partially reduced interferon-α (IFN-α) was cross-linked with thymosin α1 (Tα1) using sulfo-succinimidyl (4-iodoacetyl) amino benzoate (SIAB), a bifunctional cross-linking reagent. The partially reduced IFN-α optimal for the cross-linking reaction was obtained by incubating native IFN-α with 0.5 mM DTT at 30℃ for 60~100 min. Tα1 was activated by incubating with sulfo-SIAB at 37℃ for 30 min to produce Tα1-IAB. The Tα1-IFN-α cross-linking was achieved by the reaction of the partially reduced IFN-α with Tα1-IAB. This cross-linking was between the sulfhydryl group of Cys1 in IFN-α and the N-terminal amino group of Tα1 through acetyl amino benzoate as a spacer. The immunological activity of the cross-linked molecule showed the same extent as that of Tα1, and most of the antiviral activity was retained compared to that of the partially reduced IFN-α.
Key words: antiviral activity, cross-linking, recombinant interferon-α, thymosin α1.

The interferons (IFNs) are a complex family of naturally occurring proteins with broad antiviral, cytotoxic, and immune-modulating activity. IFN-α is one of the three major groups (α, β, and γ) and was originally defined by their capacity to render cells resistant to viral infection (Isaacs and Lindenmann, 1957). Since its discovery, many more activities have been reported including modulation of many differentiated cellular activities, inhibition of cell growth, and antitumor effects (Gresser, 1989). It is widely accepted that IFN actions are mediated through interactions with receptors present at the cell surface (Aguet, 1980; Mogensen and Bandu, 1983; Hannigan and Williams, 1986). Recently, improved tissue-culturing methods and recombinant DNA cloning techniques have permitted large-scale production of highly purified IFN-α for clinical trials.

Thymosin α1 (Tα1), first isolated from a calf thymus preparation of thymosin fraction 5 (Low *et al.*, 1979b) is an acidic polypeptide consisting of 28 amino acid residues (Goldstein *et al.*, 1977; Low and Goldstein, 1979; Low *et al.*, 1979b). It has been reported that Tα1 is active in several assay systems for enhancement of immune function both *in vitro* and *in vivo* (Low *et al.*, 1979a; White, 1980; Zatz and Goldstein, 1985), and stimulates the production of lymphokines, such as the macrophage migration inhibitory factor (Thurman *et al.*, 1984), IFN (Svedersky *et al.*, 1982), interleukin-2, and interleukin-2 receptor (Sztein and Serrate, 1989). Also, Tα1 is able to act synergistically with IFN-α and IFN-β in stimulating natural killer cell activity (Mastino *et al.*, 1992). Clinical trials using Tα1 in the treatment of patients in immunodeficiency or cancer indicate that this agent enhances immune responsiveness and augments specific lymphocyte functions (Schulof *et al.*, 1982; Sztein and Goldstein, 1986; Garaci *et al.*, 1990). Tα1 has been successfully used in the treatment of chronic hepatitis B (Dabrowski *et al.*, 1980; Sheng, 1983), although it fails to show antiviral activities *in vitro*. In a placebo-controlled trial, Mutchnick *et al.* (1991) demonstrated that Tα1 was able to promote disease remission and cessation of HBV replication in chronic viral hepatitis patients without significant side-effects. Furthermore, a high degree of immunopotentiation was achieved by a combination therapy of Tα1 plus IFN-α in experimental animal models of immunodepression (Favalli *et al.*, 1989; Garaci *et al.*, 1990).

On the basis of these observations, we tried to make a single molecule by a cross-linking of Tα1 to IFN-α. IFN-α has two disulfide bonds; one between Cys1 and Cys98 and the other between Cys29 and Cys138. The disulfide bond strength between residues 1 and 98 is known to be weaker than that between residues 29 and 138. During the refolding of a fully reduced IFN-α to the native form, a partially reduced form with a disulfide bond between 29 and 138 is observed as an intermediate (Bae *et al.*, 1995). In the partially re-

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duced IFN-α, Cys1 is shown to be more reactive than Cys98 (DeChiara *et al.*, 1986). Taking advantage of these properties of the IFN-α, we cross-linked a sulfhydryl group of Cys1 in the IFN-α to the N-terminal group of the Tα1 through a bifunctional cross-linking reagent. As described, Tα1 has been used in the treatment of hepatitis in combination with IFN-α for a synergistic effect. Therefore, one molecule containing activities of both Tα1 and IFN-α should provide an effective means of combination therapy for hepatitis or cancer.

