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Natural Human Interferon-γ Derived from Lipopolysaccharide-stimulated Human Myelomonocytic HBL-38 Cells

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A human myelomonocytic cell line, HBL-38 cells, propagated *in vivo*, spontaneously produced interferon (IFN)-γ and IFN-α. Whereas hemmagglutinating virus of Japan (HVJ) enhanced the production of IFN-α, bacterial lipopolysaccharide (LPS) markedly enhanced the production of IFN-γ. LPS could be replaced with lipid A. Furthermore, the enahancement of production of IFN-γ by LPS was completely abolished by polymixin B. IFN-γ derived from LPS-stimulated HBL-38 cells was purified to homogeneity and characterized. The apparent molecular weight, subspecies composition, amino acid sequence and glycosylated sites were in agreement with those of the product of normal human peripheral blood lymphocytes (PBL). These results indicate that the myelomonocytic HBL-38 cells, not a T-cell line, can also produce IFN-γ identical to the product of normal human PBL.

Key words: Natural human IFN-γ — Myelomonocytic cell line — LPS-stimulation — Mass production

Interferons (IFNs) are among the cytokines that have been well studied and most widely applied in clinical trials.[1] Among the three different types of IFNs, IFN-γ has appreciably more potent antiproliferative and immunoregulatory activities than either IFN-α or IFN-β.[2-4] Furthermore, IFN-γ is known to potentiate the antiproliferative effects of other cytokines such as IFN-α, IFN-β, tumor necrosis factor and lymphotoxin.[5-8]

Recent developments in genetic manipulation techniques have allowed the large-scale production of recombinant human IFN-γ with the use of *E. coli*.[9] In many cases, recombinant IFN-γ has been employed in fundamental studies or clinical trials. Whereas the recombinant IFN-γ has no carbohydrate moiety, the natural IFN-γ is a glycoprotein. In addition, some differences between natural and recombinant IFN-γ were reported, especially in recognition by monoclonal antibodies.[10] However, the supply of natural type of IFN-γ is very limited, because the natural human IFN-γ is produced by stimulating human leukocytes.[11] Thus, the development of a method for mass production of natural IFN-γ has become a matter of urgency.

The natural IFN-γ has been considered to be producible only from T-lymphocytes by stimulating them with antigens or T-cell mitogens.[12] Recently, however, Le *et al.*[13] and Blanchard *et al.*[14] reported that bacterial lipopolysaccharide (LPS) also could induce the production of IFN-γ by T-lymphocytes in the presence of accessory monocytes and that interleukin 2 and interleukin 1 played an important role in producing IFN-γ. They found that LPS could not directly induce IFN-γ from T-lymphocytes and T-lymphocyte-like cells. We have now found that the natural human IFN-γ can be produced by human myelomonocytic HBL-38 cells, not a T-cell, by stimulating them with LPS, and we have established a system to obtain large amounts of natural human IFN-γ from HBL-38 cells prepared by *in vivo* propagation.

In this report, we describe large-scale production, purification and characterization of natural human IFN-γ derived from human

Abbreviations used are: IFN, interferon; LPS, bacterial lipopolysaccharide; Con A, concanavalin A; PHA-P, phytohemagglutinin-P; HVJ, hemagglutinating virus of Japan; ATS, anti hamster thymocyte serum; PBL, peripheral blood lymphocytes; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

myelomonocytic HBL-38 cells by stimulation with LPS.

Materials and Methods

Cells A human myelomonocytic cell line, HBL-38 cells, kindly provided by Dr. J. Minowada (Roswell Park Memorial Inst., Buffalo, NY; currently at Hayashibara Biochem. Labs. Inc., Okayama), was cultured at 37° in RPMI-1640 medium supplemented with 10% FCS in a humidified atmosphere of 5% CO_2 in air.

