Separation of Human Leukocyte Interferon Components by Concanavalin A–Agarose Affinity Chromatography and Their Characterization†

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ABSTRACT: Human leukocyte interferon (HL-IF), produced by mixed leukocytes infected with Newcastle disease virus, was resolved into three distinct fractions when chromatographed on concanavalin A–agarose. The major portion (70–75%) of interferon appeared in the breakthrough (BT fraction). The bound interferon (25–30%) was displaced from the column as two peaks: the first was eluted with 0.1 M methyl α-D-mannoside, yielding 15–20% of the interferon activity (α-MM fraction), and the second by including ethylene glycol (70%) in the eluant, yielding the remaining 5–15% of the interferon (EG fraction). No interferon was retained when HL-IF produced in the presence of glycosylation inhibitors (tunicamycin or 2-deoxy-D-glucose) was chromatographed on concanavalin A–agarose, suggesting that the fraction of interferon retained by this lectin is glycosylated. The three fractions of interferon (BT, α-MM, and EG) were characterized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, cross-species antiviral activity, and neutralization by specific antisera. The BT fraction contains exclusively the 16 000 molecular weight component of human leukocyte interferon. The majority of the α-MM fraction (90%) is the 21 000 molecular weight component. However, the EG fraction contains the 16 000 and 21 000–23 000 molecular weight components in essentially equal proportions. On the basis of cross-species antiviral activity and neutralization by specific antisera, the BT and α-MM fractions are leukocyte-type interferon and the EG fraction seems to be primarily of fibroblast type.

Human leukocyte interferon (HL-IF)[1] can be resolved into two molecular weight components by sodium dodecyl sulfate–polyacrylamide gel electrophoresis: a 16 000 and a 21 000 molecular weight component in an approximate ratio of 7:3 (Stewart & Desmyter, 1975; Törmä & Paucker, 1976; Vilček et al., 1977). These two molecular weight components have also been separated partially by polyacrylamide gel electrophoresis under nondissociating conditions (Boreckỳ et al., 1974), by isoelectric focusing in polyacrylamide gels (Havell et al., 1977), and by adsorption chromatography on a sodium dodecyl sulfate–hydroxylapatite column (Törmä & Paucker, 1976). A variety of observations reported in the literature suggest that the molecular basis for this heterogeneity is the relative extent of glycosylation of the two components. For example, upon treatment with glycosidases, the apparent molecular weight of HL-IF was reduced by 4000 (Bose et al., 1976). Stewart et al. (1977) have reported that upon periodate treatment the 21 000 molecular weight component is converted to a 16 000 molecular weight component apparently due to the removal of the carbohydrate moiety; the molecular weight of the 16 000 component remained unchanged. When HL-IF was produced in the presence of the glycosylation inhibitor tunicamycin, only a single component corresponding to 16 000 molecular weight was seen (K. C. Chadha, P. M. Grob, R. L. Hamill, and E. Sulkowski, unpublished experiments). Similarly, only one component of 16 000 molecular weight was seen when another glycosylation inhibitor, 2-deoxy-D-glucose, was used (K. C. Chadha, unpublished experiments). These observations strongly suggest that variable glycosylation of the HL-IF molecule is responsible for its molecular size heterogeneity.

Lectin affinity chromatography has been utilized to purify glycoproteins and to probe the structure of their carbohydrate moieties (Kristiansen, 1974). Concanavalin A–agarose has been used to probe the glycosylation status of mouse L-cell interferon (Besancon & Bourgeade, 1974; Davey et al., 1976b) and human fibroblast interferon (Davey et al., 1974). Initial attempts to show a specific interaction between HL-IF and concanavalin A–agarose were both affirmative (Besancon & Bourgeade, 1974) and negative (Mogensen et al., 1974; Jankowski et al., 1975). Because of these conflicting observations, we have reexamined the glycosylation status of HL-IF by using lectin affinity chromatography. We now report that HL-IF can be resolved into three distinct fractions by Con A–Sepharose affinity chromatography. Each fraction has distinct physicochemical and biological properties. Since this technique quantitatively separates the two molecular weight components of HL-IF in the absence of sodium dodecyl sulfate, the biological activities of these native molecular components can now be compared. This is of considerable importance because HL-IF, denatured by detergent, fails to fully recover its native conformation upon subsequent reactivation (Allen & Stewart, 1976).