Materials and Methods

Chemical synthesis and purification of thymosin alpha 1 (Tα1)

Tα1 was synthesized by a peptide synthesizer (Applied Biosystems, model 431A; Foster City, USA) using t-boc amino acids, and their protecting groups were cleaved by TFMSA (trifluoromethyl sulfonyl anhydride; Applied Biosystems). Synthetic peptide was purified by the reverse phase HPLC system using a C18 column (Hi-Pore RP-318, 10 mm×250 mm; Bio-Rad; Hercules, USA), and its amino acid composition was confirmed by an amino acid analyzer (Applied Biosystems, model 420A). Purified peptide was freeze-dried, redissolved in deionized water, and stored at −20℃.

T cell activation assay

T cells were isolated from venous blood of normal adult volunteers following the method developed by Kouttab (1988). Isolated T cells were suspended in Isocoves modified dulbeccos medium (IMDM; Difco; Detroit, USA) supplemented with 10% FBS and their concentration was adjusted to 2×10^6 cells per ml. 100 μl of this solution was added to each well of 96 well plates and 50 μl of phytohemagglutinin (PHA; Sigma Chemical Co., St. Louis, USA) of 40 μg/ml solution in IMDM was added for the stimulation of T cells. Then Tα1 was added to the final concentration of 1.0 μg/ml and the final volume of each well was adjusted to 200 μl with IMDM. Wells containing T cell only or T cell plus PHA were used as controls. After the incubation for 48 h in a humidified CO_2 incubator at 37℃, each well was treated with 1 μCi of [^{3}H] thymidine (27 Ci/mmole; Amersham; Amersham, UK), followed by an additional incubation for 16 h. Cells were then harvested by a cell harvester, and the amount of incorporated [^{3}H] thymidine into DNA was detected by a liquid scintillation counter (LKB; Uppsala, Sweden).

Optimization of conditions for partial reduction of IFN-α

Recombinant human IFN-α was purified from transformed yeast following the methods of Bae *et al.* (1995). Using dithiothreitol (DTT; Sigma; St. Louis, USA) as a reducing agent, the optimal reducing condition for the production of a partial form was tested. Different concentrations of DTT were added to purified IFN-α dissolved in 10 mM potassium phosphate (pH 7.2) containing 0.15 M NaCl, and each tube was incubated in 30℃ for 20, 60, and 100 min, respectively. Then the reaction was quenched by adding a 1/10 volume of IAA (iodoacetic acid; Sigma; St. Louis, USA). Samples were analyzed on a non-reducing SDS-PAGE and the extent of reduction was evaluated by determining the relative density of bands on a densitometer (Bio-Rad, Hercules, USA).

Reaction of Tα1 with a cross-linking reagent and purification of its product

Sulfo succinimidyl (4-iodoacetyl) amino benzoate (Sulfo-SIAB; Pierce; Rockford, USA) was used as a bifunctional cross-linking reagent. Tα1 and sulfo-SIAB were dissolved in 100 mM of sodium borate (pH 7.6), with a 1 : 5 molar ratio, and incubated at 37℃ for 2 h. After the incubation, the reaction mixture was loaded onto a Sephadex G-25 (Pharmacia; Uppsala, Sweden) column pre-equilibrated with 20 mM Tris-HCl (pH 7.0). The peak for Tα1-IAB was pooled, and subsequently applied to a DEAE-HPLC column (Protein PAK 8HR, 10 mm×100 mm; Waters; Waltham, USA) equilibrated with the same buffer. After washing with 150 mM of NaCl, elution was done by a linear gradient of 150~350 mM of NaCl. Each fraction was analyzed by incubating with partially reduced IFN-α, and running on the non-reducing SDS-PAGE.

Optimization of the reaction time of IFN-α with a Tα1-IAB

The mixtures of purified Tα1-IAB and partially reduced IFN-α were incubated at 37℃ for different lengths of time, and the reaction was quenched by adding a 1/10 volume of IAA for a 10 min interval. Each fraction was compared on the non-reducing SDS-PAGE.

