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Evidence for IFN-β Heterogeneity in a Substrain of Namalwa Cells

H. BORG, I. NORDENSSON, T. NY, AND E. LUNDGREN*

Unit for Applied Cell and Molecular Biology, University of Umeå, S-901 87 Umeå, Sweden

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ABSTRACT

A substrain of Namalwa cells, denoted substrain B, was grown in fermentors up to the 100-L scale and was induced with Sendai virus to produce interferon (IFN). The titer of the crude IFN varied extensively between different batches; part of the variation was caused by a differential expression of IFN-α and IFN-β. More than 80% of the IFN activity was IFN-β by several criteria. A two-step purification procedure was developed and the resulting preparation had a specific activity of approximately 10^6 U/mg protein. The IFN-β type was found to be heterogenous, and could be separated into several components, which probably represented post-translational modifications of one molecule.

Index Entries: Lymphoid cells, IFN-β heterogeneity in Namalwa interferon, heterogeneity of; IFN-α, in Namalwa cells; IFN-β, in Namalwa cells; IFN heterogeneity, in Namalwa cells; production of IFN, in Namalwa cells; FPLC-separation of IFN(s).

INTRODUCTION

Interferons (IFN) comprise a family of proteins, serologically defined as IFN-α, IFN-β and IFN-γ. Of these, IFN-α is made up of several members. The variability is caused by gene duplications, but an allelic variation has also been suggested (*1,2*). No accepted nomenclature exists concerning the IFN-α subtypes.

*Author to whom all correspondence and reprint requests should be addressed.

Human leukocyte interferon (IFN) produced by virus infection of primary cultures of white blood cells obtained from blood transfusion represents a mixture of several IFN-α types with IFN-α_1 (IFN-α D) and IFN-α_2 (IFN-α A) as the predominating species (*1,2*). One alternative source has been continuous lymphoid cell lines, which can be propagated at the large-fermentor scale. The most extensively studied cell line is the B-cell line, Namalwa, derived from a patient with Burkitt's lymphoma and carrying the EBV genome (*3*). Several laboratories are producing Namalwa IFN in pilot plant scale (*4–8*) and also some reports on the clinical use of Namalwa IFN (e.g., *9,10*) have appeared.

Namalwa cells produce several species of IFN-α (*11*), but also IFN-β, calculated to be about 15% of the functional activity after Sendai virus induction (*12*). The Namalwa cell line used in this study (designated substrain B) produced highly different amounts of IFN, when induced with Sendai virus. The fluctuating titer in the crude IFN turned out to arise from the constant but low production of IFN-β and a highly variable amount of IFN-α ranging from not measurable up to more than 70% of the activity. The purification method used turned out to favor enrichment of IFN-β. Since several authors have reported that IFN-β is or might be heterogeneous (*13–15*), we decided to study the IFN-β component in more detail. Our semipurified Namalwa IFN preparation, originally aimed for clinical tests, was used as a starting material. Not only IFN-α was heterogeneous, but also IFN-β, which could be separated into several components.

MATERIALS AND METHODS

Growth of Namalwa Cells

Fermentors of 7 and 40 L, Novo Paljas, were obtained from Contact-Roestvrijstaal b.v., Ridderkerk, the Netherlands. A 100-L fermentor was used in some experiments (Chemoferm AB, Stockholm, Sweden). The fermentors were equipped with automatic controls for pH, temperature, and oxygen saturation, which parameters also were individually recorded.

Namalwa cells were grown at 37°C in RPMI 1640 medium supplemented with antibiotics and 10% fetal calf serum. From batch number 77 on, 4% (vol/vol) PEG (polyethyleneglycol) 6000 precipitated human plasma (*16*), insulin, and transferrin (2.5 μg/mL each) replaced fetal calf serum. Namalwa cells were purchased from Flow Laboratories, Irvine, Scotland. One Namalwa line, here called substrain B, was obtained from Dr. Alice Adams, Karolinska Institutet, Stockholm, Sweden. The karyotype with an 8;14 translocation, a marker chromosome derived from chromosomes 1 and 10, trisomy-7, monosomy-3, and a 15 q deletion was identical with the description by Klein et al. (*5*) and occurred in both cell lines. Some minor deviations were seen in some karyotypes, but

they were not consistent. The immunoglobulin expression (IgM, kappa) was the same as in the original Namalwa line (*5*).

