Immune interferon (IFN-γ), endogenously labeled with [35S]methionine, was produced in human peripheral blood lymphocyte cultures stimulated with 12-O-tetradecanoylphorbol-13-acetate and phytohemagglutinin. 35S-IFN-γ, immunoprecipitated from the crude culture fluid with a monoclonal antibody, was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis into three monomeric forms with molecular weights of 25,000, 20,000, and 15,500, which we designate IFN-γ I, II, and III, respectively. IFN-γ I was the most, and IFN-γ III the least, abundant in both immunoprecipitated 35S-IFN-γ and chromatographically purified IFN-γ preparations. Changes in the molecular size of the monomeric forms after glycosidase treatment suggested that IFN-γ I contains more carbohydrate than IFN-γ II, and that IFN-γ III may not be glycosylated at all. Hence, the differences in the carbohydrate contents are likely to be the major cause of the molecular size heterogeneity of IFN-γ I, II, and III.

IFN-γ (formerly immune IFN) is a product of activated lymphocytes. Unlike other IFN species whose most important function is thought to be antiviral, IFN-γ is a lymphokine affecting primarily cellular functions related to immune reactions. For instance, it has recently been demonstrated that IFN-γ acts as a potent pleiotropic activator of monocyte/macrophage functions and that it is identical with a previously unidentified protein termed macrophage activating factor (1, 2). We described the properties of IFN-γ produced in cultures of human mononuclear cells stimulated by combined treatment with TPA and PHA, and purified by a sequence of chromatographic separation steps. These earlier studies showed that native IFN-γ is a glycoprotein with a molecular weight of approximately 58,000 (3). IFN-γ is an oligomer, because NaDodSO₄-PAGE resolves IFN-γ activity into two major bands with apparent molecular weights determined to be 25,000 (25K, IFN-γ I) and 20,000 (20K, IFN-γ II) (4–7). IFN-γ I and II were indistinguishable antigenically (8, 9). Existence of these two molecular weight forms was unexpected because the sequence of cloned IFN-γ cDNA predicts only a single 17.1K polypeptide (10–12). The 17K polypeptide contains two potential N-glycosylation sites, and the naturally occurring IFN-γ I and II could differ from each other by the degree of glycosylation.

Since monoclonal antibodies specific for IFN-γ have recently become available (13, 14), we used immunoprecipitation for the isolation of IFN-γ secreted by [35S]methionine-labeled lymphocyte cultures. Immunoprecipitated 35S-IFN-γ was resolved by NaDodSO₄-PAGE into IFN-γ I and IFN-γ II, and a previously undetected minor subspecies of 15.5K, termed IFN-γ III. These three forms of IFN-γ differ in the degree of glycosylation. IFN-γ I contains more carbohydrate than IFN-γ II. We present evidence that IFN-γ III probably contains no carbohydrate.

**MATERIALS AND METHODS**

**RESULTS**

Effect of TPA and PHA Treatment on the Production of 35S-labeled Proteins Secreted by Mononuclear Cell Cultures—

Cultures of mononuclear cells stimulated with TPA and PHA and control unstimulated cultures were labeled with [35S]methionine. Treatment with TPA and PHA resulted in a stimulation of IFN production which reached a level of 1280 units/ml 62 h after induction. No IFN-γ activity (<40 units/ml) was detected in uninduced control cultures. Incorporation of [35S]methionine into proteins secreted by control cultures was 4.7 x 10⁶ cpm/ml, while in TPA/PHA induced cultures it was 49% higher, i.e. 6.6 x 10⁶ cpm/ml. These [35S]methionine-labeled culture fluids, 125I-labeled chromatographically purified natural (16) IFN-γ and 125I-labeled recombinant IFN-γ were analyzed by NaDodSO₄-PAGE. Comparison of the fluorograms of control and induced proteins (Fig. 1, lanes A and C versus B and D) and their densitometer tracings (not shown) revealed numerous differences. Two novel bands, a major one of 23K and a minor one of 18.5K, were observed in the induced cultures. In addition, the intensities of five major

