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Sepharose-Immobilized Triazine Dyes as Adsorbants for Human Lymphoblastoid Interferon Purification

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Abstract

An extensive selection of immobilized triazine dyes have been examined for their potential as adsorbants for human lymphobalstoid interferon. Procion red HE7B was selected as the most suitable for preparative scale purification. Sepharose-immobilized procion red HE7B is able to bind 10^5 reference units/mL of interferon from cell supernatants and can be eluted with at least 25-fold purification and 90% yield by a KCl gradient. Further purification was obtained either by reapplying the eluted interferon after dialysis to the dye column or by gel filtration on Ultrogel AcA 34 after lyophilization and dialysis. The latter procedure gave a final activity of about 10^6 U/mg protein and approximately 75% recovery of interferon activity.

Index Entries: Sepharose-immobilized dyes, in interferon purification; immobilized dyes, in interferon purification; triazine dyes, in interferon purification; dyes, Sepharose-immobilized triazine; adsorbants, Sepharose-immobilized dyes as; human lymphoblastoid interferon purification; lymphoblastoid interferon purification, human; interferon, human lymphoblastoid; affinity chromatography.

Introduction

Interferon (IFN)‡ production and purification is currently receiving considerable attention because of the potential IFN appears to have in the therapy of virus infec-

‡Abbreviations: IFN, interferon; HuIFN alpha, human lymphoblastoid interferon; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CPE, cytopathic effect; SFV, Semliki Forest virus.

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tions and some tumors. It seems probable that human leukocyte IFN (predominantly HuIFN alpha) is more useful for clinical applications because of its stability and "non-sticky" nature when compared to fibroblast IFN, though this is still a matter of controversy (*1*).

An IFN antigenically indistinguisable from HuIFN-alpha can be prepared from lymphoblastoid cells grown in suspension culture (*2–4*), thus making possible a large-scale production process. Recently, Secher and Burke (5) have used Sepharose-immobilized mouse monoclonal antibodies against HuIFN alpha Ly to obtain a high degree of purification in one step. HuIFN-alpha has also been purified recently by more conventional chromatographic methods (Fantes et al., personal communication; *6, 7*).

Because of the facility with which it can be produced, and its high stability, we have confined our investigations to human lymphoblastoid IFN deriving from Sendai virus-induced Namalwa cells. In an attempt to provide a rapid and facile partial purification of the IFN from the induced-cell supernatant suitable for economical handling of large volumes, we have examined a variety of Sepharose-immobilized triazine dyes for concentration and partial purification of IFN. Some of these dyes have been shown to be adsorbants capable of binding a variety of proteins, as recently reviewed (*8*), and have the advantage of being low priced. In addition, the chemistry of derivatization is extremely simple in most cases and does not require special equipment or expensive reagents.

Previously, a variety of workers (*9–12*) have attempted to purify both fibroblast and leukocyte interferons on Sepharose-immobilized blue dextran and Sepharose-immobilized Cibacron blue F3GA. After acid ethanol extraction of the original material, purification of up to 1000-fold has been reported by this procedure (*10*), and complete purification of fibroblast IFN (*12*). Sepharose-blue F3GA also exhibits a degree of selectivity between different antigenic species of IFN. As yet, there is no suitable process for fractionation of the multiple forms of HuIFN-alpha Ly. Because of the variety of triazine dyes available, it may be possible to use these for large-scale separation of IFN-alpha subspecies. We have examined the potential of alternative immobilized triazine adsorbants for the initial partial purification of lymphoblastoid IFN.

Materials and Methods

VERO cells and Semliki Forest virus were purchased from the American Type Culture Collection. Media and serum for cell growth were from Flow Laboratories, while tissue culture labware was from Corning. Sepharose 6B-CL was obtained from Pharmacia and Ultrogel AcA 34 from LKB. Reagents for SDS polyacrylamide electrophoresis were supplied by Bio-Rad. All other reagents were of analytical grade from Fisher. Reference molecular weight marker proteins were purchased from Sigma.