Cross-linking of IFN-α with Tα1-IAB and purification of its product

Purified Tα1-IAB and partially reduced IFN-α were mixed in a 4 : 1 molar ratio and incubated for 30 min at 37℃. The reaction was ended by adding a 1/10 volume of IAA. The reaction mixture was loaded onto a Sephadex G-25 column preequilibriated with 20 mM of Tris-HCl (pH 7.0) and the peak for the Tα1-IFN-α complex was pooled. This pool was then applied to a DEAE-HPLC column (Protein PAK 8HR, 10 mm×100 mm; Waters) equilibrated with the same buffer. After

washing with 150 mM NaCl, elution was performed by a linear gradient of 150~450 mM NaCl. Each fraction was analyzed on the non-reducing SDS-PAGE.

Antiviral activity assay

The antiviral activity assay of IFN-α and Tα1-IFN-α was performed by determining the cytopathic effect by the method of Kim and Park (1992). Bovine kidney cell line (MDBK) was used as the target cell and vesicular stomatitis virus (VSV) was used as the challenging virus.

Preparation of anti-Tα1 antiserum

Tα1 was conjugated with bovine serum albumin (BSA; Sigma; St. Louis, USA) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC; Pierce, Rockford, USA). These conjugates were emulsified with Freunds adjuvants, and then injected into a guinea pig three times at an interval of 2 weeks. At 2 weeks after final injection, the animal was sacrificed and the whole serum was collected.

Enzyme-immunoassay (EIA)

The conjugate of Tα1 with poly L-lysine (PLL; Pierce, Rockford, USA) was used as a standard solution. Tα1 was serially diluted by 2^n fold from 0.24 μg/ml (80 mM) and coated onto a 96 well immunoplate. Tα1-IFN-α was also serially diluted by 2^n fold and coated. After sequential incubations with anti-Tα1 antiserum and anti-guinea pig IgG antibody-peroxidase conjugate, color development was performed by using o-phenylenediamine dihydrochloride (OPD; Sigma; St. Louis, USA) as substrate.

Results

Amino acid composition and T cell activation activity of synthetic Tα1

Amino acid composition was analyzed after the chemical synthesis and purification of Tα1. Amino acid analysis of the chemically synthesized Tα1 showed an agreement with the theoretical one. In order to determine the effect of Tα1 on the proliferation of T cells, the amounts of [^{3}H] thymidine incorporated into T cells were measured. The relative amount of thymidine uptake for the case of Tα1 treatment was 150% compared to the control (treatment of PHA only). The result shows that more proliferation of T cells occurred by 50% points, thereby confirming synthetic Tα1 to be fully active. The results of amino acid analysis and activity assay indicate that the synthetic peptide is an authentic Tα1.

