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Evidence for a Polypeptide Segment at the Carboxyl Terminus of Recombinant Human γ Interferon Involved in Expression of Biological Activity

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ABSTRACT: A panel of 18 murine monoclonal antibodies was raised in BALB/c mice to the full-length, 146 amino acid residue recombinant human γ interferon (rHuIFN γ-A). Two monoclonal antibodies, designated 47N3-6 and 30N47-1, were purified from ascites tumors and further characterized. Antibody 47N3-6 neutralized both the antiviral and antiproliferative activities of rHuIFN γ-A. Both Western blotting and enzyme-linked immunosorbent assays indicated that antibody 47N3-6 could bind to rHuIFN γ-A as well as to a genetically engineered truncated form lacking the first three amino-terminal residues (rHuIFN γ-D) but did not recognize a genetically engineered variant terminating at residue 131 (rHuIFN γ-B). This antibody also demonstrated binding to a 15 amino acid residue oligopeptide, designated F-1, corresponding to residues 132–146 at the carboxyl terminus of rHuIFN γ-A. Chemical cleavage of peptide F-1 with cyanogen bromide produced two fragments that were separated by reversed-phase high-pressure liquid chromatography. Dot-blot analysis indicated that antibody 47N3-6 could bind to a fragment, $KRKRSQH_{se}$, derived from residues 132–137 of rHuIFN γ-A, but could bind only weakly to the cyanogen bromide fragment corresponding to residues 138–146. It was consistent with these results that antibody 47N3-6 demonstrated binding to a form lacking the five carboxyl-terminal amino acids (rHuIFN γ-D′) but did not bind to a synthetic polypeptide corresponding to residues 138–146. Peptide F-1 exhibited neither antiviral nor antiproliferative activity, and it did not antagonize the antiviral activity of rHuIFN γ-A. However, the specific antiviral and antiproliferative activities of rHuIFN γ-B were 3–4-fold lower than the corresponding values for rHuIFN γ-A. In distinction, antibody 30N47-1, which neutralized neither antiviral nor antiproliferative activity, was observed to bind to three forms of rHuIFN γ (γ-A, -D, and -B) but not to peptide F-1. These data suggest that a portion of the carboxyl terminus of rHuIFN γ-A and, in particular, residues 132–137 may be a component of a critical domain required for the expression of its biological activity.

HuIFN[1] γ, also designated immune or type II IFN, is a product of antigen-sensitized T-lymphocytes that expresses potent antiviral, anticellular, and immunomodulatory activities [for reviews, see Epstein (1981), Georgiades et al. (1984), and Trinchieri and Perussia (1955)]. It has a low primary structural homology with type I (α and β) IFNs (Gray & Goeddel, 1982; Gray et al., 1982) and can further be distinguished from type I IFNs by differences in antigenic determinants (deLey et al., 1980) and a variety of physicochemical properties, including molecular weight, isoelectric point, and sensitivity to low pH (Yip et al., 1981; Gray et al., 1982; Gray & Goeddel, 1983). The cell surface receptors through which the biological properties of type I and type II IFNs are mediated are also distinct (Branca & Baglioni, 1981; Littman et al., 1985; Joshi et al., 1984). It is significant that a number of biological properties appear to be more effectively mediated by HuIFN γ than type I IFNs, including monocyte/macrophage activation (Pace et al., 1983) and enhanced expression of class II major histocompatibility antigens (Wong et al., 1983). The application of recombinant DNA technology has resulted in the availability of large quantities of rHuIFN γ for conducting Phase I/II clinical trials on its utility as an antineoplastic agent (Kurzrock et al., 1985; Bennett et al., 1986; Kleinerman et al., 1986).