Both partially purified and crude human lymphoblastoid IFN from Namalwa cells, which had been induced with Sendai virus, were generous gifts from Drs. Fantes and Johnston, Wellcome Research Laboratories, Beckenham, England.

Crude IFN contained 10% calf serum. Triazine dyes were purchased from Polysciences, Warrington, Pennsylvania, or were samples kindly provided by Imperial Chemical Industries (Procion dyes) or Ciba-Geigy (Cibacron dyes).

Interferon Assay

IFN was assayed by a modification of the dye uptake/CPE reduction method (*13*) The cells used were VERO and the challenge virus was Semliki Forest virus, passaged on mouse L cells. The methodology was essentially as described previously (*1*).

The normal variation of duplicates in a routine assay on the same plate was low ($\pm$ 0.3 $\log_{10}$ units). Variation of the standard between plates assayed on the same day was in general less than this range. NIH reference standard #G-023-901-527 gave a titer between 2.4 and 3.3 $\log_{10}$ units in this system depending largely on the virus concentration.

HuIFN titers described are the results of duplicates or triplicates and are relative to the standard. Errors can be considered to be $<\pm$ 0.2 $\log_{10}$ units.

Adsorbant Preparation

Dye columns for initial screening were prepared by heating Sepharose 6B-CL (2.5 g, wet weight) suspended in a solution of 2% sodium carbonate in water (1.5 mL) at either room temperature or at 60°C. Triazine dye (5 mg) was dissolved in water (0.5 mL) and added to the Sepharose slurry with stirring. The reaction was continued for two hours at room temperature in the case of "M series" Procion dyes or overnight at 60°C in the case of "H series" Procion dyes or Cibacron dyes.

Some dyes were coupled to DEAE cellulose or to Sepharose substituted with 1,3-diamino propane after CNBr activation (*14, 15*). Because of the presence of reactive amino groups on these matrices, 100% coupling of the dye was observed after approximately 1 h at room temperature.

The large-scale preparation of immobilized Procion Red HE7B was essentially similar, particular care being taken with stirring to minimize damage to the beads. A caged stirrer bar (Fisher) was very effective for minimizing bead damage while maintaining a reasonable stirring rate of the thick slurry. Sepharose 6B-CL (200 mL) slurried to a final volume of 250 mL in a final concentration of 1% sodium carbonate was heated to 60°C while being stirred and a solution of red-HE7B in water (100 mg/mL, 5 mL) was added dropwise over 5 min. After overnight reaction, the gel was washed extensively with water and 1*M* KCl to give a final dye substitution of approximately 2 mg/mL, monitored by absorption at 540 nm (Procion red HE7B, 1 mg/mL at pH 9, has an absorbance of 29 optical density units at 540 nm). Stability of the matrix was good, with less than 1% leakage of ligand when stored over 8 weeks at room temperature. To maximize flow rates, fines were removed exhaustively before using the gel in a chromatography column.

Protein Assay

Protein was assayed by the Coomassie blue method of Bearden (*16*) or by measuring the absorbance at 280 nm.

Polyacrylamide Gel Electrophoresis

The gel electrophoretic analyses were performed by the method described earlier (*17*). Acrylamide gels (12.5%) were cast (10 × 14 × 0.3 cm) in a slab gel apparatus (Bio-Rad). Gels were electrophoresed at 25 V overnight and stained for 12 h with 0.1% Coomassie blue R-250 in acetic acid/isopropanol/water (1 : 2 : 7). For IFN assay, gels were sliced into 1.5 mm sections after staining for 1 h. Slices were rinsed in Tris-HCl (10 m*M*, pH 7.4) and then homogenized in 2 vol Tris-HCl (10 m*M*, pH 7.4) and left overnight to elute. The supernatants were then assayed for IFN as described. Reference proteins used in estimating the molecular weights were bovine γ-globulin, bovine serum albumin, lysozyme, equine muscle myoglobin, and cytochrome c.

