EXHIBIT B

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Effects of Glycosidase Treatment on the Physicochemical Properties and Biological Activity of Human Interferon-γ*

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Hanna Chroboczek Kelker, Yum K. Yip, Paul Anderson‡, and Jan Vilček

From the Department of Microbiology, New York University School of Medicine, New York, New York 10016

Highly purified human interferon (IFN)-γ was treated with a preparation of mixed glycosidases in order to evaluate the effect of carbohydrate depletion on its biological activity, isoelectric point, and molecular size. Glycosidase treatment did not reduce the antiviral activity of IFN-γ in cultures of human fibroblasts and in bat lung cells. No antiviral activity was observed before or after treatment with glycosidases in pig, mink, bovine, murine, and monkey cells. The degree of neutralization of IFN-γ activity with specific antibody was also not significantly affected by glycosidase treatment. Several components of IFN-γ activity were resolved by nonequilibrium pH gradient electrophoresis, with major peaks of activity at pI 8.5 and 8.7. Glycosidase treatment of IFN-γ resulted in a reduced charge heterogeneity and a higher pI of 9.3. ^{125}I-labeled IFN-γ was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis into two bands with molecular weights of 25,000 and 20,000. Glycosidase treatment reduced the apparent molecular weight of these bands to 18,500 and 16,000, respectively. The results suggest that both the M_r = 25,000 and 20,000 bands, thought to be monomeric forms of IFN-γ, are glycosylated.

Until recently, all three major types of interferons (α, β, and γ) were thought to be glycoproteins. More recent evidence, however, suggests that most subtypes of the human IFN[1]-α family are not extensively glycosylated because direct analysis of highly purified IFN-α did not detect amino sugars (1, 2). In addition, structural analysis of the various IFN-α proteins based on cloned DNA sequences failed to reveal any potential *N*-glycosylation sites (3).

In contrast to IFN-α, the other two major IFN types are glycoproteins. Carbohydrate has been detected in purified IFN-β protein (4, 5). Moreover, one potential *N*-glycosylation site was detected in the cloned IFN-β cDNA sequence (6, 7). Indirect evidence that human IFN-γ is a glycoprotein is based on its affinity for concanavalin A-Sepharose (8–10). Further support comes from the structural analysis of cloned human IFN-γ cDNA which revealed two potential *N*-glycosylation sites (11).

The molecular weight of native human IFN-γ was found to be between 40,000 and 60,000 by molecular sieve chromatography (reviewed in Ref. 10). In native form, the molecule is thought to exist as a dimer, because when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, IFN-γ was resolved into two bands with molecular weights of 20,000 and 25,000 (12). The 20K and 25K molecular weight forms are indistinguishable antigenically (12, 13). They do not appear to be products of two different structural genes since screening of a human gene library revealed only one gene cross-hybridizing with IFN-γ cDNA (14). Sequence analysis of cloned IFN-γ cDNA predicts a single mature polypeptide consisting of 146 amino acids with a molecular weight of 17,110 (11). The difference between this predicted molecular weight and the size of the two monomeric forms determined by NaDodSO$_4$-PAGE could be due to differential glycosylation of a single polypeptide species. Alternatively, the 20K and 25K species could be glycosylated to the same extent and the difference between them might arise as a result of limited proteolytic cleavage.

In the present study, we have measured the effect of glycosidase treatment on the molecular size, isoelectric point, and antiviral activities of human IFN-γ. Glycosidase treatment was found to increase the isoelectric point of the IFN-γ molecule and decrease the molecular size of both the 20K and 25K forms. Glycosidase treatment neither reduced the antiviral activity nor did it alter the target cell specificity of IFN-γ.

EXPERIMENTAL PROCEDURES

Materials—Lymphocyte-rich plateletpheresis residues were obtained from the New York Blood Center. 12-*O*-Tetradecanoylphorbol-13-acetate was obtained from LC Services Corp. (Woburn, MA). Phytohemagglutinin was prepared in Dr. J. D. Oppenheim's laboratory at New York University Medical Center. A mixture of glycosidases from *Streptococcus pneumoniae* (15) was a generous gift of Dr. K. Zoon. ^{125}I-Bolton-Hunter reagent was purchased from New England Nuclear. Ampholines were obtained from LKB. Other chemicals were of the highest grade available from standard sources.

