Size characteristics of human leucocyte interferon under reducing and non-reducing conditions

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(Received 4 July 1980/Accepted 15 October 1980)

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was used to characterize human leucocyte interferon (HuIFN- α) under reducing and non-reducing conditions. Under non-reducing conditions HuIFN- α possesses two size forms, but under reducing conditions (r-HuIFN- α) only one is observed. The apparent molecular weight of this one form varies with the concentration of 2-mercaptoethanol used. When r-HuIFN- α is permitted to reoxidize the bimodal configuration of HuIFN- α is restored. The size heterogeneity of native HuIFN- α can be eliminated by mild treatment with NaIO₄ [HuIFN- α /IO₄; Stewart II, Lin, Wiranowska-Stewart & Cantell (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 4200–4204]. The size of the HuIFN- α /IO₄ increases after treatment with 2-mercaptoethanol (r-HuIFN- α /IO₄) and the apparent molecular weight of this component also varies with the concentration of 2-mercaptoethanol used. In the case of r-HuIFN- α the single peak observed apparently originates from both the higher- and lower-molecular-weight components.

After the discovery that interferons were stable in SDS (Stewart *et al.*, 1974), the size of interferons could be characterized by SDS/polyacrylamide-gel electrophoresis (Stewart, 1974). In this way Stewart & Desmyter (1975) found that SDS/polyacrylamide-gel electrophoresis under non-reducing conditions showed human leucocyte interferon (HuIFN- α ; Stewart *et al.*, 1980) to consist of two distinct molecular-weight species (21 000 and 15 000). However, when electrophoresed under reducing conditions the activity of the lower-molecular-weight component was completely eliminated. Under the same reducing conditions the 15 000 mol.wt species still expressed activity on heterologous cat cells (Desmyter & Stewart, 1976). Paucker and colleagues (Torma & Paucker, 1976; Paucker *et al.*, 1977) isolated two HuIFN- α components by hydroxyapatite column chromatography that were distinguishable by SDS/polyacrylamide-gel electrophoresis as a 16 000–20 000 and a 16 000 mol.wt. species. When these two chromatographed components were treated with the reducing agent 2-mercaptoethanol both components lost interferon activity to the same extent. From this observation

they concluded that, rather than one molecular-weight form becoming differentially inactivated, both species were equally inactivated.

To complicate the issue still further, with guanidine hydrochloride rather than SDS as a denaturant and 2-mercaptoethanol as the reducing agent (Mogensen & Cantell, 1974), Chadha *et al.* (1978) reported that the molecular weight (as determined by molecular-sieve-column chromatography) was apparently decreased by 5000. When the unreduced and reduced samples obtained from the sizing column were compared by SDS/polyacrylamide-gel electrophoresis the former possessed two size forms (mol.wt. 21 000 and 15 000), whereas the latter had only one form (18 000).

In view of the purification of HuIFN- α to homogeneity (Lin & Stewart, 1978; Rubinstein *et al.*, 1979) and a description of its amino acid sequence (Mantei *et al.*, 1980), attempts to synthesize the active constituent of the molecule should be forthcoming. The involvement of the disulphide bridge(s) in maintaining the activity of interferons therefore needs further clarification.

In the present paper we show that when HuIFN- α is reduced and characterized by SDS/polyacrylamide-gel electrophoresis a single species is present. However, upon subsequent dialysis of the treated material to remove the reducing agent both species reappear. Moreover, the HuIFN- α pre-treated with NaIO₄ (to remove the size and charge hetero-

Abbreviation used: SDS, sodium dodecyl sulphate.

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geneity; Stewart *et al.*, 1977) migrated when reduced at an apparently higher molecular weight, but returned to its normal lower molecular weight upon dialysis. We also show that the single component isolated under reducing conditions represents a mixture of both the higher- and lower-molecular-weight forms.

Materials and methods

Chemicals

SDS and 2-mercaptoethanol were products of Bio-Rad Laboratories (Richmond, CA, U.S.A.). The molecular-weight standards were part of the Pharmacia Low Molecular Weight Electrophoresis Calibration Kit (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.). Bisacrylamide was purchased by Eastman Kodak Co. (Rochester, NY, U.S.A.), acrylamide from Aldrich Chemicals (Milwaukee, WI) and NaIO_4 from Mallinckrodt (St. Louis, MO, U.S.A.).