Materials and Methods

Cells and Viruses. Human foreskin cells (HF604) were obtained from Dr. J. Horoszewicz. African green monkey kidney cells (CV_1) and bovine kidney cells (MDBK) were obtained from American Type Culture Collection, Rockville, MD. Cells were grown in Eagle's minimal essential medium (Eagle, 1959) containing nonessential amino acids and 5 or 10% fetal calf serum. Newcastle disease virus (NDV) was propagated in embryonated eggs and assayed on primary chick embryo cells. Vesicular stomatitis virus (VSV) was grown and plaque assayed on CV_1 cells.

Interferon Preparation and Assay. Mixed leukocytes were isolated from buffy coats obtained from normal healthy donors, and HL-IF was induced with NDV essentially according to the procedure of Pidot et al. (1972). Interferon assays were

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[1] Abbreviations used: HL-IF, human leukocyte interferon; HL-IF^T, human leukocyte interferon produced in the presence of tunicamycin; $NaDodSO_4$-gel electrophoresis, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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performed on HF604 cells by the dye uptake method of Finter (1969) using VSV as a challenge virus.

Chromatographic Procedure. All columns (0.9 × 5 cm) were operated at 4 °C at a flow rate of 20 mL/h. Eluant from the column was divided 7:3 by a stream-splitting device. The larger fractions were used for protein determination by the fluorometric method (Böhlen et al., 1973), and the smaller fractions, to be used for interferon assays, were routinely collected into a 1% solution of bovine serum albumin in 0.02 M sodium phosphate containing 0.15 M sodium chloride, pH 7.4 (PBS), unless otherwise indicated.

Sodium Dodecyl Sulfate–Polyacrylamide Gel (NaDodSO$_4$–Gel) Electrophoresis. Disc NaDodSO$_4$–gel electrophoresis was performed essentially as described by Summers et al. (1965). The details of gel preparation, buffers, sample preparation, and elution of interferon from the gel slices are described elsewhere (Chadha et al., 1978).

Antibody Neutralization. Antibody to HL-IF was obtained from the National Institute of Allergy and Infectious Diseases (National Institutes of Helath, rabbit no. 24 5/74). Rabbit antiserum to human fibroblast interferon was prepared in our laboratory by using Con A–Sepharose-purified interferon (Davey et al., 1976a) as an immunogen. For neutralization tests, interferon (30–60 units) was mixed with various dilutions of appropriate antiserum and incubated on ice for 4 h prior to assay for the remaining interferon activity. Specific details are given in the figure legends.

Chemicals and Sorbents. Acrylamide, methylenebis-(acrylamide), *N,N,N′,N′*-tetramethylethylenediamine, and bromophenol blue were purchased from Eastman Kodak, Rochester, NY. Molecular weight standards, sodium dodecyl sulfate, and ammonium persulfate were purchased from Sigma Chemical Co. Bovine serum albumin (Pentex), used to stabilize interferon activity, was purchased from Miles Laboratories, Elkhart, IN. Media for cell propagation and fetal calf serum were from Grand Island Biological Co., Grand Island, NY. Con A–Sepharose was purchased from Pharmacia Fine Chemicals, Piscataway, NJ. All other chemicals were reagent grade.

Results

Chromatography of HL-IF on Con A–Sepharose resolves the antiviral activity into three major fractions (Figure 1A). Approximately 70% of the interferon was recovered in the breakthrough (BT fraction), about 20% was displaced with 0.1 M methyl α-D-mannoside (α-MM fraction), and 10% was eluted with 70% ethylene glycol (EG fraction); less than 3% was eluted with 1 M sodium chloride. In order to be certain that these fractions constitute true chromatographic components, we dialyzed the BT, α-MM, and EG fractions extensively against PBS and individually rechromatographed them on Con A–Sepharose. Figures 1B–D illustrate, respectively, the rechromatography of the BT, α-MM, and EG fractions on Con A–Sepharose. It is evident that each of these fractions is a true chromatographic component since each rechromatographs faithfully. The specificity of carbohydrate recognition by concanavalin A was confirmed, because only an insignificant amount of bound interferon (<4%) was displaced from Con A–Sepharose when the column was eluted with 0.1 M D-galactose instead of α-MM as a competing sugar (data not shown).