Cell Cultures—The sources of cell cultures were described by Hayes *et al.* (16). Cultures were grown in Eagle's minimal essential medium containing Hepes (10 mM), gentamycin (50 μg/ml), amphotericin B (2.5 μg/ml), and either 10% or 5% heat-inactivated fetal bovine serum. FS-4 is a human diploid fibroblast strain; GM-258 is a strain of human fibroblasts trisomic for chromosome 21; EBTr, embryonic bovine trachea cells; L 929, line of mouse fibroblasts; Vero, African green monkey kidney cells.

IFN Assays—These were based on the inhibition of the cytopathic effect of encephalomyocarditis virus in FS-4 cells grown in 96-well microplates (12). The same procedure was used with the GM-258 cell strain and with cells of various other animal species, using either vesicular stomatitis virus (Indiana type) or EMC as the challenge virus. Titers of IFN-γ are reciprocals of the highest dilution inhibiting the cytopathic effect by 50%. An internal laboratory standard of

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[1] The abbreviations used are: IFN, interferon; NaDodSO$_4$, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EMC, encephalomyocarditis virus; VSV, vesicular stomatitis virus; NEPHGE, nonequilibrium pH gradient electrophoresis; K, molecular weight in thousands, for example 20K designates 20,000.

human IFN-γ was included with each assay.

Interferon Production, Purification, and Iodination—Production of IFN-γ was as described by Yip *et al.* (12). This method is based on combined stimulation of plateletpheresis residue-derived human leukocyte cultures with 12-*O*-tetradecanoylphorbol-13-acetate and phytohemagglutinin. IFN-γ was purified as described elsewhere (12) using sequential absorption and elution on silicic acid, concanavalin A-Sepharose, and DEAE-Sephacel, followed by molecular sieving on a Bio-Gel P-200 column. These steps resulted in approximately a 1000-fold purification. This preparation containing ≥80% IFN-γ protein was labeled with ^{125}I-Bolton-Hunter reagent (17) as described by Anderson *et al.* (18). NaDodSO$_4$-PAGE was carried out on linear 10–16% acrylamide slab gels as described previously (18, 19). Autoradiography was performed at −70 °C using Kodak film XA-R-5 and DuPont Cronex Xtra Life intensifying screens.

Neutralization Assay with Antibody to IFN-γ—Antisera were raised in rabbits immunized by repeated injections of purified IFN-γ (prepared as described above) mixed with incomplete Freund's adjuvant. For neutralization assay, aliquots of IFN-γ diluted to approximately 100 units/ml were incubated with serial 2-fold dilutions of rabbit antiserum to IFN-γ at 4 °C for 1 h. Neutralization titer is the highest dilution of antiserum abolishing antiviral activity measured in FS-4 cells challenged with EMC virus.

Nonequilibrium pH Gradient Electrophoresis—This was done as described by O'Farrell *et al.* (20), except that urea and Nonidet P-40 were omitted. It was carried out in cylindrical 7.5% acrylamide gels (2 × 110 mm) containing 2% each of pH 6–8 and pH 9–11 ampholines and glycerol at the concentrations indicated below. IFN-γ samples were incorporated into the middle portion of the gel (10). Glycerol concentrations in the bottom, middle, and top portions of the gel were 15, 11.5, and 6.5%, respectively. The anolyte, catholyte, and connection of electrodes were as described by O'Farrell *et al.* (20). Electrophoresis was carried out at 500 V for 3–4 h at 7 °C. Following electrophoresis, extruded gels were frozen at −70 °C, and then separated into 1-mm slices with a razor blade slicer. Each gel slice was eluted overnight at 4 °C in 300 μl of tissue culture medium (Eagle's minimal essential medium containing 0.2% human serum albumin) and the eluates were assayed for IFN activity in FS-4 cells. For pH determination, two 1-mm gel slices were placed in 1 ml of degassed distilled H_2O and allowed to equilibrate for 1 h at 4 °C.