Reagents The sources of reagents used in this work were as follows: LPS, lipid A, Con A and PHA-P from DIFCO Lab. (Detroit, MI), polymixin B from Taito Pfizer Co. Ltd. (Tokyo), collagenase (EC 3.4.24.3) from Amano Pharmaceutical Co. Ltd. (Nagoya), dispase (EC 3.4.24.4) from Godo Shusei Co. Ltd. (Tokyo), anti-human IFN-α sheep antiserum (G-026-502-568), anti-human IFN-β sheep antiserum (G-028-501-568) and anti-human IFN-γ rabbit antiserum (G-034-501-565) from NIH (Bethesda, MD), and an immobilized anti-human IFN-γ monoclonal antibody (RESELUTE-γ) from Celltech Ltd. (Berkshire). Anti hamster thymocyte serum (ATS) was prepared by immunizing rabbits with freshly prepared hamster thymocytes. Hemagglutinating virus of Japan (HVJ) was prepared in 10-day-old embryonated hens' eggs by infecting them with a strain of HVJ which was kindly provided by Dr. K. Cantel (Univ. of Helsinki, Helsinki).

Preparation of Large Amounts of HBL-38 Cells Using Immunosuppressed Hamsters In order to obtain large amounts of HBL-38 cells, *in vivo* cell propagation using immunosuppressed hamsters (the so-called Hayashibara hamster method) was applied. Briefly, 1×10^7 cells of HBL-38 maintained in culture were subcutaneously transplanted into newborn hamsters less than 24 hr old. Every 3 or 4 days, these hamsters were given 0.1 ml of ATS intraperitoneally. Four weeks later, the solid tumor masses of HBL-38 cells were harvested, rinsed thoroughly with saline and roughly minced with scissors. Then, the small tumor masses were treated with collagenase (2 units/g tumor weight) and dispase (5,000 units/g tumor weight) at 37° for 1 hr to obtain a single cell suspension.

Induction of IFNs The cell concentration was adjusted to 5×10^6 cells/ml with RPMI-1640 medium supplemented with 10% FCS, 60 μg/ml kanamycin and 25m*M* N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), pH 7.2. Thereafter, the HBL-38 cells were stimulated with 1 μg/ml of LPS, 10 μg/ml of Con A, 10 μg/ml of PHA-P for 48 hr or 100 HA/ml of HVJ for 24 hr at 37°.

Surface Marker Analysis of HBL-38 Cells The presence of surface antigens reacting with monoclonal antibodies such as OKM-1 (Ortho Diagnostic Systems Inc. NJ), My 9 (Coulter Immunology, FL), and Leu M5 and Leu 4 (Beckton Dickinson Immunocytometry Systems, CA), was investigated by the indirect immunofluorescence method using fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG. Surface membrane immunoglobulins (SmIg) were investigated by the direct immunofluorescence method using FITC-conjugated goat anti-human Igs. Sheep erythrocytes, antibody-sensitized bovine erythrocytes and complement-sensitized bovine erythrocytes were used for the E, EA and EAC rosette formation, respectively.

Purification of IFN-γ The cell-free supernatant was concentrated with an ultra-filtration membrane module (Asahi Chemical Co. Ltd., Tokyo, AIP-3013, 6,000 cut-off). The concentrate was loaded onto an anti-IFN-γ monoclonal antibody column (Celltech, RESELUTE-γ) equilibrated with 50m*M* Tris-HCl and 0.5*M* NaCl, pH 7.5. After washing of the column with 0.1*M* Tris-HCl and 1*M* NaCl, pH 8.0, the IFN-γ was eluted with 0.1*M* cyclohexyl aminopentanesulfonic acid (CAPS) and 1*M* KCl, pH 10.7. The eluate was immediately neutralized with 0.1 volume of 2*M* Tris-HCl, pH 7.5, and the fractions containing IFN-γ activity were pooled.