In addition to several of our own HL-IF preparations, reference HL-IF obtained from the National Institutes of Health (catalog no. G023-901-527) as well as a few HL-IF preparations from other investigators (Drs. K. Berg, K. Paucker, and S. Pestka) was chromatographed on Con A–

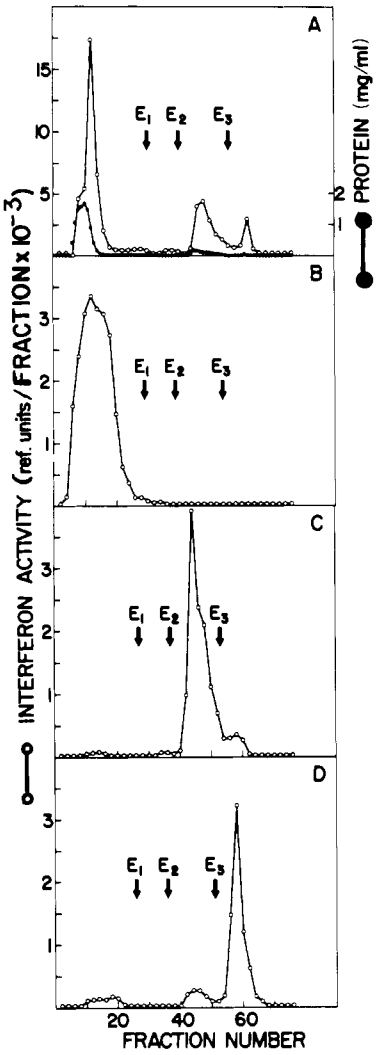

FIGURE 1: Chromatography of HL-IF on Con A–Sepharose. Each interferon preparation was dialyzed against 0.02 M sodium phosphate containing 0.15 M NaCl, pH 7.4 (PBS), and applied on a column (0.9 × 5 cm) equilibrated with PBS. (A) Chromatography of unfractionated HL-IF. An interferon sample, 5 mL, containing 155 000 units of interferon and 13.25 mg of protein was applied to a column, and the column was washed with 22 mL of equilibrating buffer (PBS). The column was then developed with 10 mL of 0.02 M sodium phosphate containing 1 M NaCl, pH 7.4 (E_1), followed by 15 mL of E_1 containing 0.1 M methyl α-D-mannoside (E_2) and finally with 20 mL of E_2 containing 70% ethylene glycol (v/v, E_3). The breakthrough fraction (BT) contained 60% of the interferon and 90% of the protein applied to the column. Approximately 2% of the interferon was eluted with 1 M NaCl. The methyl α-D-mannoside fraction (α-MM) contained 22% of the interferon and 7% of the protein. The ethylene glycol fraction (EG) contained 6% of the interferon and 3% of the protein. The overall protein recovery was 100% and the interferon recovery 90%. The BT, α-MM, and EG fractions were extensively dialyzed against PBS prior to their rechromatography on Con A–Sepharose. (B) Rechromatography of the BT fraction. An interferon sample, 12 mL, containing 55 000 units of interferon was applied to a freshly prepared column equilibrated with PBS. The column was subsequently eluted as described for Figure 1A. Nearly 100% of the interferon activity appeared in the BT fraction. Interferon recovery was 82%. (C) Rechromatography of the α-MM fraction. The interferon sample, 12 mL, containing 14 000 units of interferon was applied to a column and developed as described for Figure 1A. About 10% of the interferon activity was recovered in the BT fraction, and the remaining 90% was retained and subsequently recovered in the α-MM fraction. Interferon recovery was 100%. (D) Rechromatography of the EG fraction. The interferon sample, 15 mL, containing 2500 units of interferon was applied to the column and then developed as described for Figure 1A. Approximately 11% of the recovered interferon appeared in the BT fraction, about 19% in the α-MM fraction, and the remaining 70% in the EG fraction. Total interferon recovery was 80%. (○) Interferon; (●) protein.

