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Purification of two subspecies of human γ (immune) interferon

(lymphokines/12-*O*-tetradecanoylphorbol 13-acetate/phytohemagglutinin/liquid chromatography/sodium dodecyl sulfate/polyacrylamide gel electrophoresis)

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ABSTRACT **Interferon (IFN)-γ was produced in cultures of human leukocytes by combined stimulation with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and phytohemagglutinin (PHA). IFN-γ was purified by sequential adsorption and elution from controlled-pore glass and concanavalin A-Sepharose and by subsequent adsorptive removal of contaminating proteins on DEAE-Sephacel at pH 8.0. Treatment of such partially purified IFN-γ preparations with the anionic detergent $NaDodSO_4$ (0.1% at 20–25°C) decreased biological activity to approximately 5–20%. When analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis the bulk of IFN activity not destroyed by $NaDodSO_4$ treatment was recovered from two peaks with apparent molecular weights of 20,000 and 25,000. The two activity peaks showed close correspondence with Coomassie blue-stained bands regularly demonstrable in purified supernatants from induced cultures but absent from culture supernatants from uninduced cells. Available evidence suggests that the two bands, isolated in pure form, represent subspecies of IFN-γ. Native IFN-γ was found to have a lower affinity for alkyl agarose columns than human IFN-α or IFN-β did, suggesting that IFN-γ is a relatively hydrophilic protein. Sulfhydryl-specific binding of native IFN-γ to an Affi-Gel 501 column suggested that this IFN contains free sulfhydryl.**

Until recently virtually no information was available about physicochemical properties of the interferon (IFN) species produced in human lymphocytes upon stimulation with specific antigens (1) or with various other mitogens (2, 3). The identification of mitogen-induced IFN as IFN-γ and its differentiation from the other two major species, termed IFN-α and IFN-β (4), is based mainly on antigenic differences among the three IFN species and on the known relative instability of IFN-γ at pH 2 (3).

The scarcity of information about IFN-γ was mainly due to the lack of suitable methods for its production and purification. Recently, we reported a method of human IFN-γ production based on combined stimulation of human lymphocytes with a phorbol ester (12-*O*-tetradecanoylphorbol 13-acetate, TPA) and the T cell mitogen phytohemagglutinin (PHA) (5, 6). This method enables the production of IFN-γ preparations with relatively high initial specific activity. The use of lymphocyte-rich plateletpheresis residues as the cell source for production of IFN-γ makes it possible to prepare batches yielding several liters of starting material for purification. We showed earlier that a relatively simple three-step purification procedure led to approximately a 1,000-fold purification of IFN from this starting material (7).

Another factor that contributed to the successful purification of IFN-γ described in this paper was the recent demonstration that the biological activity of human IFN-γ was not completely destroyed by treatment with $NaDodSO_4$ (8). Residual biological activity after $NaDodSO_4$ treatment could thus be used as a marker for the identification of IFN-γ proteins under conditions precluding noncovalent interactions among protein molecules. Published estimates of the molecular weight of IFN-γ established by various modifications of molecular sieve chromatography range from about 35,000 to 70,000 (7, 9–12). However, when analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis the bulk of residual IFN activity not irreversibly destroyed by $NaDodSO_4$ treatment was recovered from two peaks with estimated molecular weights of 20,000 and 25,000 (8). Activity recovered from both peaks was identified as IFN-γ by specific neutralization with anti-IFN-γ serum. We concluded that in native form human IFN-γ may be aggregated, possibly forming dimers of the 20,000 and 25,000 species. We now report the complete purification of two protein bands with molecular weights of 20,000 and 25,000, both of which have IFN-γ activity associated with them.

MATERIALS AND METHODS

Production of Human IFN-γ. Details of the production method have been described elsewhere (6, 7); the method is based on the enhancing effect of TPA on IFN-γ production induced by PHA in human lymphocyte cultures (5). Briefly, plateletpheresis residues (kindly provided by A. Waldman and M. Wiebe of the New York Blood Center) were washed and seeded at 6×10^6 leukocytes per ml in serum-free RPMI 1640 medium containing TPA (20 ng/ml). After 2- to 3-hr incubation at 37°C in a humidified CO_2 incubator, PHA was added to a final concentration of 5 μg/ml, and the incubation was continued for 48–72 hr. The culture medium containing IFN-γ was then collected and centrifuged to remove cells and cell debris. Crude IFN-γ preparations were stored at 4°C until further processing.