Each fermentor run was initiated by thawing an ampule of frozen cells. When cells reached a density of 2–3 × 10^6 cells/mL, they were diluted by fresh medium to 2 × 10^5 cells/mL in progressively larger vessels. For larger volumes (i.e., 7 up to 100 L), fermentors were used.

Production of Interferon

At final cell density (2–3 × 10^6 cells/mL), 7 or 50 L of Namalwa cells were primed for 2 h with 100 U/mL with purified leukocyte IFN (1 × 10^6 U/mg protein). Sendai virus was then added.

After 2 h the cells were diluted by the same volume of plasma-free medium and the temperature was reduced to 35°C. IFN-containing supernatant was collected 24 h after induction by filtration through a 1.2 μm filter (Gelman, Ann Arbor, Mich, USA).

Viruses and Interferon Preparations

Sendai virus was originally kindly obtained from K. Cantell, Helsinki, Finland. The virus was grown in allantoic cavity of 11-d-old embryonated eggs and harvested after 3 d at 33°C. Each virus batch was tested for optimal IFN production before use. The virus was stored at −80°C until used, usually within 3-8 wk.

An IFN-β preparation (C-411) with a specific activity of 10^6 U/mg protein was obtained from Y.H. Tan, Calgary, Canada. IFN-α from buffy coat leukocytes (PIF) with a specific activity of 10^6 U/mg protein, was obtained from K. Cantell, Helsinki, Finland, or produced by standard procedures from human buffy coats.

Assays

IFN titration was performed by a modified method described by Havell and Vilcek (*17*) using VSV as challenge virus. Bovine MDBK cells, human Wish amnion cells, human amnion U cells, or human embryonic fibroblasts (HEL) for the assays. IFN was calibrated against the international reference preparation 69/19. The IFN-β preparation gave a 300-fold lower activity on MDBK cells than on Wish cells, which allows for the calculation of the proportions of IFN-α and IFN-β by measurements of the activity on both cell lines, considering that practically all activity measured on MDBK cells is IFN-α only. Wish or HEL displayed similar sensitivities to both IFN-α and IFN-β.

Protein concentration was assayed by equating the absorbance at 280 nm of 1.0 = 1.0 mg of protein/mL.

Anti-Interferon Sera

A sheep antiserum against IFN-α (Liivar) (*20*) with a neutralizing titer of 1/450,000 against IFN-α and 1/3000 against IFN-β was obtained as a generous gift from K. Cantell. A rabbit antiserum against IFN-β (EBAB 4640), with a neutralizing titer of 30 U/mL against IFN-α and 300,000 U/mL against IFN-β, was purchased from Enzo Biochem Inc., New York, NY, USA. Neutralizing titer of the antisera was assayed according to Kawade (*21*) on MDBK cells against IFN-α (PIF) and on HEL cells against IFN-β (C411). In samples where a 1000-fold excess of antiserum against IFN-α tested on HEL cells did not neutralize all activity, the presence of IFN-β was demonstrated in the following way. Graded amounts of anti IFN-β antiserum was added in the presence of a 1000-fold excess of anti IFN-α antisera, and the remaining titer was determined on HEL cells. The proportion of IFN-β was estimated by titration on HEL cells in the presence of a 1000-fold excess of anti IFN-α antibody.

Purification of Interferon

Batch Adsorbtion to CM-Sephadex C-50

To 100 L of crude IFN, 2.25 L 2*M* sodium acetate was added to give a final concentration of 0.044*M* sodium acetate. The pH was adjusted to pH 4.8 with about 950 mL concentrated acetic acid. An 800 g quantity of preswollen and equilibrated CM-Sephadex C-50 (Pharmacia, Uppsala, Sweden) was added and the crude IFN was stirred for 2 h at +4–6°C in the cold room. The CM Sephadex C-50 gel was allowed to sediment and the supernatant was then sucked off. About 5 L of supernatant and the ion exchanger was transferred to a Büchner funnel and washed separately with 0.05*M* sodium acetate, pH 4.8. The washed ion exchanger was packed into a column, 4.4 × 60 cm, further washed with 0.05/*M* sodium acetate, pH 4.8, and 0.1*M* sodium acetate 0.15*M* NaCl, pH 5.4. The IFN activity was then desorbed with a buffer containing 1*M* ammonium acetate and 0.014*M* sodium phosphate, pH 7.2. The volume of the eluate was normally about 2 L. The IFN activity was precipitated with ammonium sulfate, 490 g/L, for 20 h. The precipitate was collected by centrifugation at 4000*g* for 45 min, dissolved in about 90 mL 0.5*M* ammonium acetate, and dialyzed against 0.5*M* ammonium acetate for 5 h.