Glycosidase Treatment—Treatment of IFN-γ was performed essentially as described by Zoon *et al.* (15). The glycosidase preparation contained β-galactosidase (9.4 units/ml), *N*-acetyl-β-galactosaminidase (38 units/ml), and neuraminidase (1.4 units/ml). A mixture of purified unlabeled IFN-γ and radiolabeled IFN-γ was adjusted to pH 6.0 by the addition of 50 mM citric acid and divided into two equal portions. To one of the samples an aliquot of glycosidase preparation was added and this sample, together with the control untreated sample, was incubated for 4–5.5 h at 37 °C. At this time, aliquots were removed for IFN assay, analysis by NaDodSO$_4$-PAGE, and isoelectric focusing. Additional experimental details are described in Table I and Fig. 1.

Protein Determination—This was done with the aid of the Bradford reagent (21).

RESULTS

Effect of Glycosidase Treatment on the Biological Activity of IFN-γ—Antiviral activity of untreated and glycosidase-treated human IFN-γ was tested in several cell lines challenged with EMC virus or VSV (Table I). Fibroblast strain GM-258 with trisomy 21 was more sensitive to IFN-γ than the diploid FS-4 cell strain. Antiviral activity was also detected in bat cells, although it was significantly lower than in human cells. No activity was detected in pig, mink, monkey (Vero), mouse (L 929), or bovine (EBTr) cells. Glycosidase treatment did not reduce the antiviral activity; in fact, a slight increase in activity was noted in most instances after the enzyme treatment. Glycosidase treatment did not alter the target cell specificity of this IFN.

We also tested the effect of glycosidase treatment on the degree of neutralization of IFN-γ activity by a rabbit antiserum to IFN-γ. The neutralization titer against both the control and the glycosidase-treated IFN-γ preparations was 1:128.

TABLE I

Effect of glycosidase treatment on the antiviral activity of IFN-γ in various cells

Experiment No.	Assay system		No treatment	Glycosidase-treated
	Cell line	Challenge virus		
			IFN units/ml	
1[a]	FS-4	EMC	115,000	156,000
2[b]	FS-4	EMC	287,000	585,000
3[c]	FS-4	EMC	480,000	560,000
		VSV	112,000	112,000
	GM-258	EMC	3.8×10^6	4.4×10^6
		VSV	560,000	640,000
	Bat lung	VSV	640	480
	Pig lung	VSV	<100	<100
	Mink lung	VSV	<80	<80
	EBTr	VSV	<40	<40
	L 929	VSV	<40	<40
	Vero	VSV	<40	<40

[a] Experiment 1. Each of the samples contained in a 60-μl final volume purified IFN-γ (1.0×10^4 units, 8 μg of protein), and ^{125}I-labeled IFN-γ (7.7×10^5 cpm). The treated sample received 2 μl of the mixed glycosidase preparation (15). IFN activity was assayed after 4.5 h of incubation at 37 °C. Recovery of IFN activity was 63 and 84.5% for the control and glycosidase-treated sample, respectively.

[b] Experiment 2. Each of the samples (425 μl final volume) contained 1.1×10^5 units of IFN in 172 μg of protein and 3.10×10^6 cpm of ^{125}I-labeled IFN. To one of the samples was added 15 μl of glycosidase preparation. Activity was assayed after 5.5 h of incubation. Recovery of IFN activity was 110 and 225% for control and glycosidase-treated samples, respectively.

[c] Experiment 3. Each of the samples contained 1.2×10^5 units of unlabeled IFN-γ (140 μg of protein) in a volume of 135 μl. To the experimental sample was added 7 μl of the glycosidase preparation. After 5 h of incubation, antiviral activity was assayed, using cell lines and viruses listed in the table.

IFN-γ—Analysis of IFN-γ by NEPHGE revealed considerable charge heterogeneity (Fig. 1*A*). In agreement with our earlier findings (10), IFN-γ activity was mainly associated with species in the pI range of 8.5–8.7. In addition to these major activity species, we observed other species of IFN-γ with the pI of 9.15, 9.1, 8.2, 7.9, 7.25, and 6.8. Similar analysis of glycosidase-treated IFN-γ revealed a shift in the pI to approximately 9.3 (Fig. 1*B*). The carbohydrate-depleted preparation was less heterogeneous in charge, with approximately 93% of the activity recovered from a broad peak with a pI 9.5–9.1 and with only two minor peaks of activity at pI 8.95 and 8.65. Although the position of minor peaks of IFN-γ activity observed after NEPHGE in the control and glycosidase-treated preparations differed somewhat from experiment to experiment, a generally similar pattern was observed in repeated experiments.