Purification of Human IFN-γ. Human IFN-γ was purified by sequential adsorption and elution on controlled-pore glass (CPG) and concanavalin A (Con A)-Sepharose as described (7). Instead of using molecular-sieve chromatography as a subsequent purification step as described in the original procedure (7), we employed a simpler method based on the adsorptive removal of contaminating proteins on DEAE-Sephacel (Pharmacia). IFN-γ eluted from Con A-Sepharose was equilibrated to pH 8.0 by exhaustive dialysis in 20 mM Tris·HCl. DEAE-Sephacel equilibrated with the same buffer was added to the IFN-γ sample at a ratio of 1:10 (vol/vol). The mixture was allowed to equilibrate at 4°C for 15 min with occasional gentle mixing, and DEAE-Sephacel was then removed by centrifugation at $1,000 \times g$ for 10 min.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Electrophoresis was carried out on linear 10–16% acrylamide gradient slab gels, using the Laemmli procedure (13). IFN-γ samples

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Abbreviations: IFN, interferon; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PHA, phytohemagglutinin; CPG, controlled-pore glass; Con A, concanavalin A.

used for electrophoretic analysis were purified by the three-step procedure outlined above. Samples were then equilibrated to pH 6.8 by dialysis against 5 mM Tris·HCl, and concentrated by dialysis in Aquacide II (Calbiochem). For treatment with $NaDodSO_4$, sample solutions were adjusted to contain 0.1% (wt/vol) of the detergent by adding the appropriate volume of a 1% stock solution. Samples were incubated for 10–60 min at 20–25°C before being applied to the gels for electrophoresis. Slab gels with sample wells at least 1 cm in width were always used; this experimental design was intended to facilitate direct correlation of the IFN activity profile with the Coomassie blue-stained protein profile. Upon completion of electrophoresis, the gel slab was cut into 1-mm slices with the aid of a gel slicer using conventional razor blades as cutting edges (Hoefer, San Francisco, CA). About three-quarter portions of each slice were used for assay of IFN activity; the remaining cut portions, still attached to the gel slab with the molecular weight markers, were stained with Coomassie blue (see Fig. 1 for illustration). The procedure for elution of IFN for assay of activity was similar to that described for cylindrical polyacrylamide gels (15), except that freezing of the gel slab was not necessary before slicing.

Chromatography on Affi-Gel 501. Affi-Gel 501 (Bio-Rad) was packed in a plastic column and equilibrated to pH 6.0 by washing with 10 mM sodium acetate buffer. CPG-purified IFN-γ, equilibrated with the column buffer, was used for analysis of the sulfhydryl-specific binding on this column. After sample application, the column was washed with 4 vol of the column buffer. IFN-γ was eluted by a step-concentration gradient of dithiothreitol in the column buffer (see Fig. 3 for details).

Chromatography on Alkyl Agarose Columns. The hydrophobic chromatography kit as supplied by Miles consisted of six plastic columns each packed with 1 ml of agarose beads with immobilized hydrocarbon arms containing 0, 2, 4, 6, 8, and 10 carbon atoms, respectively. Before sample application, each column was equilibrated to pH 6.0 with 10 mM sodium acetate buffer containing 0.5 M NaCl. Partially purified human IFN-α (provided by B. Horowitz of The New York Blood Center), partially purified IFN-β (prepared by Rentschler Arzneimittel, Laupheim, Federal Republic of Germany), and CPG-purified IFN-γ prepared in this laboratory, each equilibrated with the column buffer, were used for binding to the alkyl agarose columns. A fresh set of columns was used for each of the IFNs.

Assay for IFN-γ Activity. The antiviral activity of IFN-γ was assayed by inhibition of the cytopathic effect of encephalomyocarditis virus in human FS-7 fibroblasts in 96-well plastic tissue culture plates (7). Titers of IFN-γ are expressed in laboratory units. The assay for IFN-α and IFN-β was performed by a similar method using FS-7 fibroblasts and vesicular stomatitis virus. IFN-α and IFN-β titers are expressed in international units.

