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EXISTENCE AND UNIQUE N-TERMINAL SEQUENCE OF ALPHA II (OMEGA) INTERFERON IN NATURAL LEUKOCYTE INTERFERON PREPARATION

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Human alpha interferon produced in Sendai virus-stimulated leukocytes was purified by immunosorbent affinity chromatography and subjected to subtype analysis. All subtypes in leukocyte culture supernatant were confirmed in purified preparation. One of these subtypes was reactive in Western blotting with antibody specific to alpha II (omega). N-terminal amino acid sequence analysis of this particular subtype showed addition of two amino acids to the N-terminus of alpha II predicted through cDNA studies. The apparent cleavage site for leader sequence of alpha II was different from the site common to all other alpha subtypes. This is the first report on the isolation of natural alpha II and its unique N-terminal amino acid sequence.

The class 1 interferon (IFN) genes were known to exist in two families, alpha and beta, with several characteristics such as subtype genes and pseudogenes and absence of introns in mammals (1, 2). In humans, one beta-type IFN and more than 20 subtypes of alpha-type IFN (leukocyte interferon) were discovered through advances in molecular biology and gene technology (3) and more than 70% homology on amino acid sequence has been shown in subtypes of human IFN-alpha (4). In 1985, a new family of class 1 interferon was reported by several groups independently (5, 6, 7). Although this new type of IFN was designated omega and uniquely possessed the N-glycosilation site, it can be considered a subfamily of IFN-alpha, IFN-alpha II, in the same sense as producer cells and in the degree of homology of amino acid sequence, and because on the same chromosome both alpha II and the rest of alpha (alpha I) genes were found (8).

Despite the information gained through genetic studies, almost no information has been provided on naturally existing IFN-alpha II itself. In this communication, we describe the purification of IFN-alpha II from Sendai virus-stimulated human leukocyte culture and then show the drastic difference

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between alpha II and IFN-alpha I in their N-terminal sequences, a difference that has not yet been reported. The nomenclature alpha II is used throughout this paper for omega type interferon following the suggestion of Adolf (8).

MATERIALS AND METHODS

Preparation of antibody, specific to IFN-alpha II. The peptide Lys-Asp-Arg-Asp-Leu-Gly-Ser-Ser corresponding to the C-terminal region of human interferon alpha II, which was designed from an unique region of alpha II, was chemically synthesized on a Model 430A peptide synthesizer (Applied Biosystems Inc., Foster City, CA). This peptide was conjugated to bovine serum albumin (BSA) as carrier protein by the maleimido method (9). The ratio of hapten to carrier was 100 to 1 in molecular base. This peptide BSA complex was then used to raise antisera in rabbits. After injection of 2 mg of complex with complete adjuvant, 1 mg of complex was injected at the second, fourth and sixth week with incomplete adjuvant. Ten days after the last injection, rabbits were bled and harvested antisera were purified to IgG, in order to avoid background interference in Western blotting, by means of affinity chromatography on agarose gel to which this peptide was immobilized.

Titration of IFN activity. The activity of IFN was measured by a cytopathogenic effect (CPE) assay using FL cells (10) and vesicular stomatitis virus in 96 well microplates. After virus infection, surviving cells were stained with neutral red, extracted with 50% ethanol (11), and scanned on a microplate reader (V max, Molecular Devices, Menlo Oaks, CA). Titer was expressed in international reference units through out these experiments.

Purification of IFN-alpha II. The crude human leukocyte IFN was prepared using the method described by Cantell (12) with slight modification (13, 14), treated with low pH (pH 2.0) for virus inactivation and then subjected to affinity chromatography using polyclonal antibody, which was highly specific to leukocyte interferon (14). Purified leukocyte IFN with subtypes was fractionated by means of reversed-phase high-performance liquid chromatography to isolate each subtype including alpha II.

SDS PAGE and Western blotting. Purified leukocyte IFN or isolated subtypes were separated on a 15% polyacrylamide gel under reducing condition with 0.1% sodium dodecyl sulfate (SDS) (15). Proteins on gel were transferred onto nitrocellulose membrane using a semidry electroblotting apparatus (Sartoblot II, Sartorius, West Germany) (16) and then processed as described elsewhere (17) using alkaline phosphatase-labeled anti-rabbit IgG and NBT/BCIP (18). Chemicals for electrophoresis and blotting were purchased from Bio Rad (Richmond, CA) or Promega (Madison, WI).

Reversed-phase high-performance liquid chromatography (RP-HPLC). Affinity-purified human leukocyte IFN was subjected to RP-HPLC using a Hi-Pore RP-318 C 18 column (ID 4.6 mm x 250 mm, Bio Rad). Subtypes were eluted with a linear gradient of acetonitrile (25% to 70%) in 0.1% trifluoroacetic acid (TFA) (14).