Results and Discussion

Interferon Adsorption

Initial screening of 0.7 mL immobilized dye packed in polypropylene columns (Bio-Rad) to which 0.7 mL crude IFN (approximately 10^3 ref. units) had been applied at room temperature is shown in Table 1 (a). The void volumes and washings (3 mL) of the individual columns were assayed for IFN, and the adsorbants giving greater than 80% retention of IFN were rescreened with fresh gels using 10 mL (20,000 ref. units) crude IFN. The percentage of interferon retained on these columns is shown in Table 1 (b). More than 85% of the applied interferon was adsorbed by red HE7B, red HE3B, violet H3B and blue F3GA.

Interferon binding to immobilized HE3B and HE7B did not appear to be highly dependent on temperature. However, capacity did appear to be up to twofold higher under some conditions when these columns were run at room temperature rather than at 4°C. As a result, these columns were run at room temperature, the stability of HuIFN-alpha making this no particular disadvantage.

A single bead of Sepharose-immobilized HE7B or HE3B, to which IFN had been adsorbed and unbound material washed off, was adequate to ensure complete protection of confluent VERO cells from virus induced CPE when placed in a well of a microtiter plate. No such protection was observed with control beads that had not been exposed to IFN or with beads that had been exposed to IFN, but had been eluted with 1 *M* KCl, 0.1% SDS, or 1 *M* potassium thiocyanate, indicating that IFN attached to the beads is still free to interact with its receptor, either directly or by leakage off the matrix.

Interferon Elution

IFN could be eluted quantitatively from Sepharose-immobilized red HE7B or HE3B by either 0.1% SDS, 10 m*M* sodium dithionite, 1 *M* thiocyanate, or 1 M KCl.

TABLE 1
The Adsorption of Interferon to a Variety of Immobilized Triazine Dyes[a]

Dye	Interferon bound, % (a)	(b)	Dye	Interferon bound, % (a)	(b)
Procion H series			*Procion M series*		
Red-H8B	93	86	Red-MX5B	50	
Red-H8BN	68		Red-MX8B	68	
Red-H3B	60		RV-MXB	90	55
			Yellow-MXR	29	
Red-H3B (c)	37		Yellow-MX6G	50	
Red-H3BN	60		Yellow-M4RF	44	
Red-HE3B	94	94	Yellow-M4RAN	37	
Red-HE7B	95	95	Yellow-MXGR	72	
Brown	44		Yellow-MX4R	80	
Black	72		Yellow-MX4G	—	72
Orange-H2R	72		Orange-MXR	60	
Turquoise-H5G	80		Orange-MX2R	77	
Violet-H3R	89	89	Blue-MXR	92	82
Cibacron dyes			Blue-MXR (c)	0	
F3GA	86	89	Unsubstituted	0	0
F3GA (d)	0		Sepharose		
BRP	84	84	6B-CL		
BRP (d)	0				
4GP		61			
4GP (d)	60				

[a]All column dimensions were 0.7 × 1.8 cm. (a) Approximately 1000 units of crude IFN applied in 0.7 mL. (b) Approximately 20,000 units of crude IFN applied in 10 mL. (c) Coupled to propylamine–Sepharose. (d) Coupled to DEAE–cellulose.

Gradient elution on a semipreparative-scale column (2.5 × 40 cm) of immobilized HE7B to which approximately 10^7 ref. units of IFN had been adsorbed gave a single broad peak of protein, with the interferon activity eluting after the major portion of the peak (Fig. 1). The pooled IFN activity in the peak gave a specific activity of approximately 10^5 reference units/mg protein from an original activity of 3×10^3 reference units/mg.

Application of partially purified IFN to a 10 mL Sepharose-immobilized HE7B or immobilized HE3B column, followed by stepwise elution of the column with 0.1*M* KSCN and 0.5*M* KSCN indicated a clear difference in the retention characteristics between these two matrices (Table 2). On the HE3B column, the majority of adsorbed IFN was eluted with 0.1*M* thiocyanate, whereas on the HE7B column, 0.5*M* thiocyanate was necessary to elute the IFN. Because of the greater affinity of the immobilized HE7B for IFN, this matrix was used for further analysis of the purification process.