Gel Filtration

The concentrated CM-Sephadex eluate was gel filtrated on Ultrogel AcA 54 (LKB, Bromma, Sweden), as detailed later in the legend to Fig. 2. Fractions with IFN activity were pooled and initially lyophylized and later on concentrated by ammonium sulfate precipitation (490 g/L). The precipitate was dissolved in 0.014*M* phosphate buffer, pH 7.2, 0.8% NaCl, 1.5 mg human albumin/mL, and dialyzed against the same buffer without albumin for 20 h. The IFN solution was filtered through 0.22 μm filter and put into ampules, 3×10^6 U/ampule in 2 mL.

TABLE 1
IFN Titer Obtained in MDBK and HEL Cells from Namalwa Cells and Namalwa Substrain B cells[a]

Preparation	Titer, U/mL		Proportion, %	
	MDBK	HEL	IFM-α	IFN-β
PIF	10,000	10,000	100	<1
C-411	25,000	8,000,000	<1	100
Namalwa[b]	5,800	6,700	87	13
Substrain B[b]	125	3,000	4	96
TV 81-4[c]	675,000	3,600,000	19	81

[a]For comparison reference preparations of IFN-α (PIF) and IFN-β (C-411) are included.
[b]The figures represent the mean of assays performed on six independent 1-L cultures.
[c]Partially purified IFN from substrain B with a specific activity of 10^6 U/mg protein.

Other Separation Methods

The SDS-PAGE gel electrophoresis was performed according to the method of Laemmli (*19*), with a 15% acrylamide separating gel. Reference molecular marker proteins were purchased from Pharmacia (Uppsala, Sweden). Ion exchange chromatography was performed on the FPLC system, Pharmacia, using the cation exchange column mono-S. The FPLC system and isoelectric focusing on granulated Sephadex (LKB, Bromma, Sweden) were performed according to the manufacturers' instructions.

RESULTS

Production of IFN

Table 1 shows the IFN produced by the original Namalwa cells compared to substrain B on a small laboratory bench scale. Taking the difference in sensitivity for IFN-α and IFN-β into account for the two test cell lines as described in Materials and Methods, the proportion of IFN-β can be calculated to be 13%, well in agreement with published figures (*12*). Substrain B on the other hand has an IFN-β proportion of 96% in the experiment shown in the table. In more than 20 small-scale experiments during a time period of 2 yr, an IFN-β proportion of more than 80% was always found.

The cumulative results from fermentors 10 to 160 are shown in Fig 1. The mean yield was 2300 U/mL. However, a considerable variation was noted ranging between 300 and 40,000 U/mL. When high and low yield batches were compared, the proportion of IFN-α shifted from nondetectable levels of IFN-α to more than 70% in certain batches, as