IFN Assay Antiviral activity of IFN was assayed in terms of the inhibition of the cytopathic effect of Sindbis virus on FL cells. IFN-α, IFN-β and IFN-γ activities were estimated in the presence of anti-IFN-β antiserum + anti-IFN-γ antiserum, anti-IFN-α antiserum + anti-IFN-γ antiserum and anti-IFN-α antiserum + anti-IFN-β antiserum, respectively. Titers of IFN-γ were expressed with reference to the NIH IFN-γ standard (Gg23-901-530).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Electrophoresis was performed as described by Laemmli.[18] The gel was stained with either Coomassie Brilliant Blue (CBB) or periodic acid-Schiff (PAS) as described by Fairbanks *et al.*[19]

Separation of Subspecies of Purified IFN-γ Purified IFN-γ was loaded onto a reversed-phase HPLC column (Bio-Rad, CA, RP-318) in 0.5*M* ammonium acetate, pH 7.0, and eluted with a linear gradient (0–60%) of dioxane in 0.5*M* ammonium acetate. The peaks were collected.

Tryptic Peptide Mapping of Subspecies of IFN-γ Tryptic digestions were performed in 0.1*M* $NaHCO_3$, pH 8.5, at 37° for 15 hr by using 2% L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma, MO). The resultant peptides were separated on a reversed-phase HPLC column (Bio-Rad, RP-318) in 0.1% trifluoroacetic acid (TFA) and eluted with a linear gradient (0–45%) of acetonitrile in 0.1% TFA. The peaks were collected.

Cleavage of Pyroglutamate Residues of Blocked N-Terminal Peptide The blocked N-terminal peptide was treated with 1*N* NaOH at 100° for 20 min and neutralized. The neutralized peptide was purified on a reversed-phase HPLC column (Bio-Rad, RP-318) with TFA and acetonitrile as described above.

Determination of Amino Acid Sequences Automated Edman degradation of peptides was performed with a gas phase protein sequenator (Applied Biosystems Inc., CA, 470A). The resulting phenylthiohydantoin derivatives of amino acids were then identified on a reversed-phase HPLC column (Senshu Scientific Co. Ltd., Tokyo, Senshu Pak SEQ-4).

Construction and Screening of a cDNA Library Poly(A)$^+$ RNA was prepared from LPS-stimulated HBL-38 cells, and purified by sucrose gradient centrifugation as described by Weening *et al.*[20] The cDNA was synthesized as described by Gubler and Hoffman.[21] The cDNA obtained was inserted into the pUC9 by G-C tailing and transformed into *E. coli* JM83. Recombinant plasmids containing IFN-γ cDNA were identified by a colony hybridization method with synthetic oligonucleotide probes (5′TGGCTGTTACTGCCAGGACCCATATGTA3′, 5′AAACGAGATGACTTCGAAA3′ and 5′CATGAACTCATCCAAGTGA3′).

Determination of Nucleotide Sequence of IFN-γ cDNA The nucleotide sequence of IFN-γ cDNA was determined by the dideoxy chain termination method.[22]

Results

Large-scale Proliferation of HBL-38 Cells In order to obtain large amounts of HBL-38 cells, a human myelomonocytic cell line, we used an *in vivo* cell propagation method with immunosuppressed hamsters. Four weeks after the transplantation of HBL-38 cells into hamsters, 50–60% of the hamsters were still alive and all of the surviving hamsters accepted the cells. The transplanted HBL-38 cells grew to a solid tumor mass (about 15 g) and we could obtain about 2×10^9 cells of HBL-38 cells with 80% viability from one hamster.

Surface Markers of HBL-38 Cells Grown in Immunosuppressed Hamsters As shown in Table I, a large population of HBL-38 cells grown in immunosuppressed hamsters reacted with monoclonal antibodies specific to myelomonocytic cells, such as OKM-1, My 9 and Leu M5, but not with antibody specific to T-cells, such as Leu 4. The cells lacked surface immunoglobulins and were negative for E, EA and EAC rosette formation. These results suggest that HBL-38 cells grown in immunosuppressed hamsters have retained the features of myelomonocytic cells.