Effect of Glycosidase Treatment on the Molecular Size of IFN-γ—Analysis of a highly purified ^{125}I-labeled IFN-γ preparation by NaDodSO$_4$-PAGE revealed that approximately 80% of the applied radioactivity was present in the 20K and 25K molecular weight forms (Fig. 2, *lane A*). A less intense band of approximately 40K seen on the gel might represent IFN-γ in the form of undissociated dimer (12, 18, 22). In addition, this preparation contained some minor contaminating protein bands with different molecular weights. A similar pattern was seen with material that had been first fractionated by NEPHGE (Fig. 2, *lanes B* and *C*), except for the sample with a pI of 6.8 which contained additional protein bands in the high molecular weight region (*lane D*). Glycosidase treatment resulted in the disappearance of the 20K and 25K bands

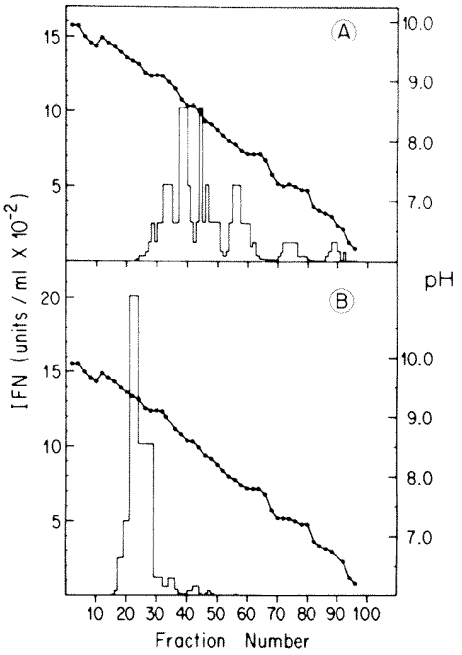

FIG. 1. **Nonequilibrium pH electrophoresis of native (*A*) and glycosidase-treated (*B*) IFN-γ.** IFN preparations and conditions of enzyme treatment were as described in Table I, Experiment 2. In *A*, 9,200 units of control IFN sample was applied and 47% of the biological activity was recovered. In *B*, 15,000 units of glycosidase-treated sample was applied and 21% of the biological activity was recovered.

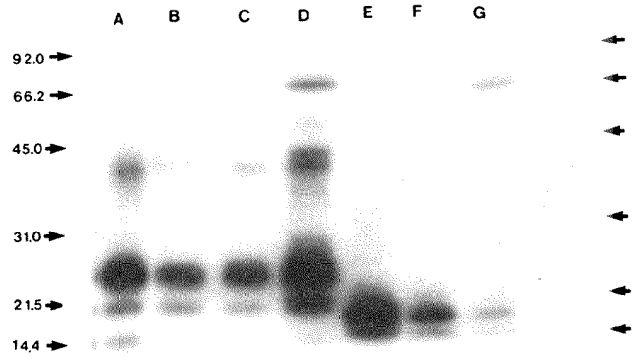

FIG. 2. **Effect of glycosidase treatment on the molecular weight of IFN-γ.** IFN-γ preparations and conditions of glycosidase treatment were as in Table I, Experiment 1. Control and glycosidase-treated samples were first fractionated by NEPHGE (not shown). Focusing gels were sliced and eluted as described under "Experimental Procedures." Aliquots of the eluted fractions containing significant amounts of IFN activity were then separated by $NaDodSO_4$-PAGE and the position of radiolabeled bands was determined by autoradiography. *Lane A* contains a preparation of ^{125}I-labeled purified IFN-γ not treated with glycosidases and not fractionated by NEPHGE. *Lanes B*, *C*, and *D* are NEPHGE fractions of control IFN-γ with pI 8.35, 7.85, and 6.8, respectively. *Lanes E*, *F*, and *G* are NEPHGE fractions of glycosidase-treated IFN-γ with pI of 9.3, 8.35, and 7.85, respectively. Positions of molecular weight markers are indicated by *arrows*.

and in their replacement by radiolabeled bands with molecular weights estimated to be 18.5 ± 0.5K and 16.0 ± 0.5K, respectively (Fig. 2, *lanes E*, *F*, and *G*). Both of these new lower molecular weight bands have antiviral activity (data not shown). The intensity of the 25K and 18.5K radiolabeled bands is greater than that of the 20K and 16K bands, suggesting that the 18.5K band represents the carbohydrate-depleted product of the 25K band and that the 16K band is a deglycosylated form of the 20K band. This suggests that both the 25K and 20K forms of IFN-γ are glycosylated. The 25K form may contain more carbohydrate since its molecular weight was decreased to a greater extent by the enzyme treatment. It is interesting that IFN-γ fractions with different isoelectric points all contain similar relative proportions of the larger and smaller molecular weight components.