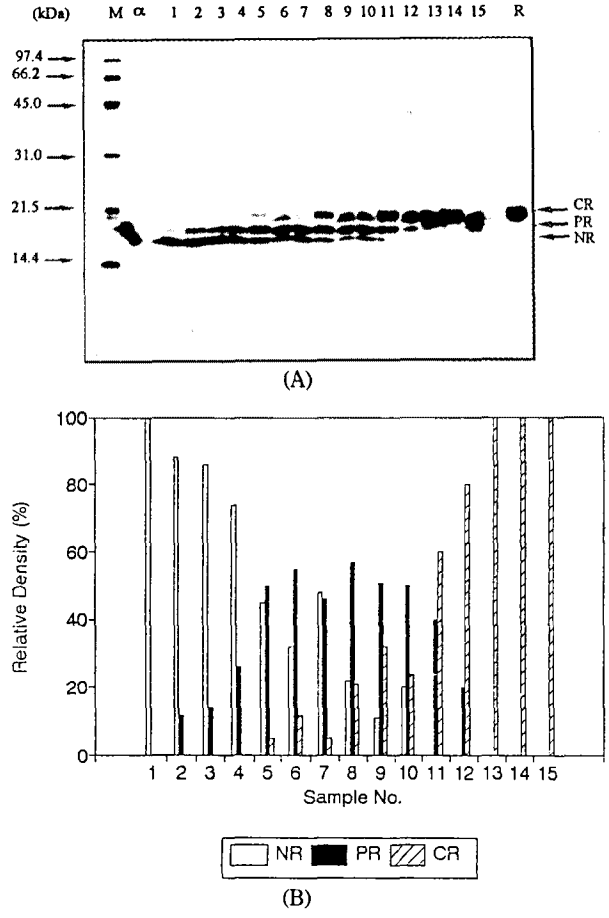


Fig. 1. Optimization of the conditions for the partial reduction of IFN-α. After the non-reducing SDS-PAGE (A), the relative ratio of each band was determined by scanning the coomassie stained gel using a densitometer (B). The extent of reduction is indicated in NR (non-reduced), PR (partially reduced), and CR (completely reduced). (Lane M: molecular weight standards; Lane α: native IFN-α; Lane 1, 2, 3: incubated with 0.1 mM DTT for 20, 60, 100 min; Lane 4, 5, 6: incubated with 0.5 mM DTT for 20, 60, 100 min; Lane 7, 8, 9: incubated with 1.0 mM DTT for 20, 60, 100 min; Lane 10, 11, 12: incubated with 2.0 mM DTT for 20, 60, 100 min; Lane 13, 14, 15: incubated with 10.0 mM DTT for 20, 60, 100 min; Lane R: completely reduced IFN-α).

Production of partially reduced IFN-α

Native IFN-α was incubated with various concentrations of DTT for different lengths of times (Fig. 1). The incubation of IFN-α with 1 mM DTT for 60 min (#8 in Fig. 1) produced the maximum amount of partially reduced IFN-α, but also a relatively large amount of completely reduced form. In this case, further cross-linking with Tα1 would result in a complication caused by the unwanted reaction of completely reduced IFN-α with Tα1, thereby making the purification of the cross-linking product difficult. The incubation of IFN-α with 1 mM DTT for 20 min (#7 of Fig. 1) produced the relatively high ratio of partially reduced IFN-α to

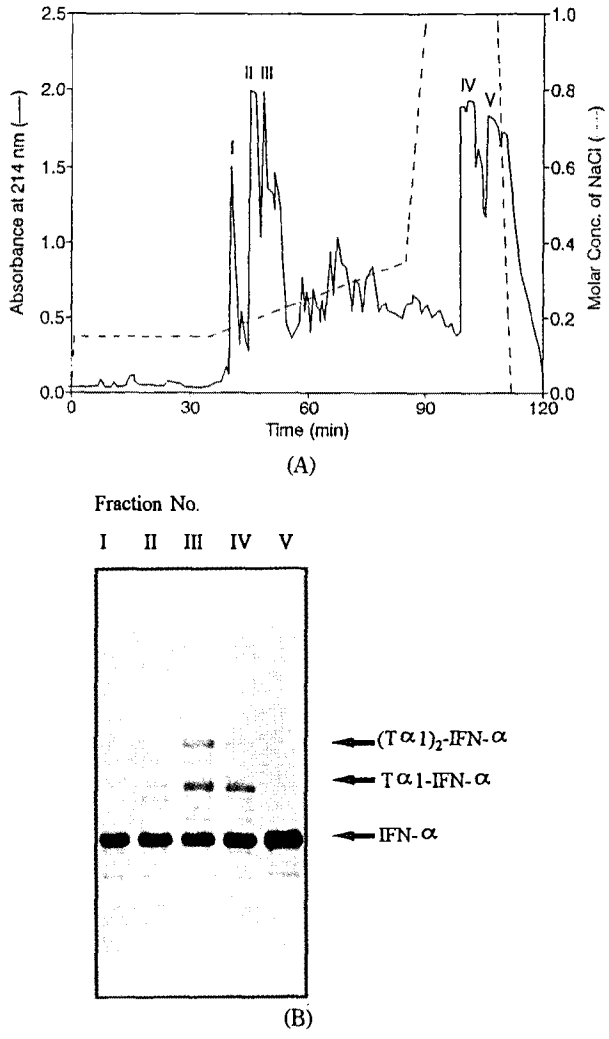


Fig. 2. Separation of Tα1 reacted with sulfo-SIAB. (A) DEAE-HPLC elution profile after the reaction of Tα1 with sulfo-SIAB. The reaction mixture was loaded onto DEAE-HPLC column preequilibrated with 20 mM Tris-HCl (pH 7.0). After washing with 150 mM NaCl, elution was done by a linear gradient of 150~350 mM NaCl. (B) Analysis of the fractions eluted from the DEAE-HPLC column. Five major fractions [I~V of (A)] were analyzed by incubating with partially reduced IFN-α, followed by running on the non-reducing SDS-PAGE.

completely reduced IFN-α. However, in this case, because of the high concentration of DTT, it was also necessary to maintain an exact incubation time. A lesser amount of partially reduced IFN-α was obtained when the native IFN-α was incubated with 0.5 mM DTT for 60 and 100 min (#5 and #6 in Fig. 1), but in this case, a far less amount of completely reduced IFN-α was produced. Therefore 0.5 mM of DTT and a 60~100 min incubation time was employed for the production of partially reduced IFN-α.