RESULTS

Association of IFN Activity with Coomassie Blue-Stained Bands on $NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Earlier experiments from this and other laboratories showed that the activity of human IFN-γ was irreversibly inactivated after treatment with the anionic detergent $NaDodSO_4$ (6, 12). Inactivation by $NaDodSO_4$ precluded molecular weight determination by $NaDodSO_4$/polyacrylamide gel electrophoresis. In contrast, $NaDodSO_4$/polyacrylamide gel electrophoresis proved very useful for analytical work with IFN-α and IFN-β species because the biological activity of these IFNs is not irreversibly inactivated after such treatment (3).

Recently we observed that treatment of highly concentrated and partially purified IFN-γ preparations with 0.1% $NaDodSO_4$ at a temperature of 20–25°C did not cause complete inactivation (Table 1). Up to 20% of the original biological activity was preserved after such treatment. It thus became possible to ana-

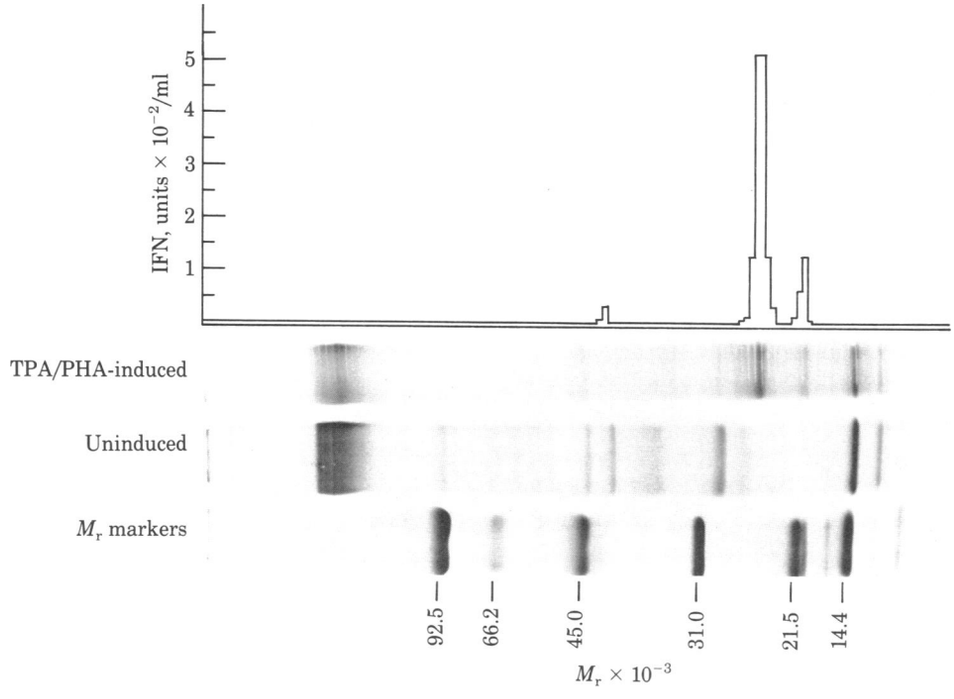

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoresis analysis of partially purified proteins from uninduced and TPA/PHA-induced cultures. Media from both cultures were purified by the three-step procedure described in *Materials and Methods*. Protein concentration of the partially purified and Aquacide II-concentrated samples was determined by the Lowry method (14). IFN activities of the TPA/PHA-induced sample before and after $NaDodSO_4$ treatment were 198,400 and 32,720 units/ml, respectively; the uninduced sample had no detectable IFN activity before or after $NaDodSO_4$ treatment. Approximately 50 μg of protein from each sample (1,840 residual IFN units from the TPA/PHA-induced sample) was applied for electrophoresis. Recovery of IFN activity was approximately 1,340 units from the M_r 25,000 band, 210 units from the M_r 20,000 band, and 40 units from the M_r 40,000 position.

Table 1. Recovery of human IFN-γ activity after $NaDodSO_4$ treatment

Exp.	IFN, units/ml: Starting material	IFN, units/ml: After $NaDodSO_4$ treatment	% of starting activity
1	25,600	160	0.6
2	6,400	20	0.3
3	198,000	15,360	7.7
4	768,000	20,480	2.7
5	409,600	10,240	2.5
6	460,000	15,360	3.3
7	793,000	30,720	3.8
8	198,400	30,720	15.5
9	172,800	7,360	4.2
10	2,048,000	409,600	20.0

$NaDodSO_4$ was added to the IFN-γ sample to a final concentration of 0.1%. The incubation was at temperatures between 20°C and 25°C for 1 hr in Exps. 1–6, and for 10 min in Exps. 7–10.

lyze IFN-γ by $NaDodSO_4$/polyacrylamide gel electrophoresis. In an earlier study we established that IFN-γ activity after $NaDodSO_4$/polyacrylamide gel electrophoresis was recovered mainly from two regions of the gel, corresponding to molecular weights of 20,000 and 25,000 (8). However, no attempt was made in our earlier study to correlate IFN activity with protein bands demonstrable by Coomassie blue staining.