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1 The abbreviations used are: IFN, interferon; NaDodSO₄, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; MEM, minimal essential medium; TPA, 12-O-tetradecanoylphorbol-13-acetate; PHA, phytohemagglutinin; NP40, Nonidet P-40; HAT, hypoxanthine, aminopterin, and thymidine; K, molecular weight in thousands; for example, 20K represents 20,000.
Three Monomeric Subspecies of IFN-γ

FIG. 1. Effect of TPA and PHA on apparent molecular weight of proteins produced by [35S]-labeled mononuclear cells. Culture fluids of lymphocytes labeled with [35S]methionine from uninduced control (A and C) or TPA/PHA induced (B and D) culture were subjected to NaDodSO4-PAGE. The amount of radioactivity applied on lane A and B was 5 x 10^5 cpm, and on lane C and D was 1 x 10^6 cpm/lane. Lanes E and F contain aliquots of chromatographically purified natural human 125I-IFN-γ (16) and Escherichia coli-derived recombinant 125I-IFN-γ, respectively. Gels were processed for fluorography, and x-ray film was exposed for 4 days (lanes A-E), or 2 weeks (lane F).

bands, with molecular weights of 45K, 38K, 36K, 35K, and 12K, were increased in the induced culture. On the other hand, treatment with TPA and PHA suppressed the synthesis of several proteins which were more pronounced in the control cultures, including two proteins with molecular weights greater than 100K and proteins with apparent molecular weights of 30.5K, 27K, and 15K. Natural IFN-γ, (Fig. 1E) showed several bands of approximately 40 to 50K, which may represent residual undissociated oligomeric forms, the 25K IFN-γ I and 20K IFN-γ II bands, and a minor band with an apparent molecular weight of 15.5K. The NaDodSO4-PAGE profiles of immunoprecipitated 35S-IFN-γ and of purified 125I-IFN-γ differ in the higher molecular weight components. Some of the higher molecular weight components present in the chromatographically purified IFN-γ preparation might be antigenically unrelated contaminants.

Ratio of Antiviral Activity to Radioactivity in the Three IFN-γ Forms—To ascertain whether the immunoprecipitated 15.5K protein is indeed a form of IFN-γ, we have subjected chromatographically purified IFN-γ to NaDodSO4-PAGE and examined the resulting fractions for antiviral activity. A minor peak of antiviral activity was detected with the apparent molecular weight of 15.5K (Table I). We therefore conclude that the 15.5K band represents an IFN-γ subspecies, and we designate it IFN-γ III.

Identification of [35S]-labeled IFN-γ—Fig. 2 shows the NaDodSO4-PAGE profiles of immunoprecipitates of the culture fluids from induced (A) and control cultures (B), compared with 125I-labeled chromatographically purified IFN-γ (C). The immunoprecipitates of the induced culture revealed [35S]-labeled bands similar to those observed in the chromatographically purified IFN-γ preparation, i.e. IFN-γ I and IFN-γ II bands as well as a band with an apparent molecular weight of 15.5K. The NaDodSO4-PAGE profiles of immunoprecipitated [35S]-IFN-γ and of purified 125I-IFN-γ differ in the higher molecular weight components. Some of the higher molecular weight components present in the chromatographically purified IFN-γ preparation might be antigenically unrelated contaminants.
IFN-γ I and IFN-γ II forms. The minor 15.5K IFN-γ III form comprised 4.0% of total radioactivity, but only 0.2 to 0.5% of antiviral activity. The fact that the percentage of recovered antiviral activity in the IFN-γ III subspecies is 10-fold lower than that of radioactivity is not likely due to the presence of contaminating radiolabelled protein, since we observed similar ratios of radioactivity in IFN-γ I, II, and III after immunoprecipitation with monoclonal antibody B3 (21).

Effect of Glycosidase Treatment on the Molecular Size of IFN-γ—A chromatographically purified preparation of IFN-γ, untreated or treated with mixed glycosidases, was fractionated by NaDodSO₄-PAGE. The resulting fractions were assayed for antiviral activities. In the control preparation (Fig. 3A), four peaks of antiviral activity, a peak of the oligomeric form of IFN-γ (40 to 45K), as well as peaks of IFN-γ I, II, and III were observed. In the glycosidase-treated sample (Fig. 3B) a reduction in the molecular size of the oligomeric peak as well as a reduction in the size of the two larger monomeric subspecies was observed. It is evident that the apparent size of IFN-γ I was reduced to 18.5 ± 0.5K, the size of IFN-γ II to 15.5 ± 0.5K and that of IFN-γ III was not altered by glycosidase treatment. All carbohydrate-depleted IFN-γ subspecies retained their antiviral activity.