Table I: Fractionation of Human Leukocyte Interferon on Con A-Sepharose

expt[a]	interferon act. (% distribution) BT fraction[b]	α-MM fraction	EG fraction
1	78	18	4
2	67	18	15
3	77	14	9
4	74	15	11
5	56	28	16
6	66	26	8
7[c]	78	16	6
8[d]	77	10	13
9[e]	74	22	4
10[f]	73	23	4

[a] Each experiment utilized a different interferon preparation; overall recovery of interferon was 85–100%. [b] The interferon in the breakthrough and 1 M NaCl fractions was summed in this tabulation. [c] NIH reference HL-IF. [d] HL-IF gift of K. Berg. [e] HL-IF gift of K. Paucker. [f] HL-IF gift of S. Pestka.

Sepharose. The HL-IF preparations obtained from other investigators were prepared somewhat differently than our own preparations; Sendai virus was used instead of Newcastle disease virus as interferon inducer or casein was substituted for human serum during interferon production. The results are included in Table I. Although there was some variation in the chromatographic distribution of interferon activity, every interferon preparation analyzed on Con A–Sepharose contained a BT, α-MM, and EG interferon fraction. Since a particular fraction of HL-IF could be selectively lost during purification, all studies were conducted with HL-IF produced in our laboratory without prior treatment.

Molecular weight estimates of the interferon present in the BT, α-MM, and EG fractions obtained from Con A–Sepharose chromatography are shown in Figure 2. Figure 2A represents $NaDodSO_4$–gel electrophoresis of native unfractionated HL-IF. Interferon activity appeared as two components corresponding to molecular weights of 21 000 (I) and 16 000 (II). The relative distribution of interferon activity between these two components varied in different preparations, but component I generally contained 40% of the activity and component II 60%. The BT fraction contained only one component of 16 000 molecular weight (Figure 2B). The α-MM fraction predominantly, 90%, contained 21 000 molecular weight interferon (Figure 2C). However, the EG fraction contained both the 16 000 and 21 000–23 000 molecular weight components in roughly equal proportions (Figure 2D). These data indicate that the majority of bound interferon has a molecular weight of 21 000 and is apparently the glycosylated component of HL-IF.

We have recently observed that HL-IF produced in the presence of 2 μg/mL tunicamycin (HL-IF^T), a specific inhibitor of glycosylation, contains only one component of 16 000 molecular weight (K. C. Chadha, P. M. Grob, R. L. Hamill, and E. Sulkowski, unpublished experiments). Since the 16 000 molecular weight component of HL-IF is apparently nonglycosylated, it was of interest to examine the chromatographic behavior of HL-IF^T on Con A–Sepharose. The results of such an experiment are shown in Figure 3. The top panel (Figure 3A) represents Con A–Sepharose chromatography of HL-IF produced in the absence of tunicamycin. The lower panel (Figure 3B) shows the elution profile of HL-IF^T on Con A–Sepharose. Essentially all of the HL-IF^T interferon was recovered in the breakthrough fractions. This suggests that the 16 000 molecular weight component either is nonglycosylated or lacks specific sugar residues and as a result