In the experiment shown in Fig. 1, two materials were analyzed in parallel on a $NaDodSO_4$/polyacrylamide gel. One material was a preparation of IFN-γ produced by the usual method employing TPA and PHA induction. This material was purified by sequential processing on CPG, Con A-Sepharose, and DEAE-Sephacel before $NaDodSO_4$/polyacrylamide gel electrophoresis. Another control material was prepared in parallel by using cultures established from the same batch of human plateletpheresis residues. These cultures were incubated for the same period of time and under the same conditions as the induced cultures except that no TPA or PHA was added. The supernatant from the control cultures was then processed in exactly the same way as the supernatant from TPA and PHA-induced cultures. Multiple stained bands were discernible on the gels run with induced or uninduced purified culture supernatants. Several bands apparent in the material from the uninduced cultures were absent or reduced in the induced supernatant, suggesting that induction with TPA and PHA suppressed the synthesis of some proteins. In contrast, some bands were present only in the induced culture supernatant. One was a 20,000 molecular weight stained band showing complete correspondence with IFN activity recovered from the same region of the gel. Another, greater, peak of IFN activity with an apparent molecular weight of approximately 25,000 also showed good correspondence with a relatively heavy Coomassie blue-stained band in the same position. A small peak of IFN activity was found in gel fractions corresponding to a molecular weight of approximately 40,000. This higher molecular weight material, quite regularly demonstrable on $NaDodSO_4$/polyacrylamide gel electrophoresis, probably represents traces of undissociated native IFN-γ (8). No stained protein band corresponding to this IFN activity peak was discernible.

Isolation of Two Subspecies of IFN-γ. To further substantiate the association of IFN-γ activity with Coomassie blue-stained bands, another preparation of IFN-γ purified by sequential processing on CPG, Con A-Sepharose, and DEAE-Sephacel was processed by $NaDodSO_4$/polyacrylamide gel electrophoresis, A portion of the gel slab was sliced in order to determine the position of IFN activity. Another small portion of the gel with molecular weight markers was stained with Coomassie blue. The remaining portion of the gel was kept frozen until the positions of the 20,000 and 25,000 molecular weight bands of IFN activity were determined by biological assay. Thereafter, proteins eluted from gel slices containing the 20,000 and 25,000 IFN activity bands were re-electrophoresed separately on another slab gel (Fig. 2). On the second Na-