Table I. Surface Phenotype of HBL-38 Cells Grown in Immunosuppressed Hamsters

OKM-1	positive
My 9	positive
Leu M5	positive
Leu 4	negative
E rosette	negative
EA rosette	negative
EAC rosette	negative

Table II. IFNs from HBL-38 Cells Stimulated with Various Stimulants

	Interferon activities from HBL-38 cells stimulated with[a]				
	None	HVJ (100 HA/ml)	LPS (1 μg/ml)	ConA (10 μg/ml)	PHA-P (10 μg/ml)
None	1,500	18,000±2,500	38,000±4,000	1,500	1,500
Anti-IFN-α+β	1,350	1,500±200	35,000±3,000	nd[c]	nd
Anti-IFN-β+γ	130	15,500±1,800	1,800±150	nd	nd
Anti-IFN-α+γ	<10	700±100	<10	nd	nd
Anti-IFN-α+β+γ	<10	50±8	<10	nd	nd
pH 2.0 treatment[b]	nd	15,000±1,200	1,500±100	nd	nd

a) HBL-38 cells (5.0×10^6 cells/ml) freshly prepared from tumors grown in hamsters were stimulated with various inducers as described in "Materials and Methods." Forty-eight hours later, the culture supernatants were harvested and assayed for antiviral activities in the presence or absence of antisera.

b) Culture supernatants were dialyzed overnight against 0.1*M* glycine-HCl buffer, pH 2.0, then adjusted to neutral pH and assayed.

c) Not done.

Each value is the mean±SD for 5 experiments.

Induction of IFNs from HBL-38 Cells Table II shows the results of production of IFNs from HBL-38 cells by stimulation with various agents. Even under unstimulated conditions, HBL-38 cells produced 1,500 units/ml of IFNs. In the case of stimulation with LPS or HVJ, the cells produced large amounts of IFNs, 38,000 units/ml or 18,000 units/ml, respectively. However, T-cell mitogens such as PHA-P or Con A did not show any stimulating effect on the production of IFNs from HBL-38 cells.

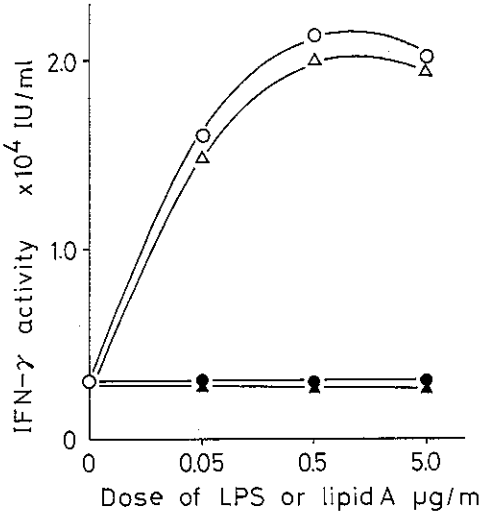

Fig. 1. LPS-dependency of IFN-γ production of HBL-38 cells. HBL-38 cells (5×10^6 cells/ml) were stimulated with the indicated doses of LPS (○, ●) or lipid A (△, ▲) in the presence (●, ▲) or absence (○, △) of polymixin B (10 μg/ml). After 48 hr, produced IFN-γ was measured in the presence of anti-IFN-α and anti-IFN-β antibodies.

By neutralization with antibodies and from the stability at acidic pH, the types of IFNs produced by HBL-38 cells were determined. The cells spontaneously produced IFN-α and IFN-γ. The LPS-stimulated and HVJ-stimulated cells produced mainly IFN-γ and IFN-α, respectively. These results indicate that LPS markedly enhanced the production of IFN-γ from the cells and HVJ enhanced that of IFN-α.