We have also determined by NaDodSO₄-PAGE the molecular weight of control and glycosidase-treated 125I-labeled IFN-γ forms isolated by immunoprecipitation with monoclonal antibody B3 (not shown). The conclusions from this experiment were the same as those reached on the basis of an analysis of biological activity, i.e. the apparent molecular weights of IFN-γ I and II were reduced to 18 and 16K, respectively, while that of IFN-γ III was not reduced at all. Autoradiographs of the immunoprecipitates analyzed by NaDodSO₄-PAGE did not reveal bands smaller than 15.5K even on extended exposure (21). We have also treated 125I-labeled recombinant IFN-γ with the glycosidase preparation and found no change in its apparent molecular weight as determined by NaDodSO₄-PAGE (data not shown). This result indicates the absence of proteolytic activity in the glycosidase preparation and confirms our conclusion that the observed size reduction of natural human IFN-γ monomers is indeed a result of carbohydrate depletion.

DISCUSSION

Earlier studies suggested that natural human IFN-γ produced in peripheral blood leukocyte cultures stimulated with TPA and PHA, exists as an oligomer which by NaDodSO₄-PAGE can be separated into 20K and 25K monomeric subspecies (5, 6, 8). The same two monomeric subspecies were also observed in human IFN-γ produced spontaneously by a T cell hybridoma (7) and by Chinese hamster ovary cells transfected with a human IFN-γ cDNA containing plasmid (24). Taken together these results indicate that the two monomeric forms are naturally occurring subspecies, derived by post-transcriptional or, more likely, post-translational modification of a single gene product. We propose to term the 25K and 20K subspecies IFN-γ I and II, respectively.

Experiments described in this paper revealed the presence of a previously undetected 15.5K monomeric subspecies which we propose to term IFN-γ III. The results of glycosidase treatment of IFN-γ suggested that IFN-γ III may be a naturally occurring unglycosylated IFN-γ polypeptide. Moreover, since IFN-γ II and III became indistinguishable in size after deglycosylation, the difference in molecular weight between IFN-γ II and III may be due entirely to the presence of carbohydrate in the IFN-γ II molecule. Our present as well as earlier (4) results show that IFN-γ I contains more carbohydrate than IFN-γ II. However, it is not yet known whether the molecular weight difference between the 18.5 and 15.5K forms (products of deglycosylation of IFN-γ I and II, respectively) is due to the presence of glycosidase-resistant oligosaccharide in IFN-γ I or to a difference in the protein moieties.

The molecular weight of the unglycosylated human IFN-γ polypeptide, calculated from the cDNA sequences (after subtraction of the presumed signal peptide sequence), is 17.1K. Although factors other than molecular size might affect migration rates of proteins on NaDodSO₄-PAGE, detection of a naturally occurring form of IFN-γ with an apparent molecular weight of 15.5K suggests that in addition to signal peptide cleavage, the IFN-γ polypeptide may undergo further proteolytic processing in human lymphocytes. Such processing was shown to occur during the synthesis of IFN-α, since three biologically active IFN-α species were reported to lack up to 10 COOH-terminal amino acids predicted from the cDNA sequences (25). To determine whether a similar processing occurs during IFN-γ synthesis it will be essential to analyze the amino acid sequence of the three IFN-γ forms. It is unlikely that the 15.5K polypeptide is derived from the 17.1K IFN-γ polypeptide by proteolytic cleavage after secretion, since no change in the apparent molecular weight of IFN-γ I, II, and III could be detected by NaDodSO₄-PAGE after its incubation with TPA/PHA incubated lymphocyte culture fluid (data not shown). It has been recently reported that removal of up to 11 carboxyl-terminal amino acids from human IFN-γ did not abolish antiviral activity (26).