Reaction of Tα1 with cross-linking reagent and purification of its products

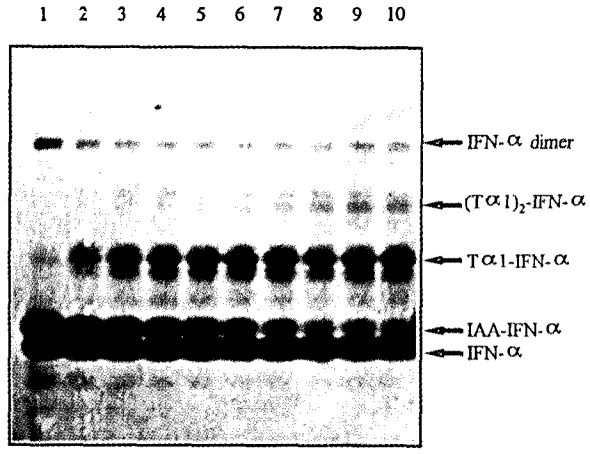


Fig. 3. Optimization of the reaction time of IFN-α with Tα1-IAB. The optimum reaction time was determined by the incubation of partially reduced IFN-α with Tα1-IAB for different length of time, followed by running on the non-reducing SDS-PAGE. (Lane 1: 0 min; Lane 2: 10 min; Lane 3: 20 min; Lane 4: 30 min; Lane 5: 40 min; Lane 6: 50 min; Lane 7: 60 min; Lane 8: 70 min; Lane 9: 80 min; Lane 10: 90 min).

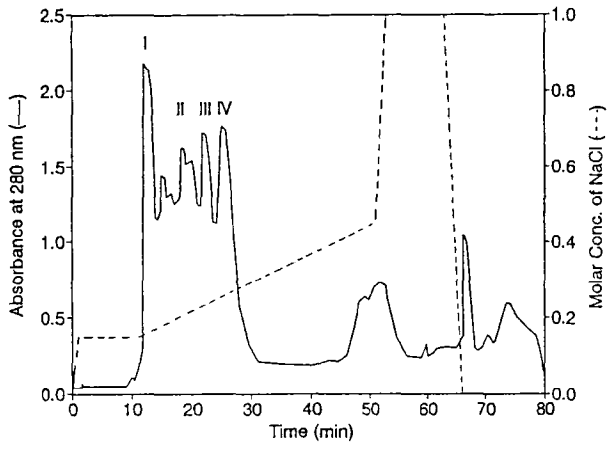
After the reaction of Tα1 with sulfo-SIAB, DEAE-HPLC column chromatography was conducted (Fig. 2A), and the elution resulted several peaks. Although sulfo-SIAB binds to the N-terminal amino group selectively, nonspecific reactions may also occur as in most chemical reactions. These nonspecific reactions may be the reason for the elution of more than the expected number of peaks. In order to determine the target fraction, 5 major peaks (peak I~V in Fig. 2A) were reacted with partially reduced IFN-α. This reaction was not optimized to obtain the cross-linking molecule having a molar ratio of 1 to 1. The result of non-reducing SDS-PAGE (Fig. 2B) indicated that peak III was the highest reactivity of the cross-linking, thereby this fraction was Tα1-IAB.

Optimization of the reaction time of IFN-α with Tα1-IAB

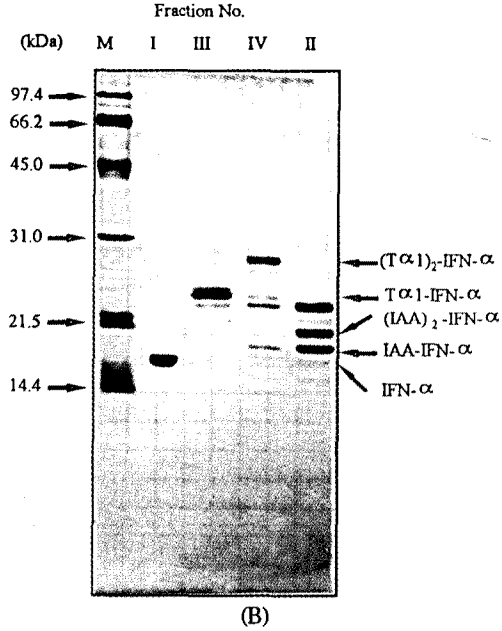
The reaction of partially reduced IFN-α with Tα1-IAB proceeds immediately after mixing. Binding of one molecule of Tα1 to IFN-α was completed within 30 min, and after that the second molecule of Tα1 starts binding to Tα1-IFN-α (Fig. 3). The reaction condition in which IFN-α reacts with Tα1 in a 1 : 1 ratio was adopted in order to retain the biological activity of each cytokine and minimize the alteration of the three dimensional structure of IFN-α.