Interferons

Human leucocyte interferon (HuIFN- α) was produced by Dr. V. G. Edy of the Sloan-Ketterings Institute's Interferon Production Laboratory at the Theodor Kocher Institute (Berne, Switzerland) by the method previously described by Cantell *et al.* (1974). The preparations used in this investigation had a specific activity of 1.0×10^6 units/mg of protein.

Interferon samples were assayed on human cells trisomic for chromosome 21 (GM-2767; Mammalian Genetic Mutant Cell Repository, Camden, NJ, U.S.A.) in a modification of the microtitration assay against vesicular stomatitis virus (Havell & Vilcek, 1972). In this assay 1 unit was equivalent to approx. 1 NIH HuIFN- α unit (reference reagent no. G-023-901-527).

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed as previously described (Braude *et al.*, 1979). When required $10 \mu\text{l}$ of either as 10 or 1% (v/v) solution of 2-mercaptoethanol was added to $100 \mu\text{l}$ samples.

Periodate oxidation of HuIFN- α

Periodate oxidation of HuIFN- α was as previously described (Stewart *et al.*, 1977). The preparations used in this investigation had a specific activity of 1.0×10^6 units/mg of protein.

Results

HuIFN- α appeared as two distinct size forms when analysed by SDS/polyacrylamide-gel electrophoresis under non-reducing conditions, as higher-

(21 000–23 000) and a lower-molecular-weight species (15 000–18 000, Fig. 1a). However, when the sample was characterized under reducing conditions [r-HuIFN- α , final concn. of 2-mercaptoethanol was 1.0% (w/v)] only a higher-molecular-weight form (21 000–25 000) was observed (Fig. 1b, continuous line).

As native HuIFN- α seemed to consist of two species, whereas r-HuIFN- α appeared as only one, we found it important to determine whether one species was irreversibly inactivated or if it could be recovered upon reoxidation of the sample. As shown in Fig. 1(b) (broken line) when r-HuIFN- α was allowed to reoxidize, by exhaustive dialysis, and was examined by SDS/polyacrylamide-gel electrophoresis it showed both the higher- (21 000–25 000) and nearly all of the lower-molecular-weight components (18 000).

Similar results were obtained when HuIFN- α was reduced with 10 times less 2-mercaptoethanol. In this instance, however, the peak of r-HuIFN- α activity was broad (mol.wt. 19 000–23 000; Fig. 1c, continuous line). In addition, more of the lower-molecular-weight form reappeared when the r-HuIFN- α was subsequently reoxidized (Fig. 1c, broken line). Thus, although after reduction native HuIFN- α showed only one peak, both molecular-weight components reappeared subsequent to reoxidation.

The size and charge heterogeneities usually associated with HuIFN- α can be eliminated by mild treatment with NaIO_4 (Stewart *et al.*, 1977). We therefore thought it of interest to study the effects of 2-mercaptoethanol on an apparently size-homogeneous preparation of HuIFN- α (i.e., pretreated with NaIO_4 : HuIFN- α /IO $_4$).

HuIFN- α /IO $_4$ when characterized under non-reducing conditions by SDS/polyacrylamide-gel electrophoresis ran as a single peak of activity (mol.wt. 18 000–19 000, Fig. 2a). The same sample examined under reducing conditions (r-HuIFN- α /IO $_4$) also ran as a single peak, but now had a molecular weight ranging from 26 000 to 30 000 (Fig. 2b, continuous line). When r-HuIFN- α /IO $_4$ was permitted to reoxidize the activity still remained as one peak, but had a molecular weight ranging between 19 000 and 22 000 (Fig. 2b, broken line).