LPS-dependent Enhancement of IFN-γ Production of HBL-38 Cells To ascertain whether LPS is really the enhancer of IFN-γ production by HBL-38 cells, polymixin B (which binds to and inactivates LPS) was added to the cells just before the addition of LPS. Produced IFN-γ was measured in the presence of anti-IFN-α and anti-IFN-β antibodies. Figure 1 shows that the enhancement of production of IFN-γ was dependent on the dose of LPS and that lipid A could replace LPS. Polymixin B (10 μg/ml) completely abolished the enhancing effect of LPS and lipid A on IFN-γ production. These results suggest that LPS plays an important role in the enhancement of IFN-γ production by HBL-38 cells.

Purification and Characterization of IFN-γ Produced by LPS-stimulated HBL-38 Cells IFN-γ produced by LPS-stimulated HBL-38 cells was purified on an ultrafiltration membrane module followed by a monoclonal antibody column. The scheme of the purification and the yield at each step are shown in Table III. Eighty liters of culture supernatant containing 3.0×10^9 IU of IFN-γ activity was prepared from 4.0×10^{11} cells obtained from 200 hamsters bearing solid tumors of HBL-38 cells. The IFN-γ was purified with a high

Table III. Purification Scheme for IFN-γ of HBL-38 Cell Origin

Purification step	Volume (ml)	Total protein[a] (mg)	Total act. (IU)	Yield (%)	Specific act. (IU/mg)
Culture supernatant	80,000	281,000	3.0×10^9	100	1.1×10^4
Concentrated supernatant	15,000	214,000	2.4×10^9	80	1.1×10^4
RESELUTE-γ eluate	50	158	1.6×10^9	53	1.0×10^7

a) The protein concentration was determined by Bio-Rad dye protein assay with human serum albumin as the standard.

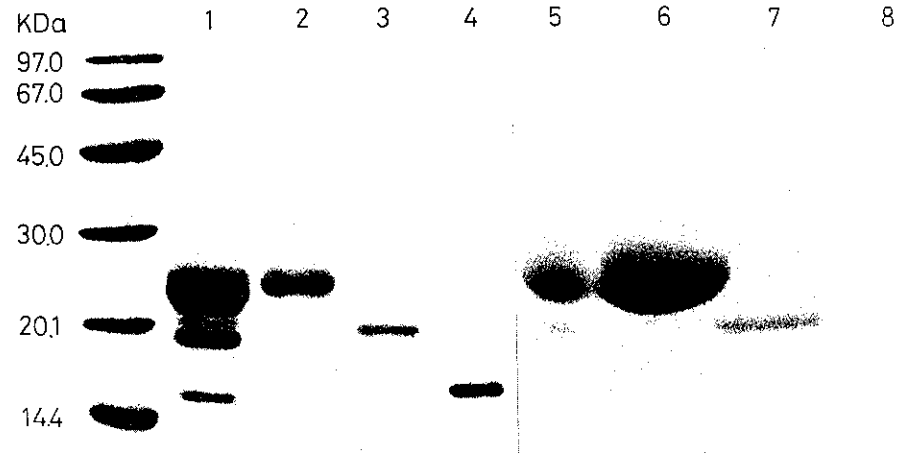

Fig. 2. SDS-PAGE analyses of purified IFN-γ from HBL-38 cells. Separated subspecies of IFN-γ from HBL-38 cells were analyzed on a 12.5% polyacrylamide gel. Lanes 1–4, stained with CBB; lanes 5–8, stained with PAS. Lanes 1 and 5, unseparated IFN-γ; lanes 2 and 6, separated 25 kDa; lanes 3 and 7, separated 20 kDa; lanes 4 and 8, separated 17 kDa subspecies. Molecular weight markers used were phosphorylase *b* (97.0 kDa), bovine serum albumin (67.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

recovery of 53% and it had a high specific activity of 1×10^7 IU/mg.

The gel permeation HPLC pattern of the purified IFN-γ showed that the apparent molecular weight was 40–45 kDa (data not shown). On the other hand, as shown in Fig. 2, analyses on SDS-PAGE showed that IFN-γ consisted of three subspecies with molecular weights of 25, 20 and 17 kDa. These results suggest that IFN-γ produced by LPS-stimulated HBL-38 cells exists in a dimeric form in non-denaturing solution. PAS staining analyses of the SDS-PAGE revealed that 25 and 20 kDa, but not 17 kDa, subspecies are glycosylated (Fig. 2, lanes 5–8).