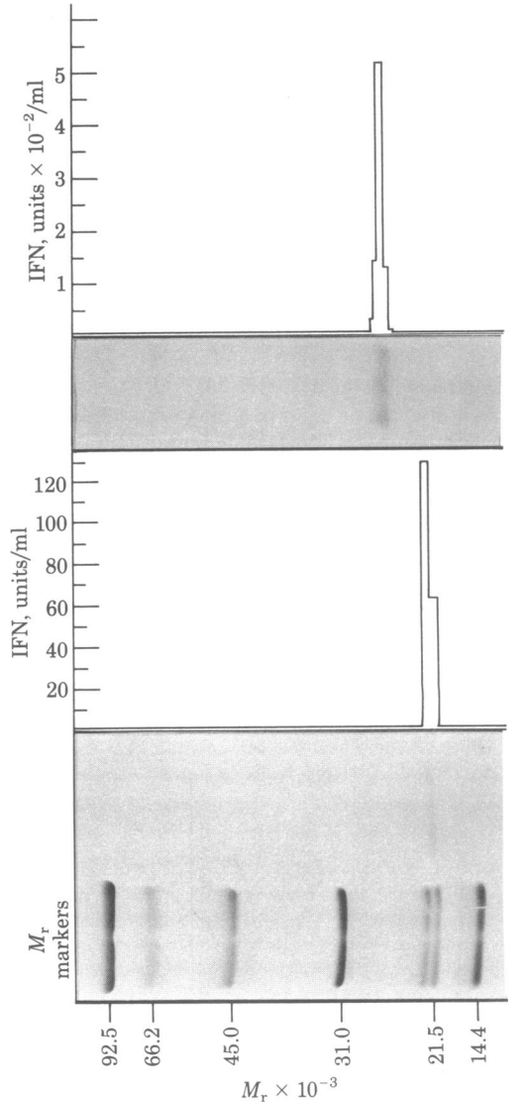

FIG. 2. Isolation of the 20,000 and 25,000 molecular weight subspecies of IFN-γ by $NaDodSO_4$/polyacrylamide gel electrophoresis. Human IFN-γ processed by the three-step procedure was treated with 0.1% $NaDodSO_4$, and 0.6 ml of the sample solution, containing a total of 4,400 residual IFN units, was applied to a preparative slab gel 1.5 mm in thickness with sample well of 9 × 2 cm. Upon completion of electrophoresis, a strip of the gel approximately 1.5 cm in width was sliced for assay of IFN activity and for staining of proteins; the remaining portion of the gel was kept frozen at −70°C without fixing or staining. After the positions of IFN activity recovered (1,890 units from the 25,000 M_r region and 550 units from the 20,000 M_r region) had been identified the corresponding slices from the frozen gel were excised for re-electrophoresis on a second $NaDodSO_4$/polyacrylamide gel. Gel slices from the 25,000 M_r region and from the 20,000 M_r region were pooled separately and homogenized with a mortar and pestle. The homogenized gel pieces were taken up in 0.2 ml of 5 mM Tris·HCl, pH 6.8, containing 0.1% $NaDodSO_4$ for transfer to the sample wells. Each sample was run in duplicate, one for the assay of IFN activity and the other for the staining of proteins. Final recovery of IFN activity was 1,100 units from the 25,000 M_r band and 340 units from the 20,000 M_r band.

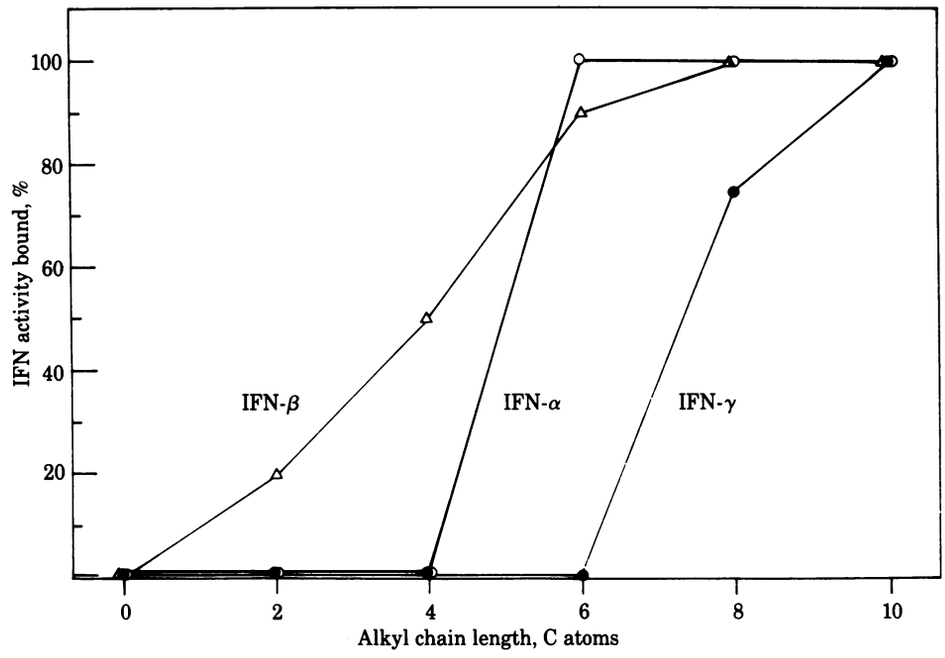

FIG. 3. Chromatography of human IFN-α, IFN-β, and IFN-γ on alkyl agarose columns. IFN samples, 1 ml each, were applied to each alkyl agarose column. IFN-α = 819,000 units/ml; IFN-β = 102,400 units ml; and IFN-γ = 51,200 units/ml. After application of the samples, IFN activity was determined in the flowthrough and the amount of IFN activity bound to each column was calculated.