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Three Monomeric Subspecies of IFN-γ

J. Immunol., in press


Supplemental Material to
Three Molecular Variants of Human Interferon-γ Revealed by Immunoprecipitation with Monoclonal Antibody

Renee Christine Buhler, Joanne Le, Benoit P. Rubin, Tom Ring, Sidney Negari, and Jack Fink

MATERIALS AND METHODS

Materials. E-(-)[3H]methionine (translational grade), specific activity of 350 Ci/mmol, and [125I]-labeled Ram bute were purchased from New England Nuclear. First-year infant human fibroblasts were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. The cells were obtained from the New York Blood Center and cultured up to 5x10^6 cells/ml. 1x10^6 cells were mixed with 1x10^6 lymphocytes and washed five times with PBS containing 0.1% gelatin. The cells were suspended in DMEM containing 10% fetal bovine serum, 1% penicillin-streptomycin, and 2.5mM glutamine. The cells were labeled with 1.25 Ci of [3H]-methionine for 3 hours in the presence of 5% CO2 in a humidified incubator at 37°C. The cells were harvested and washed twice with PBS containing 0.1% gelatin. The cells were resuspended in 10% SDS sample buffer containing 0.5% mercaptoethanol and boiled for 5 minutes. The supernatant was analyzed by SDS-PAGE and immunoprecipitated with monoclonal antibody 2F12. The immunoprecipitated IFN-γ was analyzed by autoradiography.


Results

Production of IFN-γ in transiently transfected mammalian cells. IFN-γ producing cells were transfected with pVSV-IFN-γ plasmid DNA using the calcium phosphate method. The transfected cells were cultured in the presence of 10% fetal bovine serum and 1% penicillin-streptomycin. The cells were harvested and washed twice with PBS containing 0.1% gelatin. The cells were resuspended in 10% SDS sample buffer containing 0.5% mercaptoethanol and boiled for 5 minutes. The supernatant was analyzed by SDS-PAGE and immunoprecipitated with monoclonal antibody 2F12. The immunoprecipitated IFN-γ was analyzed by autoradiography.


Table 1. Antigenic activity and radiolabeled IFN-γ with different specific activities

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Table 2. IFN-γ radiolabeled with different specific activities

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Fig. 1. Effect of glucocorticoid treatment on the monoclonal binding of Recombinant IFN-γ with different specific activities

An aliquot of [3H]-labeled IFN-γ was adjusted to pH 5.0 and treated with a mixture of glucocorticoid as described (6). For each glucocorticoid and glucocorticoid mixture treatment, 38,353 IFN units were utilized. After 0.5 h of incubation control (A) and glucocorticoid (B) binding were measured. The binding activity was decreased to 1.0% of the total binding activity of the control treatment. The binding activity of the glucocorticoid-treated sample was determined by a competitive binding assay using 125I labeled control IFN-γ as a standard. The binding activity of the glucocorticoid-treated sample was 0.32 of the control activity. The binding activity of the glucocorticoid-treated sample was determined by a competitive binding assay using 125I labeled control IFN-γ as a standard. The binding activity of the glucocorticoid-treated sample was 0.32 of the control activity.

Fig. 2. Effect of glucocorticoid treatment on the monoclonal binding of Recombinant IFN-γ with different specific activities

An aliquot of [3H]-labeled IFN-γ was adjusted to pH 5.0 and treated with a mixture of glucocorticoid as described (6). For each glucocorticoid and glucocorticoid mixture treatment, 38,353 IFN units were utilized. After 0.5 h of incubation control (A) and glucocorticoid (B) binding were measured. The binding activity was decreased to 1.0% of the total binding activity of the control treatment. The binding activity of the glucocorticoid-treated sample was determined by a competitive binding assay using 125I labeled control IFN-γ as a standard. The binding activity of the glucocorticoid-treated sample was 0.32 of the control activity. The binding activity of the glucocorticoid-treated sample was determined by a competitive binding assay using 125I labeled control IFN-γ as a standard. The binding activity of the glucocorticoid-treated sample was 0.32 of the control activity.
Three molecular weight forms of natural human interferon-gamma revealed by immunoprecipitation with monoclonal antibody.
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