SDS–Polyacrylamide Gel Electrophoresis of Purified Human Leucocyte Interferon and the Antiviral and Anticellular Activities of the Different Interferon Species

(Accepted 16 May 1980)

SUMMARY

Human leucocyte interferon (HuLeIF) was purified by a series of techniques involving precipitation, gel filtration, Cu-chelate-, blue dextran- and antibody-affinity chromatography. The two major species of HuLeIF were identified in SDS-PAGE as two clearly separable and stainable proteins representing 85% of the biological activity. Three more species of HuLeIF representing 15% of the biological activity were also demonstrated. The specific activity of pure interferon proteins was approx. 10⁸ IFU/mg protein. Recovery was about 50%, and the purification factor exceeded 350000. All five species of HuLeIF had definite anticellular activities when tested with Daudi cells (inhibition of thymidine uptake).

It is well known that interferons, in addition to inducing an antiviral state, have other effects on cells, including the so-called anticellular effects (Paucker et al. 1962; Stewart II, 1979a). Complete purification of all species of human leucocyte interferon (HuLeIF) without the use of any chemical modification procedures (Stewart II et al. 1978) or hydrophobic selection procedures (Rubinstein et al. 1979) should make it possible to determine whether the various biological effects attributed to preparations of different purity are due to all types of interferon molecules. For example, Dahl & Degre (1975) and Dahl (1977) reported that partially purified HuLeIF could be separated into an anticellular fraction (with no antiviral activity) and an antiviral fraction (with no anticellular activity). In contrast, Stewart II et al. (1976) argued that the two activities were always carried by the same (interferon) molecules.

We report here on the purification of five species of HuLeIF in their native form with a high final recovery (50%). The preparations were homogeneous by SDS-PAGE. The five species were also assayed for anticellular activity, measured as inhibition of thymidine uptake of Daudi cells (Hilfenhaus et al. 1976; Heron & Berg, 1978).

Interferon was titrated in a micro-assay system using Vero cells and vesicular stomatitis virus (VSV) as the challenge virus (Berg, 1977). Results were expressed in international interferon units (IFU) in terms of the international reference human leucocyte preparation 69/19 B. Anticellular activity was determined by incubating 4 × 10⁴ Daudi cells in 0.02 ml of culture medium with and without interferon (quadruplicate cultures). DNA synthesis was quantified by adding of 1 μCi of tritium-labelled thymidine in 20 μl 0.9% NaCl per culture for the last 16 h of a 96 h culture period.

Crude human leucocyte interferon was prepared according to published methods (Cantell & Hirvonen, 1978; Christophersen et al. 1978) by incubating leucocytes in 2% human serum and using Sendai virus as the interferon inducer. The specific activity of the crude interferon was 5 × 10⁸ IFU/mg protein. It was concentrated by precipitation with 0.5 m-KSCN at pH 4.5, re-dissolved in 1/20 of the original volume (in PBS) and dialysed against PBS (pH 7.2) containing 1 m-NaCl and 25% ethylene glycol. The crude interferon was generously provided by K. Osterh (Alfred Benzon A/S, Copenhagen, Denmark).
Interferon (4 x 10^6 IFU in 10 ml) was first purified 200-fold by gel filtration on a 100 cm long Ultrogel Aca-5/4 column at 4 °C using PBS (pH 7.2), 1 M-NaCl and 25% ethylene glycol at 4 °C as a buffer. The gel-filtered interferon (3.5 x 10^6 IFU) was further purified three- to sixfold on a Cu-chelate column (Porath et al. 1975; E. Sulkowski, personal communication) followed by a thorough dialysis against 20 mM-phosphate buffer (PB), pH 7.2. The specific activity of the interferon was now 1 to 5 x 10^6 IFU/mg protein with final recovery of 50% (1.8 x 10^6 IFU). After a thorough dialysis against 20 mM-PB, pH 7.2, the interferon was purified on a blue dextran Sepharose column (Bollin et al. 1978) equilibrated with 20 mM-PB, pH 7.2. After washing, the interferon was eluted with 0.6 M-NaCl (1.8 x 10^6 IFU together with 20 µg of proteins) and loaded directly to an anti-interferon column coupled with highly absorbed anti-interferon immunoglobulins, as described by Berg et al. (1978). The pure interferon proteins (about 1 µg together with 1.8 x 10^6 IFU) were eluted by lowering the pH to 2.4 and collected in the presence of 0.1% SDS (final concentration; if SDS was omitted, more than 85% of the biological activity was lost immediately. For further details see Berg & Heron, 1980).