N-terminal amino acid sequence. Applied Biosystems Model 477A/120A was used for sequence analysis. All reagents for sequencing were purchased from Applied Biosystems Inc. The IFN-alpha II isolated by RP-HPLC was electrophoresed on a 15% polyacrylamide gel containing 0.1% SDS and transferred to polyvinylidene difluoride membrane (Immobilon, Millipore, Bedford, MA). After staining with Ponceau S, a band 25 kDa in size was cut and applied to the sequencer (19).

Chemicals. Unless otherwise noted, chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), or were of equivalent grade.

RESULTS AND DISCUSSION

Leukocyte IFN was purified from Sendai virus-stimulated human leukocyte culture without apparent loss of biological activity and 100 million units/mg protein of specific activity (10,000-fold purification) was achieved by this one step. We have already reported that this preparation possessed all subtypes detected in culture supernatant (14). This preparation was then subjected to RP-HPLC. The elution profile of protein and activity is shown (Fig. 1). More than 15 peaks of activity can be seen. The ratio of the IFN activity of each subtype is summarized in Table 1. This preparation was precipitated with ethanol as described by Cantell et al. (20) with modification in order to ensure inactivation of possible virus contamination and has been utilized for clinical trial in Japan. In addition, it has already been confirmed that this ethanol precipitation step did not deplete any subtype existing in affinity-purified preparation (data not shown).

These results prompted us to investigate whether IFN-alpha II was existing in our preparations. Alpha II was originally reported by Capon et al and other groups in 1985 and they clarified the cDNA gene for this molecule (3, 4, 5).

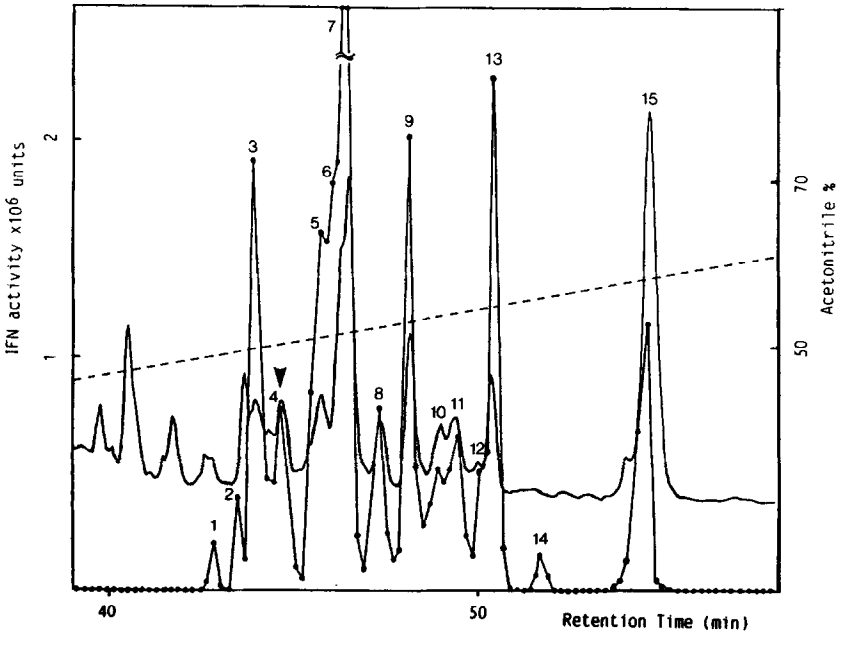

Fig. 1. RP-HPLC elution profile of human leukocyte IFN preparation.
2.5×10^7 units of human leukocyte IFN was loaded onto a C 18 column (Hi-Pore RP-318), washed with 25% acetonitrile, 0.1% TFA, then eluted with a linear gradient of acetonitrile (25% to 75%) in 0.1% TFA at a flow rate of 1.0 ml/min and fractionated to isolate each subtype. IFN activity in each fraction (0.2 ml/0.2 min) was measured by CPE assay. The expanded portion where the activities were eluted is shown. Absorbance at 280 nm, acetonitrile concentration, and IFN activities are indicated by solid lines, broken lines and closed circles, respectively.

Table 1. Summary of subtype composition analysis of IFN-alpha by RP-HPLC

Peak	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Retention Time (min)	43.3	43.8	44.4	45.1	46.0	46.4	46.6	47.5	48.2	49.1	49.5	50.1	50.5	51.6	54.4
Mw (kDa)	27.9	26.2	26.2	25.2	21.2	21.2	21.2	19.4	20.2	27.0	19.8	18.5	27.4	?	18.0
IFN activity (%)	1.2	2.1	10.1	4.5	(8.7)	(9.9)	18.9	4.4	11.1	2.9	3.7	(3.0)	12.1	0.8	6.6
Reactivity to anti-synthetic peptide	-	-	-	+	-	-	-	-	-	-	-	NT	-	NT	-

Molecular weights were estimated by SDS PAGE under reducing condition.
?, not detected on SDS PAGE; (), shoulder peak in RP-HPLC; NT, not tested.