Similar results were obtained when HuIFN- α /IO $_4$ was reduced with 10 times less 2-mercaptoethanol. The r-HuIFN- α /IO $_4$ analysed by SDS/polyacrylamide-gel electrophoresis now had a very broad peak of activity (mol.wt. 20 000–27 000; Fig. 2c, continuous line). However, when allowed to reoxidize more of the r-HuIFN- α /IO $_4$ activity now appeared within the same molecular-weight range as HuIFN- α /IO $_4$ (Fig. 2c, broken line). These results show that when a homogeneous population of HuIFN- α was reduced the molecular weight of

Fig. 1. *SDS/polyacrylamide-gel electrophoresis of reduced, non-reduced and reoxidized HuIFN- α*

Non-reduced (0.1 ml) (a), reduced (b and c, continuous lines) and reoxidized (b and c, broken lines) HuIFN- α were analysed by SDS/polyacrylamide-gel electrophoresis. The acrylamide gel concentration was 10% (w/v). All samples were incubated with 1.0% (w/v) SDS (final concn.) at 100°C for 1 min. Also included in some samples was 1.0% (v/v) (b) and 0.1% (c) (final concns.) of 2-mercaptoethanol (2-ME) (No 2-ME, without 2-mercaptoethanol). The molecular-weight markers are ovalbumin (OA, 45000), carbonic anhydrase (CA, 30000), soya bean trypsin inhibitor (SBTI, 20100) and α -lactalbumin (LA, 14400).

Fig. 2. *SDS/polyacrylamide-gel electrophoresis of reduced, non-reduced and reoxidized HuIFN- α /IO $_4$*

Non-reduced (0.1 ml) (a), reduced (b and c, continuous lines) and reoxidized (b and c, broken lines) HuIFN- α /IO $_4$ were analysed by SDS/polyacrylamide-gel electrophoresis. The acrylamide gel concentration was 10% (w/v). All samples were incubated with 1.0% (w/v) SDS (final concn.) at 100°C for 1 min. Also included in some samples was 1.0% (b) and 0.1% (c) (final concns.) of 2-mercaptoethanol (2-ME) (No 2-ME, without 2-mercaptoethanol). The molecular-weight markers are described in Fig. 1.

Fig. 3. Interferon activity profile of HuIFN- α gel isolates treated or untreated with 2-mercaptoethanol

HuIFN- α (0.1 ml) was electrophoresed by SDS/polyacrylamide-gel electrophoresis. The acrylamide concentration was 10% (w/v). All gel isolates were incubated with 1.0% (w/v) SDS (final concn.) at 100°C, for 1 min. The 2-mercaptoethanol (2-ME) treated [1.0% (v/v) final concn., broken line] and untreated (continuous line) gel isolates were then assayed for activity. The molecular-weight markers are described in Fig. 1.

almost the entire population was increased. However, when reoxidation was permitted the molecular weight of interferon activity returned to its unreduced size.

As described above (Fig. 1), native HuIFN- α , that possesses two different molecular-weight forms was converted into only one form on reduction. That single form could consist exclusively of the higher- or lower-molecular-weight species or could be derived from both species. In an attempt to distinguish between these three possibilities HuIFN- α was subjected to SDS/polyacrylamide-gel electrophoresis and individual gel isolates were treated with 2-mercaptoethanol [final concn. 1.0% (w/v)] in the same manner as the samples loaded on the gels. As shown in Fig. 3, the activity profiles for treated (broken line) and untreated (continuous line) interferons were very similar. Thus, rather than 2-mercaptoethanol differentially inactivating one molecular-weight species versus another, the single peak of r-HuIFN- α activity apparently represents a mixture of both forms.

Discussion

The salient features of the results presented can be summarized as follows: (i) on reduction HuIFN- α ,

which usually possesses two size forms, is isolated only as a higher-molecular-weight form; (ii) re-oxidation of r-HuIFN- α restores the activity profile to its bimodal configuration; (iii) when the size heterogeneity of interferon is first eliminated by periodate, and the product is then reduced, interferon activity appears as a higher-molecular-weight species and the interferon is restored to its normal lower size on reoxidation; (iv) in the case of r-HuIFN- α the single peak observed is apparently composed of both the higher- and lower-molecular-weight components.

The observation that when more 2-mercaptoethanol was used the molecular weight of either r-HuIFN- α or r-HuIFN- α /IO₄ increased can best be explained as follows: when a protein's disulphide bridge(s) is broken the protein appears larger than its native size in SDS/polyacrylamide-gel electrophoresis because the molecule becomes less compact. Since HuIFN- α is likely to possess two disulphide bridges (Mantei *et al.*, 1980) perhaps the lower concentration of 2-mercaptoethanol breaks only one bridge, whereas in the higher concentration the second is also broken, leaving the protein still more unfolded and apparently larger.