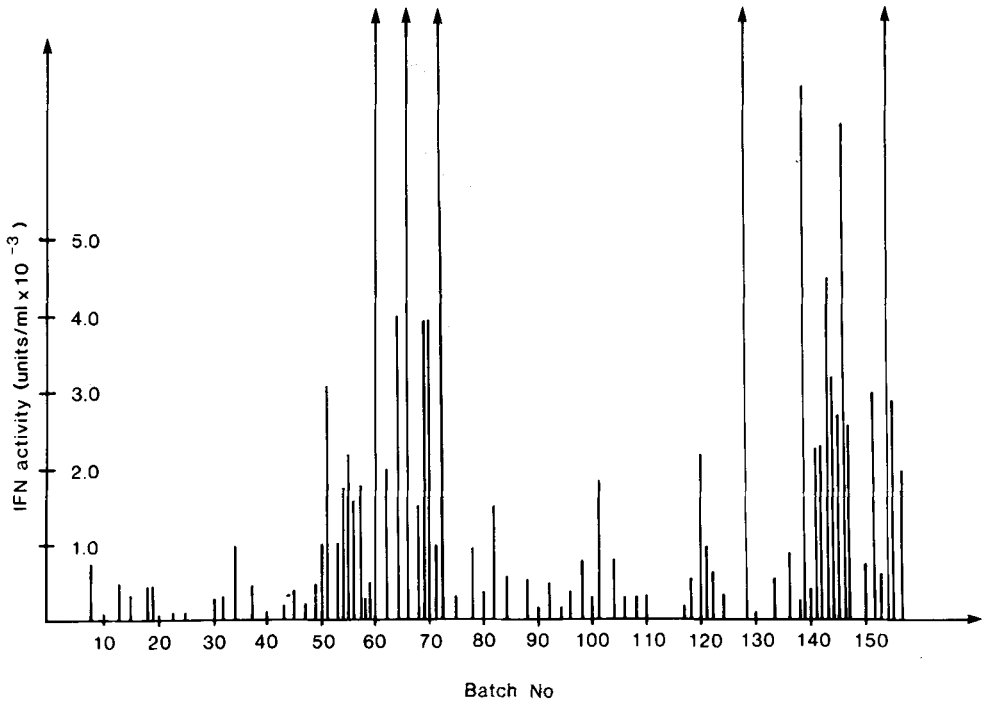

Fig. 1. IFN Production from Namalwa cells, substrain B, in 160 consecutive fermentors. The bars represent means of three independent determinations in triplicate. The cells were grown, primed, and induced with virus, as indicated in the Materials and Methods section. Bars with activity less than 100 U/mL are omitted.

exemplified in Table 2. The reason for this fluctuation was not understood, thus it was not further explored. However, it affected yield upon batch adsorption to CM-Sephadex, as IFN-β was completely absorbed in contrast to IFN-α.

Partial Purification of Namalwa IFN

For a more careful biochemical characterization batches 144-152 were pooled and partially purified to give a preparation designated TV 81-4. A

TABLE 2
Comparison of IFN-α and IFN-β Content in Selected Batches

	Titer, U/mL		Estimated titer		IFN-β,
Batch	MDBK	HEL	IFN-α	IFN-β	%
140	250	3500	280	3220	92
143	6000	12000	6000	6000	50
153	6000	9000	6030	2970	33

TABLE 3
Partial Purification of IFN Produced by Sendai Virus-Infected Substrain B Namalwa Cells[a]

Purification Step	Volume, L	Total activity, U × 10^6	Spec. act., U/mg	Purific. factor	Yield, % Step	Yield, % Acc.
Crude Namalwa IFN	100	309	0.01	1	100	100
CM-Sephadex	2	203	0.06	6	66	66
Ammonium sulfate precipitation	0.04	130	0.33	33	64	42
Gel filtration	0.35	72	0.8	80	55	23

[a]The figures represent a mean of 13 consecutive batches, 144–152.

general outline of the procedure used is shown in Table 3. The final yield was approximately 25%, and specific activity around 10^6 U/mg protein. The procedure can be detailed as follows.

In pilot tests it was found that most of the IFN was bound to the ion exchanger after 30 min and after 2 h no additional binding could be achieved. To avoid losses of activity, washing of the CM-Sephadex C-50 gel had to be performed within about 5 h after batch adsorbtion had been completed.

The CM-Sephadex eluate was concentrated by precipitation with ammonium sulfate according to Materials and Methods. The chromatographic pattern is shown in Fig. 2, showing a broad peak in the MW range of 15,000–30,000 daltons. The main contaminants were high molecular weight proteins. SDS-polyacrylamide gel electrophoresis under reducing conditions confirmed that the molecular weight of the IFN produced varied between 15,000 and 30,000 (not shown).

Characterization of Partially Purified Namalwa IFN

Isoelectric focusing of TV 81-4 gave four main peaks with IFN activity at PI 4.7, 5.2, 6.1, and 7.3 (Fig. 3). The two lower PI peaks seemed to be mainly IFN-α, according to the antibody neutralization data shown in Table 4. The two higher PI peaks could not be quantitated on MDBK cells. These components were more sensitive to the anti-IFN-β serum, measured on Wish cells, than the two low PI components. Thus, the two low PI components were IFN-α, whereas the high PI components were IFN-β. The proportion of IFN-α in isoelectric focusing of TV 81-4 (Fig. 3) was calculated to be 32%, which is in reasonable agreement with the proportion of 19% calculated in TV 81-4 (Table 1) based on the differential sensitivity of the cell lines used.