Cross-linking of IFN-α with Tα1-IAB and purification of the cross-linking product

After the cross-linking reaction of IFN-α with Tα1-IAB, DEAE-HPLC column chromatography was conduct-



(A)



(B)

Fig. 4. Separation of IFN-α reacted with Tα1-IAB. (A) DEAE-HPLC elution profile after the reaction of partially reduced IFN-α with Tα1-IAB. The reaction mixture was loaded onto DEAE-HPLC column preequilibrated with 20 mM Tris-HCl (pH 7.0). After washing with 150 mM NaCl, elution was done by a linear gradient of 150~450 mM NaCl. (B) Analysis of the fractions eluted from the DEAE-HPLC. Four major fractions [I~IV in (A)] were analyzed on the non-reducing SDS-PAGE. [Lane M: molecular weight standards, same as in the Fig. 1 (A)]

ed, and each peak was analyzed on non-reducing SDS-PAGE (Fig. 4). Theoretically, four types of reaction products can be obtained by the cross-linking reaction of partially reduced IFN-α with Tα1 : Tα1 and unreacted IFN-α; IAA-bound IFN-α; one molecule of Tα1 bound IFN-α; and two molecules of Tα1 bound IFN-α. Fig. 4 shows that fraction III was the cross-linking product with one molecule of Tα1 bound to one molecule of IFN-α, fraction I was unreacted IFN-α, fraction II was a mixture of reaction products between IFN-α and Tα1 or IAA, and fraction IV was the complex of two molecules of Tα1 bound to IFN-α.

Table 1. Antiviral activity assay of IFN-α and its derivatives.

Sample	Specific Activity (IU/mg)	Relative Activity
Native IFN-α	2.30×10^8	100
Partially reduced IFN-α	1.60×10^8	70
Fraction III in Fig.6	1.36×10^8	59
Fraction IV in Fig.6	7.22×10^7	31
Tα1	No Activity	

Specific activity was obtained by determining cytopathic effect using a bovine kidney cell line (MDBK) and vesicular stomatitis virus (VSV), followed by dividing it with the amount of protein.

Antiviral activity assay

Native IFN-α and cross-linking products were assayed for antiviral activities (Table 1). The recombinant IFN-α showed its specific activity of 2.3×10^8 IU/mg, which is comparable to the known value, whereas Tα1 itself showed no antiviral activity in vitro as reported. Partially reduced IFN-α had a 70% antiviral activity of the native form, and the cross-linked form of IFN-α and Tα1 in a 1 : 1 ratio (fraction III in Fig. 4) had a 59% antiviral activity of the native form. The antiviral activity of the Tα1-IFN-α complex was 84% compared to that of partially reduced IFN-α. Fraction IV, which was the cross-linking form of IFN-α and Tα1 in a 1:2 ratio, had an antiviral activity of only 31% of the native form.

Enzyme-immunoassay (EIA)

In order to confirm that the cross-linking to IFN-α does not disturb the Tα1, EIA was conducted. The calibration curve was constructed by plotting the average absorbance values of standards (y-axis) versus the logarithmic dilution factor ($\log_2 D$) of standards (x-axis). Then the dilution factor of the sample (fraction III in Fig. 4) can be determined from the calibration curve. The $\log_2 D$ values of 2^4 fold diluted and 2^5 fold diluted samples were 1.49 and 1.92, respectively. These values correspond to 28.47 nM and 21.16 nM of molar concentration of the standard solution, which correspond to 0.567 μM of the mean molar concentration of the undiluted sample. This molar concentration was converted to 11.91 μg/ml on the assumption that the molecular weight of Tα1-IFN-α was 21,000. By the BCA protein assay, the protein concentration of the fraction III was measured to be 10.15 μg/ml, which was similar to the concentration determined by EIA. Therefore, it was proved that Tα1 had almost the same immunological activity even after cross-linking with IFN-α.