An alternative explanation for the apparent increase in the size of interferons after treatment with higher concentrations of 2-mercaptoethanol could be that after the addition of more 2-mercaptoethanol less of the lower-molecular-weight form of interferon remains active, thereby giving the appearance of a single peak with a higher molecular weight. This explanation can be discounted for three reasons. First, the peak widths are the same for samples treated with both concentrations of 2-mercaptoethanol. This would not be so if the lower concentration of 2-mercaptoethanol left more of the lower-molecular-weight species active. Secondly, the molecular weight for the interferon sample treated with 1.0% (v/v) 2-mercaptoethanol was greater (21 000–25 000) than the size of the higher-molecular-weight form (21 000–23 000) of native HuIFN- α . Lastly, the results in Fig. 3 indicate no appreciable loss of the lower-molecular-weight component's activity.

It is difficult to be sure why there appears to be only one peak of interferon activity after reduction of a sample containing a mixture of both the higher- and lower-molecular-weight forms. However, two possible explanations can be considered. The first postulates that the higher-molecular-weight form of interferon is resistant to 2-mercaptoethanol, whereas the lower-molecular-weight form is susceptible; thus the former remains the same size whereas the latter, owing to the breakage of one or more of its disulphide bridges, appears larger. Alternatively, the disulphide bridges of both forms are susceptible to breakage by 2-mercaptoethanol, but respond differ-

ently to its effects. The data do not allow us to decide between these alternatives. However, both interpretations suggest that some structural dissimilarities must exist between the two forms.

Chadha *et al.* (1978) reported the isolation after reduction of a 18000 mol.wt. species by SDS/polyacrylamide-gel electrophoresis. The discrepancy between their estimation for the molecular weight of r-HuIFN- α and ours may be due to differences in procedure. Samples of r-HuIFN- α tested by Chadha *et al.* (1978) before examination by SDS/polyacrylamide-gel electrophoresis were dialysed. This would have permitted the r-HuIFN- α to reoxidize and resume its lower-molecular-weight form. What is puzzling in their report is the absence after dialysis of the higher-molecular-weight form. Perhaps the use of guanidine hydrochloride as a denaturant irreversibly inactivated this form.

The controversy concerning the composition of r-HuIFN- α can now be resolved. Stewart & Desmyter (1975) demonstrated that normally HuIFN- α (when characterized by SDS/polyacrylamide-gel electrophoresis) migrates as a bimodal configuration, but only the higher-molecular-weight species was observed after treatment with a reducing agent. They concluded that the lower-molecular-weight component must therefore have been eliminated. Based on their characterization of two size forms isolated by hydroxyapatite chromatography Paucker and colleagues (Torma & Paucker, 1976; Paucker *et al.*, 1977) reasoned that, since both forms were susceptible to the effects of 2-mercaptoethanol, the single peak of r-HuIFN- α isolated by SDS/polyacrylamide-gel electrophoresis must be derived from both of the size forms.

Our results demonstrate that, similar to the observation by Stewart & Desmyter (1975), r-HuIFN- α when analysed by SDS/polyacrylamide-gel electrophoresis was isolated as a single peak. However, in light of the evidence obtained with reoxidized r-HuIFN- α (where both peaks of activity were restored, Fig. 2) and the results where individual gel isolates were treated with 2-mercaptoethanol (and where both the higher- and lower-molecular-weight species were apparent) we must agree with the interpretation of Paucker and

colleagues (Torma & Paucker, 1976; Paucker *et al.*, 1977).

We gratefully acknowledge the help of Dr. Kingsley Sanders for his editorial assistance. This work was supported in part by grant 1-R01-AI-16439 awarded by the National Cancer Institute, and Institutional Core grant NCI-CA-08748-13. Irwin A. Braude was supported by an NIH training grant (no. T32 CA-09193-01A1). William E. Stewart II was supported by U.S. Public Health Service Research Career Development Award AI-00219 from the National Institute of Allergy and Infectious Diseases.

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