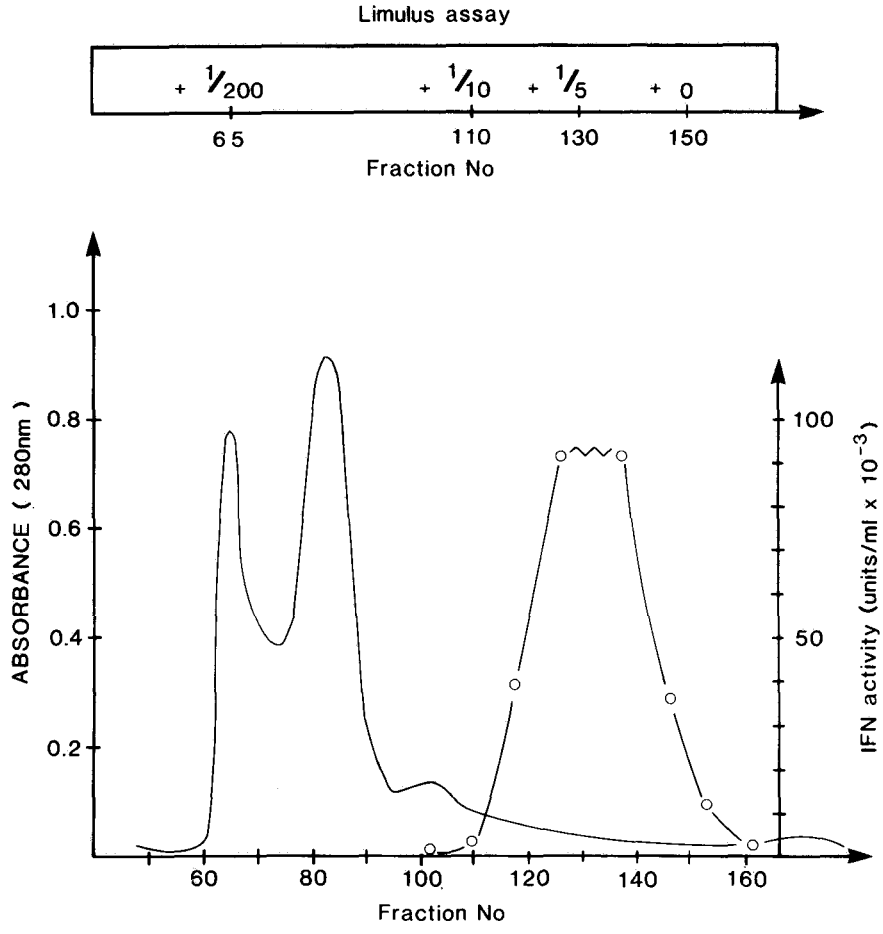

Fig. 2. Gel filtration of CM-Sephadex purified and ammonium sulfate-precipitated Namalwa IFN. Greatest positive Limulus titer in marked fractions are indicated. Column size: 5.0 × 87 cm; Gel: Ultrogel AcA-54; Buffer: 0.5*M* ammonium acetate; Sample volume: 45 mL. Fraction size: 10 mL. Flow rate: 98 mL/h.

Separation of Several IFN-β Components from Partially Purified Namalwa IFN

TV 81-4 was chromatographed on FPLC mono-S at pH 4.6 (Fig. 4). IFN activity from each fraction was analyzed on both HEL and MDBK cells. Three peaks could be detected; two of these were not possible to measure on MDBK cells. Their IFN-β character was confirmed as the activity was completely blocked by the antiserum directed against IFN-β (not shown). These peaks comprised 84% of the activity, again in good agreement with the data on TV 81-4 in Table 1.