Discussion

Native IFN-α has two disulfide bonds. In the reduction process, two different forms are known to exist. One is a partially reduced form in which 1-98 disulfide bond is broken, and another is a completely reduced form in which both 1-98 and 29-138 disulfide bonds are broken. The antiviral activity of completely reduced IFN-α is shown to be only 10% of that of the native form. Partially reduced IFN-α, on the other hand, has a similar extent of activity to that of the native form (Wetzel R. *et al.*, 1982). Our result (Table 1) shows that partially reduced IFN-α is 70% active compared with the native form, as expected. Furthermore, the cross-linked IFN-α retains its antiviral activity of 84% compared to partially reduced IFN-α (59% compared to the native form), in spite of the cross-linking with relatively large peptides. This observation suggests that the three dimensional structure of IFN-α for antiviral activity was essentially undisturbed even after cross-linking with Tα1. The slight loss of activity of the cross-linked IFN-α may be due to impurities in the cross-linked molecule preparation. Fraction III used in the antiviral activity assay is not completely pure and may contain cross-linking reaction by-products (Fig. 4B). Those impurities appear to decrease the specific activity of the cross-linked IFN-α. The band appearing just below Tα1-IFN-α (fraction II and III in Fig. 4B) may be another form of Tα1-IFN-α in which the attached site of Tα1 is not the N-terminal. In addition to the N-terminal amino group, the ε-amino group of lysine in the Tα1 can react with sulfo-SIAB. In this case, the cross-linked molecule becomes more compact, which causes its mobility in the electrophoresis faster than expected.

It is known that Cys1 is more reactive than Cys98, and Cys98 is located in a nearby receptor binding site. Table 1 shows that the antiviral activity of the cross-linking product between one molecule of IFN-α and two molecules of Tα1 dramatically decreased. This indicates that the binding of a relatively large oligopeptide such as Tα1 at Cys98 greatly influences the structure of IFN-α. Therefore, it was indirectly proved that the location of attached Tα1 in fraction III was Cys1 of IFN-α.

The activity assay of Tα1 moiety in the cross-linked molecule could not be reliably measured by T cell activation assay because of the cytostatic effect of IFN-α. Although T cells were activated by Tα1, it seems that the uptake of [^{3}H]thymidine does not increase because of from the effect of IFN-α in the cross-linked molecule. It has been reported that the N-terminal of Tα1 is not crucial for its biological activity, that is, if the C-terminal part of the Tα1 moiety of the cross-linking molecule is exposed to the surface, it would be worth its function. Tα1 in the cross-linked molecule was proved to have almost the same immunological property as Tα1 alone. The same immunological property of the free and the cross-linked Tα1 indicates that the Tα1 moiety in the cross-linking molecule is exposed to the surface. Therefore, it is a reasonable assumption that Tα1 attached to IFN-α has its own biological activity. At the same time, the cross-linking molecule between IFN-α and Tα 1 in the same molar ratio retains the biological activity of the authentic IFN-α, implying that the three dimensional structure of IFN-α for the biological activity is not disturbed.

In addition to the T cell activation assay, there are several other assay methods to determine the biological activity of Tα1, such as *in vitro* terminal deoxynucleotidyl transferase (TdT) suppression assay and *in vitro* specific macrophage migration inhibitory factor (MIF) assay (Thurman *et al.*, 1984). Further works would be those biological activity assays of Tα1 moiety in the cross-linking molecule.

At present, IFN-α and Tα1 are being proceeded in a combination therapy for various purposes, and some positive results are continually reported. In addition to the treatment of hepatitis B, which is one of the most active trials, several clinical trials are in progress, such as the treatment of autoimmune hepatitis, cancer, and AIDS in a combination therapy with zidovudine (Garaci *et al.*, 1994). In such cases, it must be the new merit of IFN-α and Tα1 being in a single molecule. Because oligopeptide drugs such as Tα1 are known to have a relatively short life time in a physiological condition, the dose or the frequency of injection must be increased, which may be solved by attaching peptides to macromolecules. On the basis of such experiments, this cross-linking method can be applied to other cytokines, such as IFN-β, IFN-γ, TNF, EPO, and the interleukin series. In addition, the expression of cross-linked proteins in transformed cells by recombinant DNA technology is in progress.

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