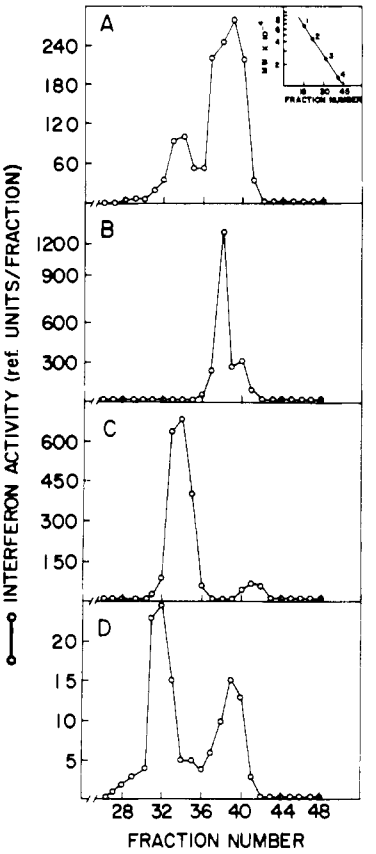

FIGURE 2: $NaDodSO_4$–gel electrophoresis of unfractionated HL-IF and HL-IF fractions (BT, α-MM, and EG) obtained from Con A–Sepharose column chromatography. Unfractionated HL-IF, BT, α-MM, and EG fractions were dialyzed individually against 0.002 M sodium phosphate, pH 7.5, and lyophilized. They were resuspended in sample buffer (0.1 M sodium phosphate, pH 7.5) containing 1.0% sodium dodecyl sulfate and heated at 100 °C for 2 min in a water bath, and then 100–200-μL aliquots were applied on 10% polyacrylamide disc gels. The gels were run for 16 h at 5 mA/gel. Gels containing protein standards were stained with Coomassie brilliant blue and destained with a glacial acetic acid–methanol mixture until clear. Gels containing interferon were sliced into 0.2-cm segments and placed in 1 mL of culture media with 10% fetal calf serum. Following overnight elution at 4 °C on a shaking platform, samples were assayed for interferon. (A) Unfractionated HL-IF. (B) BT fraction; (C) α-MM fraction; (D) EG fraction. Molecular weight standards were (1) bovine serum albumin (67 000), (2) ovalbumin (45 000), (3) chymotrypsinogen A (25 000), and (4) bovine pancreatic ribonuclease A (13 700).

Table II: Cross-Species Antiviral Activity of Fractions of HL-IF Obtained from Con A–Sepharose Column Chromatography

	interferon titer HF604	bovine	% cross-species act.
BT	700	1300	185
α-MM	40	25	62
EG	250	4	1.5
HL-IF[a]	3800	2000	52
NIH ref HL-IF[a]	185	110	59
HF-IF[a]	6200	28	0.5

[a] Unfractionated.

is unable to bind to Con A–Sepharose. HL-IF produced in the presence of 2-deoxy-D-glucose, another inhibitor of glycosylation, gave the same chromatographic elution profile as HL-IF^T when analyzed on Con A–Sepharose (data not shown).

The heterospecies antiviral activity of the two molecular weight components of HL-IF has been extensively documented (Borecký et al., 1974; Stewart et al., 1977; Paucker et al., 1977). Table II shows the homologous and heterologous an-