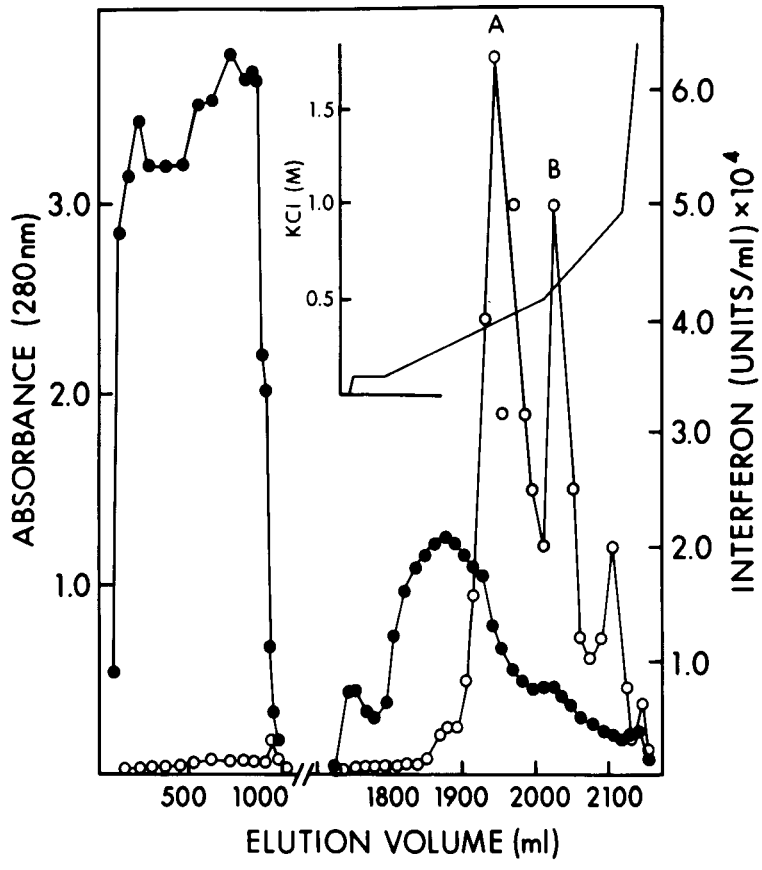

Fig. 1. Initital purification of human lymphoblastoid IFN on Sepaharose 6B-CL immobilized Procion red HE7B. Crude IFN (800 mL, 1.3 × 10^4 ref. units/mL from virus induced Namalwa cell supernatant was applied to a 1.6 × 40 cm Sepharose-HE7B column at room temperature, with a flow rate of 80 mL/h. The column was washed with 600 mL of 10 m*M* Tris-HCl buffer pH 7.4, followed by 0.1 *M* KCl in Tris-HCl buffer (80 mL) and was eluted with a 0.1–1 *M* KCl gradient with a total volume of 350 mL. Fractions (13.5 mL) were assayed for IFN (○), the absorbance at 280 nm was measured (●), and the conductivity measured to give KCl concentration (solid line). Peaks A and B were used for detailed analysis on SDS-PAGE (Fig. 2) and on gel filtration on Ultrogel AcA 34 (Fig. 3).

Electrophoretic Analysis of Eluted Interferon

SDS-Polyacrylamide gel electrophoresis analysis of the two IFN activity peaks after dialysis and lyophilization (fractions A and B in Fig. 1) on a 12.5% slab gel, indicated that all the major contaminants consisted of proteins of molecular weight greater than 40,000. The IFN activity in fraction B (Fig. 1) migrated with a molecular weight in the region of 21–23,000 (Fig. 2). The IFN activity present in the earlier fraction (fraction A) appeared to have a slightly higher molecular weight and was rather more heterogeneous than that in the later fraction (B). It was also observed that bovine serum albumin (the major band in the crude material) was not

TABLE 2
A Comparison of Immobilized HE7B and HE3B as Absorbants for HuIFN-alpha Ly

Experimental conditions	Units of Interferon,%	
	HE3B	HE7B
Breakthru fractions	2.2×10^5 (17.4%)	1.3×10^5 (15.2%)
Eluted with 0.1 *M* KSCN	8.9×10^5 (70.6%)	3.8×10^5 (25.2%)
Eluted with 0.5 *M* KSCN	1.5×10^5 (11.9%)	10×10^5 (66.2%)