$DodSO_4$/polyacrylamide gel each of the two isolated proteins yielded a single peak of IFN activity and a single stained band with apparent molecular weights of 20,000 and 25,000, respectively. As in the first $NaDodSO_4$/polyacrylamide gel electrophoresis analysis (not shown), more IFN activity and more stainable protein was present in the 25,000 molecular weight band than in the 20,000 molecular weight band. Available evidence suggests that these two bands represent pure subspecies of IFN-γ (see also *Discussion*).

Relative Hydrophobicity and Binding of IFN-γ to a Sulfhydryl-Specific Affi-Gel 501 Column. Earlier work by Sulkowski and coworkers (16, 17) showed that human IFN-α and IFN-β differ in their relative hydrophobicity, as demonstrable by their binding to and elution from various hydrophobic ligands. In general, IFN-β is known to be more hydrophobic than IFN-α when analyzed under similar conditions. Because no information was available about the relative hydrophobicity of human IFN-γ, we compared the binding of IFN-α, IFN-β, and IFN-

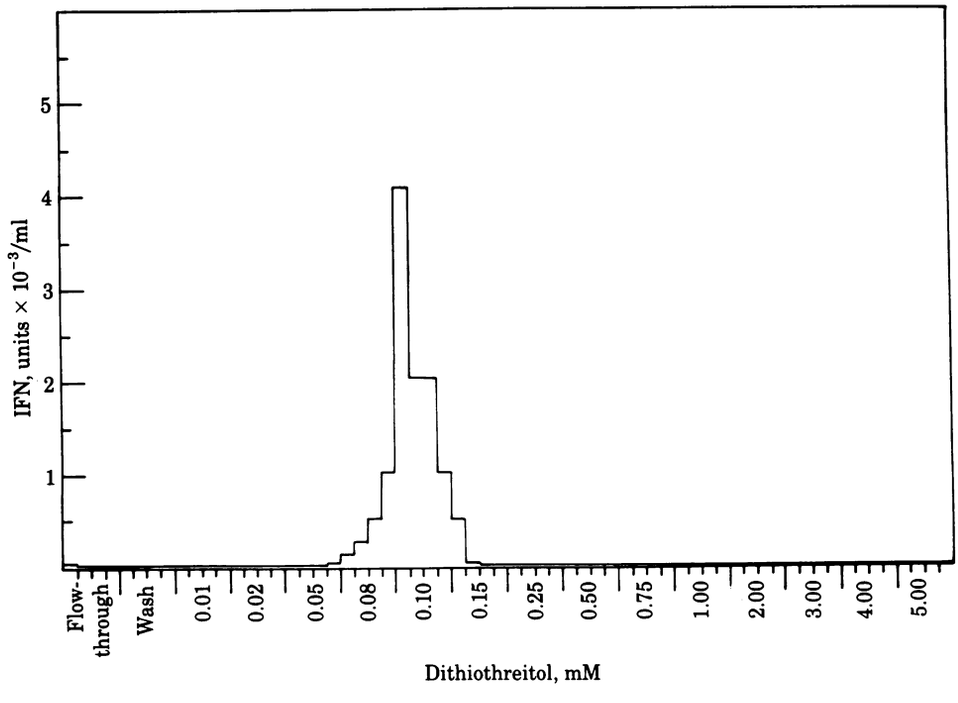

FIG. 4. Chromatography of human IFN-γ on Affi-Gel 501. A plastic column 1 cm in diameter was packed with Affi-Gel 501, 1 ml in bed volume. A 4-ml sample of CPG-purified IFN-γ containing a total of 163,840 units was applied to the column, and the flowthrough was collected in 1-ml fractions. After application of the IFN sample, the column was washed four times, each with 1 ml of the column buffer. For elution of IFN-γ, a step gradient of dithiothreitol from 0.01 to 5.0 mM was used; 1 ml of buffer of each concentration was applied to the column four times each. IFN-γ was eluted within a narrow concentration range of 0.08–0.10 mM dithiothreitol; a total of 117,360 units (72%) was recovered from the column.

γ on alkyl agarose columns with various alkyl chain lengths (Fig. 3). In general, more highly hydrophobic proteins show avid binding to agarose columns with short-chain alkyl groups, whereas less hydrophobic proteins bind only to columns with longer-chain alkyl residues. A comparison of the binding of the three human IFNs showed that IFN-γ was the least hydrophobic species, with complete binding observed only on decyl-agarose columns. Hydrophobic chromatography thus provides additional means for the purification of IFN-γ and for its separation from the IFN-α and IFN-β species.