Most of the loss of the interferon activity occurred during the dialysis (about 30% loss), after which the interferon activity remained constant throughout the purification procedures, ending with a total recovery of 50% in the last eluate (10 ml, 1.8 x 10^6 IFU and approx. 1 µg of protein).

The protein estimates were performed by comparing the intensity of the stained interferon protein bands in SDS-PAGE with the corresponding intensity of various known amounts of protein markers (Gordon, 1969). This method was found to be highly reproducible, giving deviations around 5 to 10%, and could detect a minimum of about 0.1 µg of proteins.

Based on the above findings, the specific activity of the purified human leucocyte interferon proteins (in the SDS-containing eluate) appears to be about 10^8 IFU/mg protein, which is ten times higher than that found by Rubinstein et al. (1978, 1979) for one of the human leucocyte interferon species. The discrepancy is more likely to reflect differences in the methodology of the protein determinations (which are based on fundamentally different properties of the proteins) than differences in purity.

The SDS–PAGE was carried out as follows: the interferon proteins in the SDS-containing eluate (10 ml) were precipitated quantitatively by lowering the temperature to 0 °C for 15 min (for details, see Berg & Heron, 1980) followed by centrifugation (20 min, 4 °C, 20000 rev/min in a Sorvall steel tube). The pellet was dissolved in 4 M-urea and after several dialyses against 4 M-urea, distilled water and SDS-sampling buffer, the interferon proteins were examined in an SDS–PAGE-system comprising a 20 cm long polyacrylamide slab gel (gradient: 12 to 18%) and a 5% stacking gel (Berg & Heron, 1980). The discontinuous buffer system included SDS as described by Laemmli (1970). The dialysed interferon sample (concentrated to about 60 µl in total and containing 1.8 x 10^6 IFU) was divided into two aliquots before loading on to the gel. Known markers in predetermined quantities (from 0.1 to 2 µg of each) were also included. After electrophoresis overnight at 10 °C one of the interferon-containing acrylamide strips was cut off the gel and further subdivided into 1 mm pieces. Each piece was minced with a Teflon rod, eluted with 0.4 ml of 0.01% SDS (rocking at room temperature for 5 h) and titrated for antiviral activity. The results are shown in Fig. 1 and 2. As can be seen from Fig. 2 the purified interferon preparation consisted of three visible protein bands of mol. wt. 18400, 20180 and 20400. As shown in Fig. 1, the two major peaks of interferon antiviral activity coincided precisely with the stained protein bands (see later for a more detailed discussion). Thus, the three visible protein bands in Fig. 2 – corresponding to 80% of the interferon activity – are all interferon bands.

Three more peaks of interferon antiviral activity were seen (Fig. 1, ●–●) at mol. wt. 19500, 20900 and 22130. In an experiment with a bigger interferon load of about 3.6 x 10^6
Fig. 1. Separation of purified human leucocyte interferon into different species by SDS-PAGE. About $1.8 \times 10^6$ IFU of purified HuLeIF were concentrated and dialysed against SDS-PAGE sampling buffer and then divided into two portions which were loaded into two separate slots. After electrophoresis, one of the interferon-containing gel strips was cut out, divided into 1 mm pieces, eluted and titrated for antiviral activity (○—○). The remaining part of the gel was stained, destained and dried, and the locations of the protein bands were plotted. The upper curve shows the anticytolytic activity (---) of the interferon fractions, tested at a dilution of 1:1000, in terms of inhibition of thymidine uptake, expressed as a percentage of that in control cultures (mean ct/min ± S.D. of the control cultures without interferon was 78336 ± 3790). ■, Mol. wt.