Partially purified IFN (10 mL, 0.1 mg protein/mL, and approximately 10^5 U/mL) was applied to 10 mL columns of either Sepharose-immobilized Procion red HE7B or HE3B, washed with three column volumes of buffer (10 m*M* Tris-HCl, pH 7.4) and the unbound IFN assayed as described. The columns were then eluted with two column volumes of 0.1 *M* KSCN followed by the same volume of 0.5 *M* KSCN and the eluates assayed for IFN activity as described in the text.

retained by the immobilized dye, whereas a protein with a molecular weight of approximately 40,000 was adsorbed with a high degree of specificity (the major band in Fig. 2b).

Gel Filtration Analysis of Eluted Interferon

Portions of the two IFN activity peaks (fractions A and B, Fig. 1) were lyophilized, redissolved, and further purified on an Ultrogel AcA 34 column (1.5 × 20 cm) equilibrated with 10 m*M* Tris-HCl, pH 7.4, containing 0.1% SDS. The IFN activity was separated from the majority of proteins to give a peak specific activity of at leat 1.2×10^6 reference units/mg protein (Fig. 3). Consistent with the results on SDS-PAGE, the IFN activity in the earlier fraction was slightly more heterogeneous than that in the later fraction, but the main activity peaks in both cases migrated in the same region with an apparent molecular weight of about 20,000 daltons.

Reapplication of IFN eluted from the immobilized dye column, onto the same column washed with 2*M* KCl and re-equilibrated, resulted in a further purification of the interferon (data not shown). All of the protein and IFN activity in the sample was adsorbed and could be eluted quantitatively with a KCl gradient. The majority of the contaminating proteins again eluted prior to the interferon, giving a final IFN purity of 5×10^6 reference units/mg protein.

The various triazine dyes that we have used to screen adsorption and purification of IFN have markedly different structures, the only feature in common being the presence of one or more reactive chlorine groups in the triazine residue, which participates in the coupling, and a planar series of conjugated aromatic groups, with or without associated metal ions. In all triazine dyes there are hydrophobic regions as