Determination of Primary Structure of IFN-γ Produced by LPS-stimulated HBL-38 Cells

The three subspecies were separated on a reversed-phase HPLC column and their primary structures were analyzed. The N-termini of these subspecies were considered to be blocked because automated Edman degradation gave no signals for the intact proteins. The complete amino acid sequence of IFN-γ derived from HBL-38 cells was deduced from the sequence of cDNA in a cloned plasmid pUγ853 and checked by tryptic mapping analyses of the three subspecies. The results may be summarized as follows:

1) The complete nucleotide sequence of the cDNA (data not shown) and the amino acid sequence deduced from the cDNA were identical to those in the case of IFN-γ from normal human PBL reported by Gray *et al.*[9] and Rinderknecht *et al.*[23] including two possible Asn-X-Ser/Thr N-glycosylation sites.

2) The tryptic maps of the three subspecies (Fig. 3) and amino acid sequence analyses of the tryptic peptides (Fig. 4) revealed that all three subspecies have an identical amino acid sequence. The 25 kDa subspecies was glycosylated on both the 25th and the 97th Asn, the 20 kDa subspecies was glycosylated only on the 25th Asn and the 17 kDa subspecies was not glycosylated. The results on glycosylation are in agreement with those of SDS-PAGE analyses by PAS staining.

3) The N-termini of the three subspecies were blocked by pyroglutamate, because the automated Edman degradation gave no signals for tryptic peptide No. 18, but the amino acid sequence was determined as Glu-Asp-Tyr-Val-Lys after alkaline treatment of the peptide.