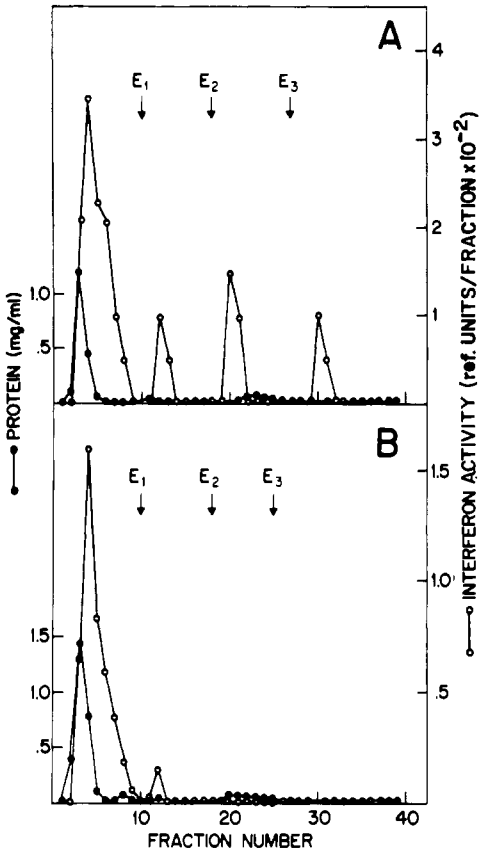

FIGURE 3: Chromatography of HL-IF and HL-IF^T on Con A-Sepharose. Interferon was dialyzed against PBS at 4 °C and applied to a column (0.9 × 5 cm) equilibrated with PBS. (A) HL-IF. An interferon sample, 2.5 mL, containing 8400 units of interferon and 6.62 mg of protein was applied to a column, and the column was developed essentially as described in Figure 1A. The BT fraction contained 68% of the interferon and over 90% of the protein recovered from the column. The α-MM fraction contained 14% and the EG fraction 9% of the recovered interferon. Overall protein recovery was 72% and interferon 52%. (B) HL-IF^T. An interferon sample, 2.5 mL, containing 1250 units of interferon and 6.2 mg of protein was applied to the column. Essentially all interferon activity (96%) was recovered in the BT fraction. Overall protein recovery was 100%, and recovery of interferon was 92%.

tiviral activity of the BT, α-MM, and EG fractions of HL-IF resolved by Con A–Sepharose chromatography. Reference HL-IF, obtained from the National Institutes of Health, and our own unfractionated HL-IF were used as controls. The reference HL-IF, the unfractionated HL-IF, and the interferon in the α-MM fraction exhibited similar ratios of antiviral activity on bovine vs. human cells, whereas the interferon in the BT fraction gave a higher ratio, indicating greater activity on bovine cells. The EG fraction, on the other hand, gave strikingly different results; bovine cells were far less protected (60-fold less) by the interferon present in the EG fraction. Since human fibroblast interferon exerts little antiviral activity on bovine cells (Gresser et al., 1974; Vilček et al., 1977), a sizable proportion of the interferon activity present in the EG fraction is apparently due to fibroblast-type interferon. Havell et al. (1975) observed that a small percentage of the interferon activity in HL-IF preparations (1–5%) is due to the presence of fibroblast-type interferon. This minor fibroblast component of HL-IF is apparently heavily enriched in the EG fraction during chromatography on Con A–Sepharose.

Both human fibroblast interferon (HF-IF) and HL-IF can be distinguished antigenically (Berg et al., 1975; Havell et al., 1975). Table III represents the neutralization of antiviral activity of various fractions of HL-IF obtained from Con A–Sepharose chromatography. The interferon activity in the BT and α-MM fractions was selectively neutralized by HL-IF antiserum; no neutralization was seen when HF-IF antisera were used instead. However, the interferon activity present in the EG fraction was preferentially neutralized (70%) by antisera against HF-IF and only 20% was neutralized when HL-IF-specific antisera were used. Complete neutralization of the interferon in the EG fraction occurred when both HL-IF- and HF-IF-specific antisera were mixed together in a neutralization test. These results suggest that the interferon activity present in the EG fraction is a mixture: the majority of interferon is fibroblast type as shown by both its lack of cross-species activity and by preferential neutralization by HF-IF antisera; the minor species is leukocyte-type interferon as shown by its neutralization by HL-IF antiserum.

Table III: Neutralization of Antiviral Activity of HL-IF Fractions Obtained from Con A–Sepharose Chromatography

	% neutralization[a]					
	column fractions			unfractionated	NIH ref	
dilutions	BT	α-MM	EG	HL-IF	HL-IF	HF-IF
HL-IF antiserum						
40	100	100	19	100	100	0
200	100	100	5	100	100	0
400	98	87	0	96	100	0
800	89	55	0	86	51	0
HF-IF antiserum						
100	0	0	67	0	0	100
200	0	0	40	0	0	69
400	0	0	10	0	0	7
800	0	0	0	0	0	0

[a] Interferon preparations contained a final concentration of 30 units/mL for unfractionated HL-IF and HF-IF, 40 units/mL for α-MM and EG, and 60 units/mL for BT and NIH reference HL-IF.