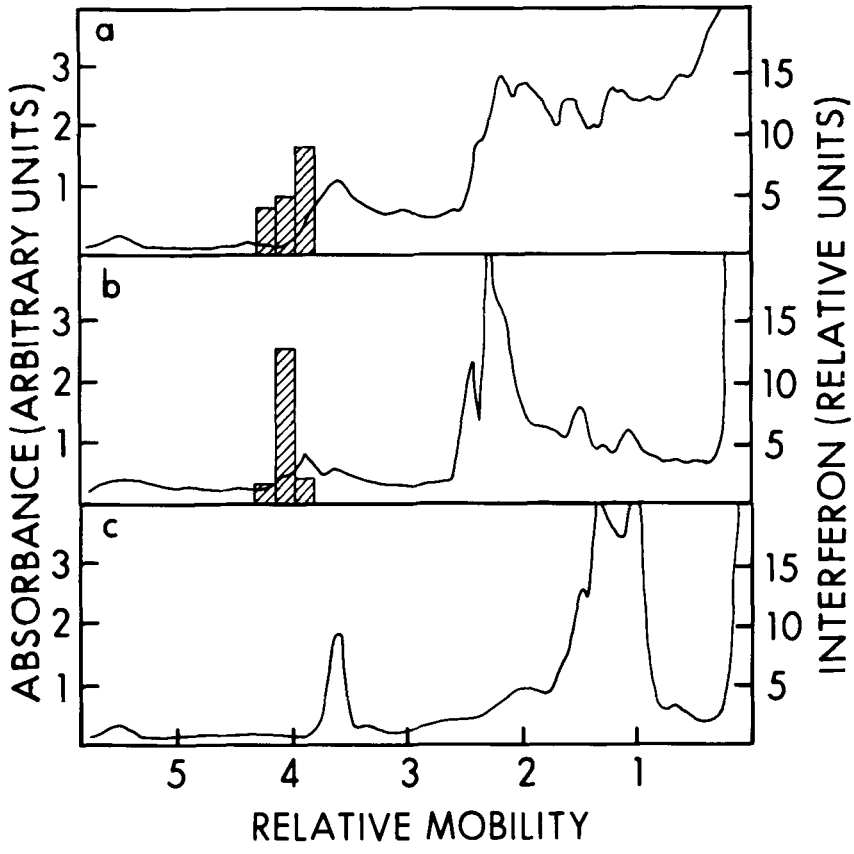

Fig. 2. SDS-PAGE analysis of fractions eluted from Sepharose HE7B. Peaks A and B eluted from immobilized HE7B (Fig. 1) were dialyzed against 5 m*M* Tris-HCl, pH 7.4, and lyophilized. Samples were then applied to 3-mm thick 12.5% polyacrylamide slab gels and electrophoresed for 20 h at 30 V. Gels were either stained with Coomassie blue G 250 or cut into 1.5 mm slices and assayed for IFN (cross-hatched area). Stained gels were scanned on a soft laser scanning densitometer (Biomed Instruments, Chicago). (a) Peak A (Fig. 1). (b) Peak B (Fig. 1). (c) Original crude IFN sample prior to adsorption on the Sepharose dye column.

well as charged residues, such as sulfonyl groups, the properties of either of which can contribute to the overall characteristics of the immobilized dye as an adsorbant. Thus, fortuitously, any particular dye may have some specificity for certain regions of a biological macromolecule.

Among the variety of dyes screened for purification of HuIFN-alpha, immobilized Procion red HE7B, and HE3B seemed to be the most promising adsorbants.

The concentration at which a purely hydrophobic ligand has been shown to be effective is in the range of 30 m*M* (*18*). Although the molecular structure and therefore the exact molarity of the dyes bound to Sepharose is not available, the ligand on the gel is probably in the range of 1–5 m*M* at the concentration used (2 mg/mL), which would suggest that the interaction is largely ionic. This is further confirmed by the elution of the IFN at high salt concentrations. The dye-IFN interaction is probably a combination of hydrophobic and electrostatic effects, the latter predom-