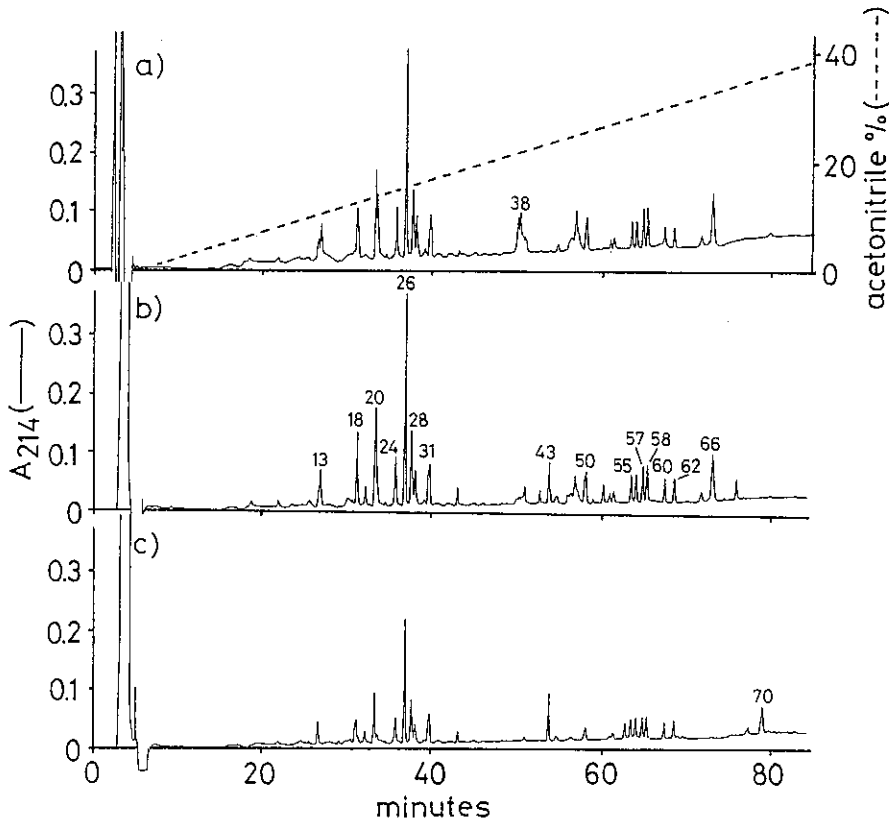

Fig. 3. Tryptic peptide maps of subspecies of IFN-γ. Tryptic peptides of each subspecies of IFN-γ were separated on a reversed-phase RP-318 column with a gradient of acetonitrile. a), 25 kDa subspecies; b), 20 kDa subspecies; c), 17 kDa subspecies.

These results are in agreement with those described by Rinderknecht *et al.*[23] and Kelker *et al.*[24]

From the results described above, we concluded that myelomonocytic HBL-38 cells could produce large amounts of IFN-γ, identical to that of normal human PBL, on stimulation with LPS.

Discussion

In this paper, we have shown that human myelomonocytic HBL-38 cells produced large amounts of IFN-γ when stimulated with LPS. In general, it has been considered that IFN-γ is a product of T-lymphocytes stimulated with antigens or T-cell mitogens, such as PHA-P or Con A.[12] However, the results of surface marker analyses of HBL-38 cells grown in immunosuppressed hamster revealed that HBL-38 cells belong to the category of myelomonocytic cells, but are not T-cells.

Recently, Le *et al.*[13] and Blanchard *et al.*[14] reported that LPS could induce IFN-γ production in human PBL and mouse splenocytes but not purified T-cells. They considered that interleukin 1 (IL-1) and interleukin 2 (IL-2) were induced from monocytes contained in PBL or splenocytes by stimulation with LPS, and that IL-1 and IL-2 played an important role in the production of IFN-γ. In our case of HBL-38 cells, it remains to be examined whether some soluble factors, such as IL-1 and/or IL-2, produced by LPS-responding cells are involved in the enhancement of IFN-γ production.

As shown in Table II, HBL-38 cells produced different types of IFN in response to different stimulants. LPS-stimulated HBL-38