After their reports, Adolf added the information on the antigenecity (6). However, the natural molecule produced in leukocytes has not been isolated and no information on that was reported.

To detect alpha II in our leukocyte IFN preparation, specific antisera were prepared. In the amino acid sequence deduced from cDNA including its leader sequence, C-terminus was apparently unique to this molecule among alpha interferons. Then peptide corresponding to this region (Lys-Asp-Arg-Asp-Leu-Gly-Ser-Ser) was synthesized and conjugated with BSA. The antisera against this peptide-BSA complex were utilized as probe for Western blotting. Although we faced the difficulty of high background in Western blotting (data not shown), use of the IgG affinity-purified on the peptide immobilized agarose could avoid this problem. All peaks with IFN activity, fractionated by RP-HPLC, were subjected to SDS PAGE and transferred to nitrocellulose electrically. The membrane was reacted with this antibody after blocking with

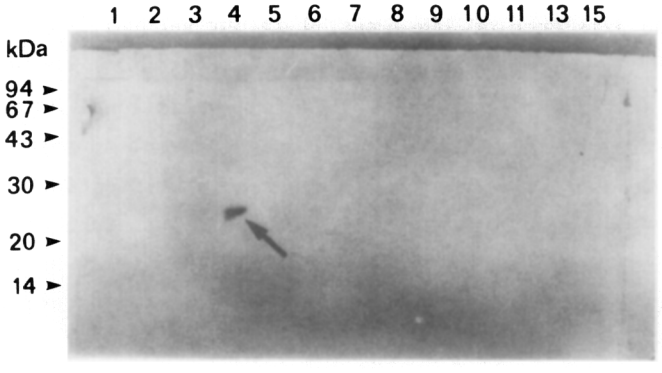

Fig. 2. Detection of alpha II IFN by western blotting.
Each subtype was electrophoresed, transferred onto nitrocellulose membrane, and reacted with antibody specific to synthetic peptide of IFN-alpha II and processed as described under Materials and Methods. Numbers on each lane show the elution order of each subtype on RP-HPLC.

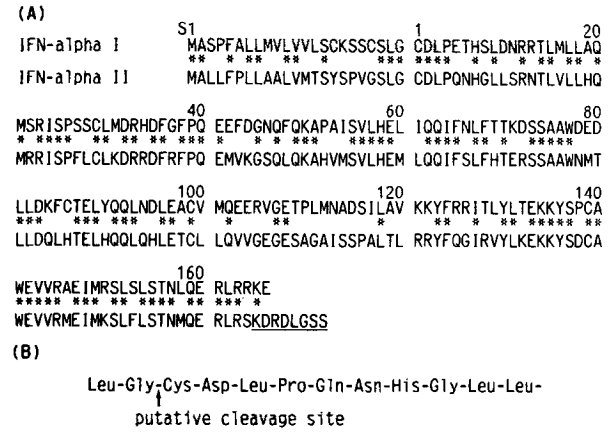

Fig. 3. **Amino acid sequence of alpha I and II IFN.**
(A) Amino acid sequence of IFN-alpha I consensus (4) and IFN-alpha II predicted through cDNA sequence (5). Stars, common amino acids; underline, synthesized peptide.
(B) Result of amino terminal sequencing of natural alpha II interferon isolated from human leukocyte interferon.

5% skim milk (17). Only one band, 25 kDa in size, was appeared to be reactive with this antibody (Fig. 2) and that was corresponding to 4th eluting peak (Fig. 1, arrowhead) in our gradient program of RP-HPLC system.

The consensus amino acid sequence of alpha and the putative sequence of IFN-alpha II are shown in Fig. 3A. It is also known that the family of human alpha interferon possessed homology to some extent and cystein for N-terminus is one of them. Our 4th peak was subjected to N-terminal amino acid sequence analysis and showed the unique property which differed from the information provided through cDNA study. N-terminus of this preparation was started from leucine and followed by glycine then cystein, which was known as consensus terminus, and consensus sequence with substitution of amino acids specific to alpha II was started (Fig. 3B). This result gave us not only the definitive evidence for the existence of IFN-alpha II in our natural leukocyte IFN preparation but also absolutely new and unique information on the property of IFN-alpha II itself among subtypes of IFN-alpha. Additional 2 amino acids were exactly correlating to that of leader sequence adjacent to reported cleavage site. In other words, cleavage site was apparently shifted for 2 amino acids into its leader sequence. This finding is suggesting the unique pathway and post translational modification for IFN-alpha II and it may be too much to say but IFN-alpha II could be carrying certain specific role(s) among more than 20 subtypes of human IFN-alpha family.

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