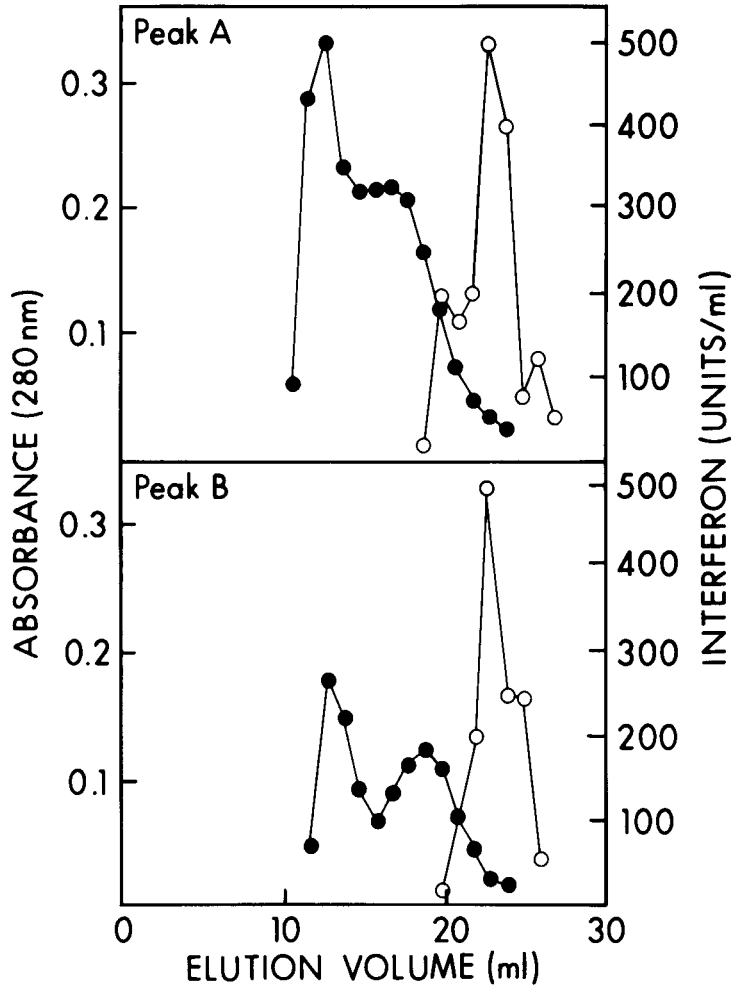

Fig. 3. Gel filtration of IFN samples A and B eluted from Sepharose HE7B. Peaks A and B eluted from the immobilized HE7B column (Fig. 1) were lyophilized, dissolved in 1 mL water, and applied separately to an Ultrogel AcA 34 column (1.5 × 20 cm), equilibrated with 10 m*M* Tris-HCl, pH 7.4, containing 0.1% SDS. Fractions (1 mL) were collected and assayed for IFN (○) and the absorbance at 280 nm was measured (●).

inating because of the large number of sulfonyl groups present in these dyes. The slightly increased capacity for IFN observed with immobilized HE7B at room temperature compared to that at 4°C may indicate that there is a degree of hydrophobicity in the interaction.

Of the two dyes (HE3B and HE7B) with the greatest potential for adsorption and purification of HuIFN-alpha Ly, HE7B was found to be better for purification purposes because of the higher salt concentration required for elution, which facilitates the separation of IFN from contaminating proteins.

Procion red HE7B immobilized to Sepharose 6B-CL at approximately 2 mg/mL has a marked affinity for human lymphoblastoid IFN (Fig. 1). While only 20% of the protein from the crude cell supernatant is adsorbed, more than 90% of the IFN

binds to the column, with a useful capacity of approximately 5×10^5 reference units IFN/mL adsorbant. Capacity may be higher when serum-free crude IFN is adsorbed (Neame & Acton, unpublished results).

Although IFN can be eluted with approximately 0.5 m*M* KCl, optimum resolution from its contaminants can be obtained by using a 0.1–1 *M* KCl gradient. This method gives at least a 25-fold purification with close to 100% recovery of IFN activity. IFN eluted by a salt gradient appears to resolve into at least two components. That these are not artefacts is indicated by the reproducibility of this observation on reloading the peaks onto the same column. The appearance of other minor peaks that are not as consistent are probably a result of the inherent lack of accuracy in the assay. Analysis of the A and B peaks on SDS-PAGE (Fig. 2) indicate little difference, if any, in the molecular weight, but a slight difference in the degree of heterogeneity of IFN based on the position of the IFN activity. This may reflect either differences in the nature and/or extent of the glycosylated residues of the IFN present in the two peaks or that there are multiple forms of IFN within any one type, as evidenced through the techniques of amino acid (*19*) and DNA (*20, 21*) sequencing.

It will be noted from the acrylamide gels that the major contaminants of the IFN containing fractions are of considerably higher molecular weight and thus further purification of IFN was achieved by gel filtration on an Ultrogel AcA 34 column (Fig. 3). Similar elution profiles are obtained whether SDS is used in the eluting buffer (as shown) or left out.

The final purity of IFN obtained in a semipreparative purification on a dye column, followed by Ultrogel gel filtration (data not shown) was in the region of 1.5×10^6 reference units/mg protein with at least 80% overall recovery. Alternatively, as mentioned earlier, the eluted IFN can be reapplied to the Sepharose-immobilized dye column to obtain a similar purity. Greater resolution, and therfore greater purity, could probably be obtained with longer columns, and can be obtained with serum-free preparations (*22*).

The data presented in this communication clearly show the potential of a few Sepharose-immobilized triazine dyes as adsorbants for IFN purification. It remains to be seen whether there is a fundamental difference in the structure or biological activity of the IFN in the two peaks resolved on the dye column. It is unlikely that either peak is HuIFN-beta (a minor component of lymphoblastoid IFN) because of their chemcial stabilities under a variety or conditions. It is possible that they represent some of the components of different gene products (*19–21*). More detailed experiments to determine the characteristics of the two components and potential of alternative dyes to separate them further are in progress.

Acknowledgments

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