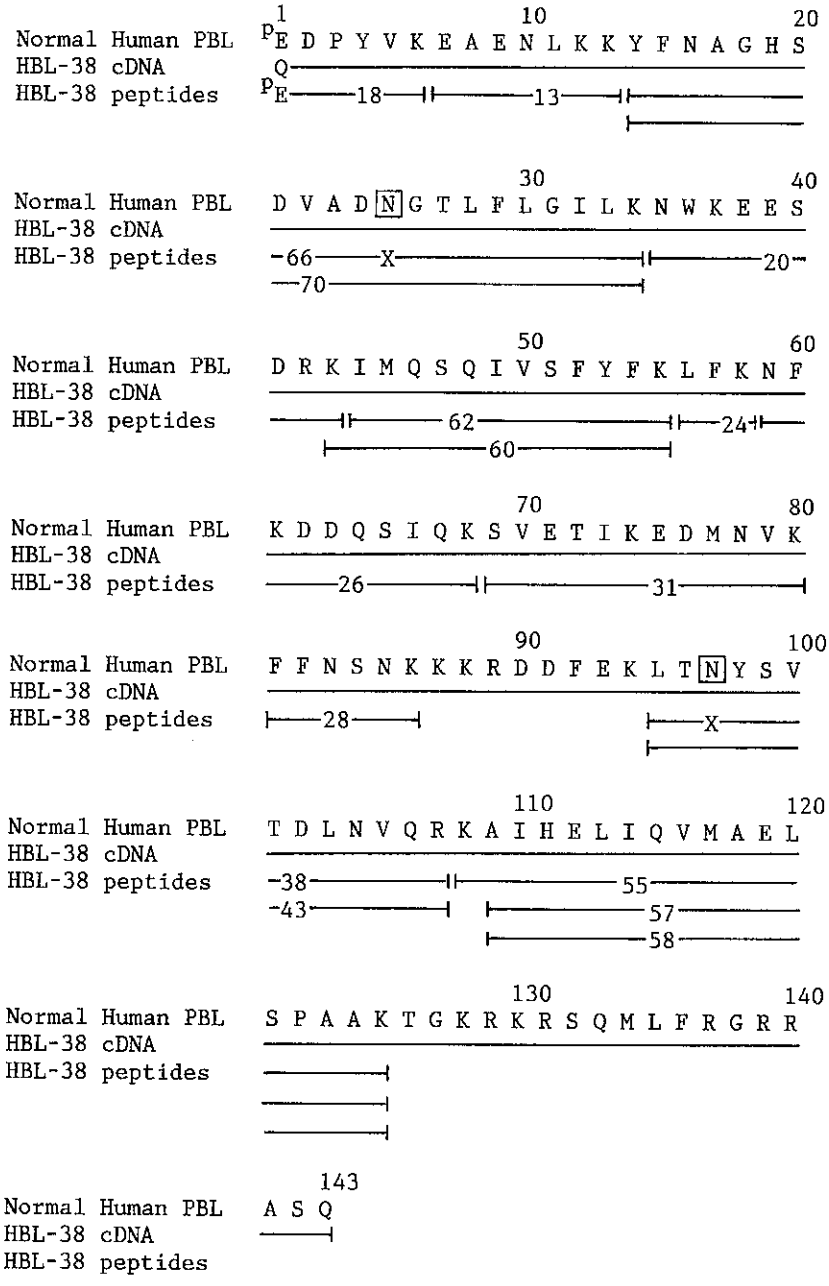

Fig. 4. Amino acid sequence of IFN-γ from normal human PBL and from HBL-38 cells. Each amino acid is expressed in one letter form. The amino acid sequence of IFN-γ from normal human PBL was reported by Rinderknecht *et al.*[23] The complete amino acid sequence of IFN-γ from HBL-38 cells was deduced from the sequence of IFN-γ cDNA in plasmid pUγ853. Tryptic peptides are indicated by lines and the numbers correspond to the peak numbers in Fig. 3. ^{p}E(Glu) represents pyroglutamate. X indicates a residue unidentified by automated Edman degradation. The squares enclosing N(Asn) indicate possible N-glycosylation sites.

cells predominantly produced IFN-γ and HVJ-stimulated cells mainly produced IFN-α. At the present time, we do not know whether the same cell clone produces IFN-γ and IFN-α in response to different stimuli. This problem will require further study.

The IFN-γ derived from human myelomonocytic HBL - 38 cells had the same physicochemical characteristics and primary structures as those of natural IFN-γ derived from normal human PBL.[23] The natural human IFN-γ exists as a dimeric form with an apparent molecular weight of 45 kDa and consists of three subspecies of 25, 20 and 15.5 kDa. The first and the second were glycosylated.[24] These data were in agreement with our results. The nucleotide sequence of cDNA for IFN-γ from HBL-38 cells was identical to that of cDNA of IFN-γ from normal human PBL.[9] Further, the amino acid sequence was identical to that of natural IFN-γ of normal human PBL origin.[23] Therefore, we conclude that IFN-γ derived from HBL-38 cells is identical to that of normal human PBL origin.

Recently, other investigators have purified recombinant human IFN-γ derived from CHO cells and determined its carbohydrate sequence.[25] In the case of natural human IFN-γ, however, the carbohydrate sequence remains undetermined due to limited availability. We have previously established a large-scale *in vivo* cell propagation method for human cells and a mass production system to obtain natural human IFN-α[15] and tumor necrosis factor[16, 17] from human cells. By the same method, we have obtained a large amount of natural human IFN-γ. This should facilitate detailed analysis of the structure and biological functions of natural human IFN-γ. Further studies on the sequence determination and biological significance of the carbohydrate moieties of natural human IFN-γ are proceeding in parallel with a comparison of biological activities and conformations between natural and recombinant human IFN-γ.

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