DISCUSSION

Earlier studies by several investigators indicated that the carbohydrate moiety of IFN is not essential for biological activity. Fujisawa and Kawade (23) reported that the antiviral activity of mouse IFN-β and IFN-α (which unlike human IFN-α is thought to be glycosylated) remained largely intact after treatment with mixed glycosidases. Similarly, Knight and Fahey (24) reported that deglycosylation of human IFN-β with the aid of a mixed glycosidase preparation did not abolish antiviral activity. Moreover, while cloned human IFN-β expressed in *E. coli* is not glycosylated, it is biologically active (25, 26).

The results of our present study indicate that the entire carbohydrate moiety is not required for the antiviral activity of human IFN-γ. Although it is not clear how much of the total carbohydrate was removed by the glycosidase treatment, the degree of reduction in molecular weight on enzyme treatment suggests that it was a substantial portion. The fact that glycosidase treatment did not abolish biological activity is not surprising, since it was shown earlier that recombinant DNA *E. coli*-derived human IFN-γ is biologically active (11). However, until now it was not known whether the presence of the carbohydrate moiety affects the specific activity and the pattern of target cell specificity of IFN-γ. Our results show that removal of a substantial portion of the carbohydrate did not produce a marked change in these parameters.

Although treatment with glycosidases did not result in a marked change in the degree or the pattern of antiviral activity at the tissue culture level, alterations in the carbohydrate moiety might significantly influence biological activity in the intact organism. Studies with other glycoproteins showed that the sugar moiety can exert a marked effect on plasma clearance rates and tissue distribution (27, 28). Removal of carbohydrate also might affect susceptibility to proteolytic degradation (29, 30), stability (31), and solubility of the molecule (32). Additional studies will be required to determine the effect of selective deglycosylation on these parameters of IFN-γ.

We have demonstrated that human IFN-γ displays a peripheral charge heterogeneity characteristic of many glycoproteins (33). Charge heterogeneity was also observed with murine IFN-γ (34). Studies with rabbit IFN (35, 36) showed that charge heterogeneity is a consequence of variable amounts of sialic acid present as terminal monosaccharide. Changes in the isoelectric focusing pattern observed after glycosidase treatment indicate that the charge heterogeneity of native IFN-γ is also due to variable carbohydrate contents. Human IFN-γ cDNA sequence (11) predicts that the unglycosylated IFN molecule is quite basic since it contains 27 basic and 19 acidic amino acids. It is therefore not surprising that glycosidase treatment resulted in a net increase in charge.

Earlier studies suggested that native human IFN-γ exists in the form of a dimer and that under denaturing conditions the native molecule dissociates into the monomeric 25K and 20K forms (12, 22). The nature of the interaction responsible for the presumed formation of dimers is not known but S-S

bonds are not likely to be involved since dissociation takes place in the absence of reducing treatment. The decrease in molecular size of both the 25K and 20K forms as a result of carbohydrate depletion indicates that both molecules are glycosylated. It appears that removal of carbohydrate decreased the size of the 25K form more than that of the 20K form (by 6.5K and 4.0K, respectively). However, the extent of carbohydrate removal obtained here is not known and it is still not clear whether the difference in the molecular size of the 25K and 20K forms rests entirely in the carbohydrate contents or whether the protein moieties also differ. Since the original demonstration of the 25K and 20K forms of IFN-γ by Yip *et al.* (12), these two forms have been found in human IFN-γ preparations from various sources (37). The fact that in different preparations the amount of the 25K form regularly exceeds the amount of the 20K form by about 3- to 5-fold (see Fig. 2 and Refs. 12 and 18) suggests that the generation of the two size classes is not random. Such distinct size heterogeneity has not been observed with IFN-β which, like IFN-γ, appears to be coded for by a single structural gene (38–40).

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