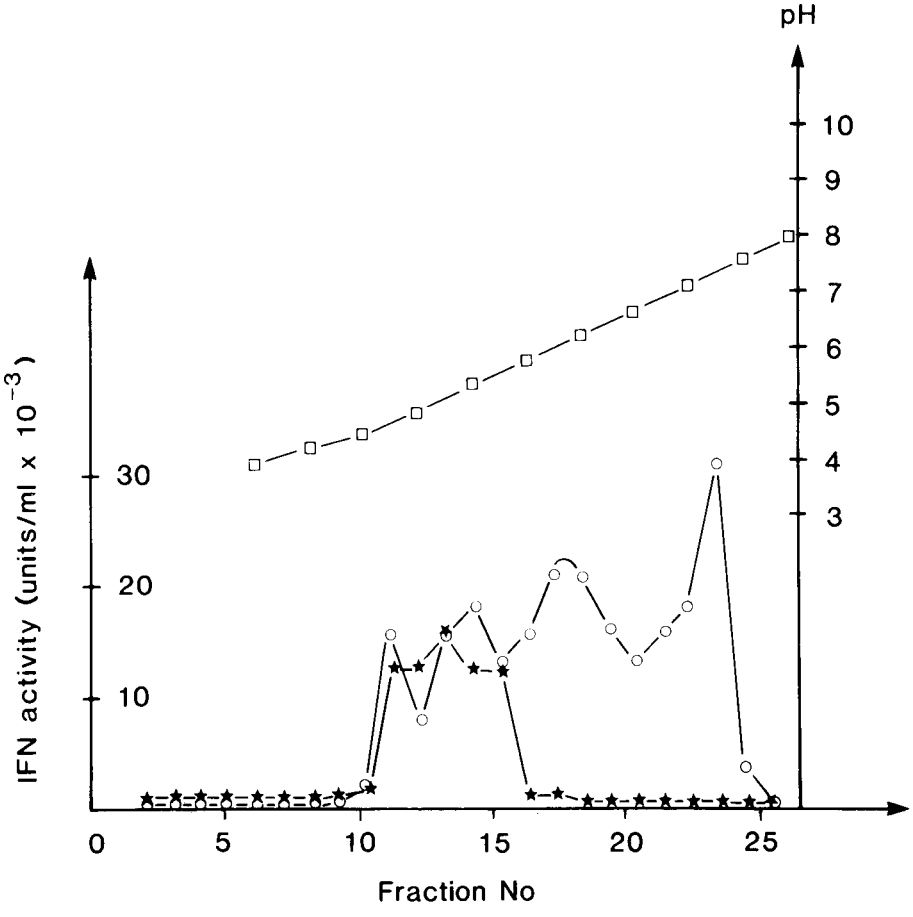

Fig. 3. Preparation flat-bed electrofocusing, pH 3.5–9.5, of partially purified Namalwa IFN. Sample: TV 81-4, 2 mL, 1.4×10^6 IU. (★) IFN-α (titrated on MDBK cells); (○) IFN-β + IFN-α (titrated on HEL cells); (□) pH.

TABLE 4
Effects of Neutralizing Antisera on Fractions Obtained from Flat-Bed Isoelectric Focusing

PI	MDBK, Anti IFN-α	Wish	
		Anti IFN-α	Anti IFN-β
4.7	<1[a]	<12.5	100
5.2	<1	<12.5	100
6.1	—[b]	75	<1
7.3	—	100	<1

[a]Figures represent the percent remaining activity for the determination of neutralizing titer; *see* Material and Methods.

[b]No activity, *see* Fig. 3.