The possible presence of a free sulfhydryl group on the molecule of human IFN-γ was analyzed indirectly by the ability of this IFN to bind to a sulfhydryl-specific Affi-Gel 501 column (Fig. 4). All IFN activity applied to the column bound and could be recovered in the eluate obtained with 0.08–0.10 mM dithiothreitol. The fact that IFN activity was recovered in a homogeneous peak suggests that all IFN-γ molecules are similar in their content of free sulfhydryl groups. This procedure could also be employed as a purification step for IFN-γ.

DISCUSSION

Although gel filtration analysis of native human IFN-γ suggested that the mass of this molecule is in the range of 40,000–60,000 daltons (7, 9–12), $NaDodSO_4$/polyacrylamide gel electrophoresis analysis revealed two major peaks of IFN-γ activity with apparent molecular weights of 20,000 and 25,000 (8). In this paper we report the complete purification of the two molecular weight forms of IFN-γ. The reason for the difference in the apparent molecular weight of human IFN-γ when analyzed by molecular sieve chromatography and $NaDodSO_4$/polyacrylamide gel electrophoresis is not yet completely understood. Other IFNs generally do not show this type of behavior. However, Knight and Fahey (18) recently reported on a 40,000 molecular weight form of human IFN-β that could be converted into the usual 20,000 molecular weight monomeric form by heating in $NaDodSO_4$ and thioglycolic acid. One obvious possibility is that in native form IFN-γ is an aggregate, possibly a dimer composed of the 20,000 and 25,000 subunits. (The dimer could be composed of identical or nonidentical subunits.)

Another possibility, which cannot be completely ruled out, is that the 20,000 and 25,0000 molecular weight species represent minor components and that the bulk of IFN-γ activity associated with the higher molecular weight form is irreversibly inactivated by $NaDodSO_4$ treatment. However, this is an unlikely possibility in view of the fact that a small amount of IFN activity could be recovered quite regularly in the 40,000 to 45,000 molecular weight region on $NaDodSO_4$/polyacrylamide gels but no corresponding stainable protein band was found in this position (Fig. 1). This finding supports the view that the 40,000 molecular weight form represents residual undissociated IFN-γ, rather than the major species irreversibly inactivated by $NaDodSO_4$. Complete correspondence of stained protein bands with the 20,000 and 25,000 molecular weight forms strongly suggests that these bands represent the major IFN-γ proteins.

In our earlier study IFN activity associated with the 20,000 and 25,000 molecular weight forms was identified as IFN-γ by neutralization with specific antisera (8). Activity eluted from the gels was completely neutralized with an antiserum against IFN-γ and not neutralized at all with antisera to IFN-α or IFN-β.

Recently we immunized rabbits with the purified 20,000 and 25,000 molecular weight bands of IFN-γ, separated by $NaDodSO_4$/polyacrylamide gel electrophoresis. Antisera produced by these rabbits showed neutralizing activity against homologous purified IFN-γ activity bands. However, both antisera also neutralized activity from the heterologous IFN-γ band (i.e., antiserum to the 20,000 molecular weight form neutralized 25,000 molecular weight IFN and antiserum to the 25,000 molecular weight form caused neutralization of 20,000 molecular weight IFN). In addition, both antisera neutralized native IFN-γ (results not shown). These findings confirm that both the 20,000 and the 25,000 molecular weight forms are, by definition, IFN-γ.

It remains to be established whether the 20,000 and 25,000 molecular weight IFN-γ forms represent products of two related genes or whether they arise by differential modification of a single gene product. It is known that α IFNs represent products of a closely related large multigene family (19, 20). There also appear to be multiple β IFNs, possibly being coded for by a less closely related family of genes (21). Therefore, it would not be surprising if IFN-γ also consisted of products of a family of related genes. This question will undoubtedly be answered in the near future by amino acid sequence analysis of the purified subspecies of IFN-γ and by gene cloning studies.

Note Added in Proof. Gray *et al.* (22) have reported the cloning of human IFN-γ complementary DNA and its expression in three different host–vector systems. The nucleotide sequence of the cloned DNA indicates that this human IFN-γ is composed of 146 amino acids with a molecular weight calculated to be approximately 17,000. The exact relationship of the IFN-γ sequence cloned by Gray *et al.* to the two IFN-γ subspecies identified by us is not yet known.

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