IFU, visible, but faint protein bands emerged at 19500, 20900, and 22130 together with the usual sharp bands at 18400, 20180 and 20400. On another occasion the single peak of antiviral activity at 20000 (Fig. 1) was further split into two peaks: a major biological peak at 20180 and a five to seven times smaller peak at 20400. The relative amounts of interferon activity in the two peaks corresponded very well to the intensity of the stain of the two bands (the distance between the bands was about 1 mm). In this instance six biological peaks (and six protein bands) were found; mostly only five biological interferon peaks were seen (six experiments). Thus, it can now be concluded unambiguously that all the stained protein bands seen in Fig. 1 and 2 are interferon proteins and that HuLeIF exists in multiple forms (five to six) in the 18000 to 24000 mol. wt. range.

The distance between the two major interferon proteins in the wet gel (Fig. 1) was always about 1 cm. When a less purified HuLeIF preparation, with sp. act. of $1 \times 10^6$ IFU/mg protein, was run on SDS-PAGE, no clear separation of the two major biological peaks was seen, probably because an excessive amount of protein was loaded on to the SDS-PAGE.
Fig. 2. SDS-PAGE of purified human leucocyte interferon. A total amount of $0.9 \times 10^6$ IFU was loaded on to the gel and about $0.5 \mu g$ was estimated in the three bands (in total; for further details see the text). Thus the specific activity of pure interferon proteins appears to be around $10^9$ IFU/mg protein.

The above results are in good agreement with several papers dealing with the heterogeneity of human leucocyte interferon (Stewart II & Desmyter, 1975; Morser et al. 1978; Lin et al. 1978). Furthermore, Stewart II et al. (1978), Stewart II (1979b) and several others (Törmä & Paucker, 1976; Morser et al. 1978) found that human leucocyte interferon gave rise to
several peaks in isoelectric focusing. Thus, to summarize the present knowledge, it can be concluded that human leucocyte interferon consists of two major proteins of mol. wt. 18,400 and 20,180, which represent more than 85% of the biological activity together with minor protein peaks (three or four in all) in the range of mol. wt. 18,400 to 22,200, accounting for 10 to 15% of the activity. Whether or not these peaks are derived from the two major peaks remains to be determined. This could be done by cross-neutralization experiments with the isolated interferon species against antisera developed against the individual interferon species, and such experiments are in progress in our laboratory.

It is well known that the antigenic determinants of HuLeIF differ from those of human fibroblast interferon, as shown for example by Havell et al. (1975) and Vilcek et al. (1976). To determine whether the five interferon species differ in their content of leucocyte and/or fibroblast antigenic determinants, interferon from each fraction (Fig. 1) was diluted (in medium) to contain about 5 to 10 IFU/ml; 100 µl of each interferon dilution were mixed with 100 µl of a solution containing sufficient rabbit anti-leucocyte interferon serum to neutralize 15 IFU of crude human leucocyte interferon by the method described previously (Berg, 1977). All the five peaks were completely neutralized. Anti-fibroblast interferon serum used in a similar manner did not neutralize any of the five peaks.

Finally, the anticellular activity of leucocyte interferon was investigated by incubating Daudi cells with 1:1000 dilutions (in medium) of each interferon species eluted from the fractions shown in Fig. 1. As can be clearly seen in Fig. 1, the 'anticellular curve' followed the antiviral curve very strictly, thus proving that all species of pure native human leucocyte interferon contain both antiviral and anticellular activity. These findings are supported by the observations made with impure interferon preparations by Gresser et al. (1970) and Stewart II et al. (1976), who argued that the antiviral activity and the anticellular activity found in the major species were most likely contained in the same molecule(s). We noted that the height of the different 'anticellular peaks' did not vary linearly with the corresponding size of the 'antiviral peaks', which probably reflects the sensitivity of the Daudi cell system (Hilfenhaus et al. 1976). The small antiviral peak at mol. wt. 19,500 did not give rise to a corresponding peak in the anticellular curve when tested at 1:1000, but when tested at 1:100, a small but distinct peak of anticellular activity was observed (data not shown).

The authors wish to express their gratitude for excellent technical assistance by Kaj Vestergård, Karin Durup and Inger Sorensen. This investigation was supported by the Danish Cancer Foundation.

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Short communications


(Received 5 October 1979)