Discussion

When chromatographed on Con A–Sepharose, HL-IF can be separated into three distinct fractions which differ in their physicochemical, antigenic, and biological properties. The three interferon fractions (BT, α-MM, and EG) vary somewhat in their relative proportions but were consistently resolved in all of our HL-IF preparations as well as in HL-IF preparations obtained from other investigators. Apparently, the viral inducer, NDV or Sendai, as well as the protein supplement, serum or casein, did not significantly influence the observed heterogeneity. Thus, the apparent variation in the relative distribution of interferon among the BT, α-MM, and EG fractions is due to the intrinsic heterogeneity of HL-IF. When the interferon from each of the BT, α-MM, and EG fractions is rechromatographed on Con A–Sepharose, the activity appears in its respective place, thereby eliminating the possibility that any one of these three fractions is a chromatographic artifact (Figure 1).

When HL-IF is chromatographed on Con A–Sepharose, ~30% of the interferon activity is retained (α-MM and EG fractions) and the majority of this bound interferon has a molecular weight of 21 000 when analyzed by NaDodSO$_4$–polyacrylamide gel electrophoresis. HL-IF produced in the presence of glycosylation inhibitors (tunicamycin or 2-deoxy-D-glucose) contains only the 16 000 molecular weight component (K. C. Chadha, P. M. Grob, R. L. Hamill, and E. Sulkowski, unpublished experiments), and this interferon is not retained when chromatographed on Con A–Sepharose. These results show that the two molecular weight components of HL-IF can be effectively separated by Con A–Sepharose

chromatography. The BT fraction contains exclusively the seemingly nonglycosylated 16 000 molecular weight component, and the α-MM fraction contains essentially the glycosylated 21 000 molecular weight component.

We have demonstrated that the BT and α-MM fractions exhibit considerable cross-species antiviral activity on bovine cells which is a widely recognized property of HL-IF (Gresser et al., 1974). The BT fraction interferon (M_r 16 000) displayed higher antiviral activity on bovine cells than the α-MM fraction interferon (M_r 21 000). In agreement with our observation, Thang et al. (1979) have reported that the 16 000 molecular weight species of HL-IF separated by polynucleotide–agarose affinity chromatography also exhibits a higher antiviral activity on bovine vs. human cells. On the other hand, Paucker et al. (1977) reported no difference in the cross-species antiviral activity of the two molecular weight components of HL-IF separated by sodium dodecyl sulfate–hydroxylapatite chromatography. The methodology employed to separate the HL-IF molecular weight components may account for these differences in the level of cross-species antiviral activity.

The EG fraction from Con A–Sepharose chromatography was also assayed on bovine cells for antiviral activity, and very little protection was observed. Therefore, on the basis of cross-species activity, the interferon in the EG fraction behaves like fibroblast-type interferon. It is important to mention that when HF-IF is chromatographed on Con A–Sepharose, the majority of interferon activity is displaced with eluant containing 50% EG [0.02 M sodium phosphate, 1 M NaCl, 0.1 M methyl α-D-mannoside, and 50% EG (v/v), pH 7.4] (Davey et al., 1974). This observation is consistent with our result that the fibroblast component of HL-IF is enriched in the EG fraction during chromatography on Con A–Sepharose. The possibility that the EG fraction largely constitutes human fibroblast-type interferon was further strengthened by conducting neutralization studies with specific antisera. The majority of interferon activity (70%) in the EG fraction was neutralized by antiserum raised against partially purified human fibroblast interferon, and only 20% was neutralized by HL-IF-specific antisera. The interferon in the EG fraction was completely neutralized when the two antisera were mixed together during the neutralization test. On this basis, it seems that although the EG fraction is largely fibroblast-type interferon, it also contains a fraction of antiviral activity which is similar to leukocyte-type interferon.

Chromatography of HL-IF on Con A–Sepharose not only resolves the two molecular weight components but also separates the fibroblast component into a separate fraction. This chromatographic procedure will facilitate the accurate assignment of biological activities to specific HL-IF interferon components since these components can now be quantitatively separated in the absence of sodium dodecyl sulfate, which when present destroys the native molecular conformation.

Acknowledgments

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