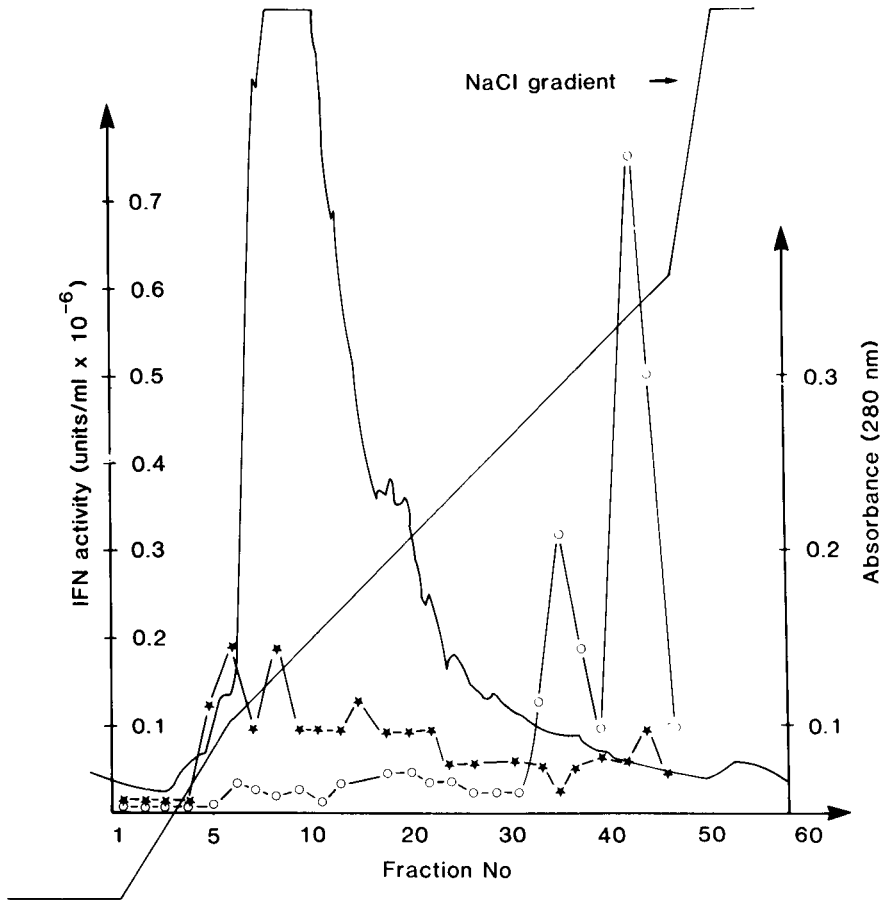

Fig. 4. Separation of partially purified IFN on FPLC mono-s. Sample TV 81-4, 2.3 mL, 1.4 × 106 IU. Flow rate 2.0 mL/min. Eluent 0.05*M* Na-acetate with NaCl gradient 0–1*M*, pH 4.6. (★) IFN-α (titrated on MDBK cells); (○) IFN-α + IFN-β (titrated on HEL cells); NaCl gradient.

DISCUSSION

The cell line used, Namalwa, has been employed by several groups for large-scale production of IFN and is known to produce mainly IFN-α, with a minor fraction of IFN-β, amounting to 15% after induction with Sendai virus (*12*). It has been reported to contain one molecular species with a molecular weight of 17,000 using only Sendai virus as an inducer, while BrdUrd pretreated Namalwa cells contained several IFN-α species ranging in molecular weight between 15,000 and 24,000 (*22*), which is the range of the IFN-α values seen in this line.

Substrain B used in this study differed from the original cell line by its high production of IFN-β. However, its identity seems to be estab-

lished by karyotyping, thus showing that it shares the immunoglobulin expression with the original line.

We here show in the Sendai virus-induced Namalwa substrain B that the fluctuating titer in the crude IFN probably depends on a low and rather constant titer of IFN-β and a variable IFN-α titer. The IFN-α and IFN-β content was determined by titration on homologous and heterologous cells and after separation by inhibition with antisera against IFN-α and IFN-β.

Namalwa cells have by now a long in vitro culture history and substrain heterogeneity is well known to occur for long-term cultured cell lines. A variant of Namalwa cells described by Shuttleworth et al. (*23*) could be an example of this phenomenon. This cell line transcribed the IFN-β gene, but no translation product was detectable.

Several reports have appeared in the literature concerning IFN-β heterogeneity. Sagar et al. (*24*) have proposed the existence of several IFN-β genes based on mRNA size heterogeneity; however, this has not been confirmed by others (*25*). A cDNA library from substrain B made in our laboratory contained eight clones hybridizing to an IFN-β probe under stringent conditions (*26*). Sequencing has revealed all of them to be identical with IFN-β.

Therefore, we conclude that only one IFN-β gene product was found in substrain B. Our result that at least two species of IFN-β was found in the used Namalwa cell line is based on the separation on FPLC mono-S combined with neutralization with specific antisera. Billeau et al. (*15*) have presented evidence for at least two IFN-β species in the MG 63 osteosarcoma cell line by Zn-chelate chromatography. In preliminary experiments, the Namalwa IFN-β component showed similar behavior.

Since the results from the cDNA studies mentioned above point to only one IFN-β gene product, the only explanation for the IFN-β heterogeneity seems to be a post-translational modification of the protein, which is presently under study in our laboratory. Since IFN-β has special pharmacokinetic properties (*27*), limiting its clinical use for systemic administration, the impact of the naturally occurring modifications of IFN-β on clinical efficacy should be explored. It could be added that serum levels more than 100 U/mL of IFN were detected after intramuscular injection of Namalwa substrain B IFN preparations.

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