Limited information is available on the relationship of the structure of rHuIFN γ to its biological activity. Rinderknecht et al. (1984) have reported that biologically active natural HuIFN γ lacks the first three amino-terminal amino acids (Cys-Tyr-Cys) and is heterogeneous at the carboxyl terminus, as evidenced by the detection of six different carboxyl-terminal amino acids. Although these authors did not measure the antiviral activity of each of the six truncated variants of rHuIFN γ, in which up to 16 amino acids were removed from the carboxyl terminus, Rose et al. (1983) have reported that a rHuIFN γ variant truncated at position 131 retained full

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[1] Abbreviations: IFN, interferon; HuIFN, human interferon; rHuIFN γ, recombinant human γ interferon; HuIFN α, human α interferon; PBS, phosphate-buffered saline; EMEM, Eagle's minimum essential medium; Tris, tris(hydroxymethyl)aminomethane; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TBS, Tris-buffered saline; EMC, encephalomyocarditis; IU, international units; ELISA, enzyme-linked immunosorbent assay; HAT, hypoxanthine, aminopterin, and thymidine; IgG, γ immunoglobulin.

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antiviral activity. It was consistent with these data that a monoclonal antibody raised to a synthetic 16 amino acid carboxyl-terminal polypeptide did not neutralize antiviral activity (Ichimori et al., 1985). The fact that polyclonal antibodies directed to a 20 amino acid residue polypeptide at the amino terminus of rHuIFN γ neutralized antiviral activity implied that the amino rather than the carboxyl terminus was critical for antiviral activity (Johnson et al., 1982). In distinction, the genetic engineering of a form of rHuIFN γ lacking the 19 carboxyl-terminal amino acid residues resulted in a significant depression in both antiviral and antiproliferative activities (Burton et al., 1985; Czarniecki et al., 1985). Similarly, Arakawa et al. (1986) have recently observed that tryptic digestion of rHuIFN γ produced a variant lacking ca. 13 residues at the carboxyl terminus and exhibiting a 1000-fold loss in antiviral activity.

Previous studies in this laboratory (Lydon et al., 1985) have demonstrated that monoclonal antibodies may be utilized for elucidating structure–function relations in rHuIFN α. Several procedures were employed for identifying the epitope to which these antibodies were directed, including binding to polypeptide fragments prepared by chemical or enzymatic cleavage and cross-reactivity with homologous IFN subtypes. Alternatively, other studies have indicated that chemically synthesized short oligopeptides corresponding to sequences in the parental molecule may be employed either for identification of epitopes or for use as immunogens to raise antibodies of predetermined specificity (Walter et al., 1980; Palfreyman et al., 1984; Jacobs et al., 1983; Leist et al., 1985). We report here obtaining a panel of murine hybridomas that produce antibodies directed to the full-length, 146 amino acid residue form of rHuIFN γ-A. An antibody that neutralized biological activity was determined to be directed to the carboxyl terminus by a novel approach on the basis of lack of cross-reactivity with a genetically engineered variant lacking the carboxyl-terminal 15 amino acids (rHuIFN γ-B) and by its ability to recognize oligopeptides corresponding to carboxyl-terminal regions. In combination with the observation of significantly reduced antiviral and antiproliferative activities of rHuIFN γ-B compared to rHuIFN γ-A, these data support that at least a portion of a domain critical for the expression of the biological activity of rHuIFN γ resides at the carboxyl terminus.

Experimental Procedures

A synthetic rHuIFN γ gene was expressed in *Escherichia coli* K-12 by standard techniques of recombinant DNA methodology, similar to that previously described (Nagabhushan & Leibowitz, 1984). Cloned variants were purified to constant specific antiviral activity by methodology similar to that described for recombinant murine IFN γ (Le et al., 1986). Oligopeptides corresponding to the nine amino-terminal and fifteen carboxyl-terminal residues of rHuIFN γ-A as well as the seven carboxyl-terminal residues of rHuIFN γ-B were synthesized by Bachem (Torrence, CA). The nine carboxyl-terminal residues of rHuIFN γ-A were synthesized by Applygene (Yllyrch, France). Monoclonal antibodies were raised in BALB/c mice as previously described (Lydon et al., 1985). The mice were obtained from Jackson Labs and supplied by IFFA Credo (L'Arbresle, France). Horseradish peroxidase conjugated goat anti-mouse immunoglobulin and 2,2′-azinobis[3-ethylbenzothiazolinesulfonate] were purchased from Kirkgaard Perry (Gaithersburg, MD). Diethanolamine, isopropyl alcohol, ammonium sulfate, PBS [NaCl (8 g/L), KH_2PO_4 (0.2 g/L), and KCl (0.2 g/L)], and Tween 20 were purchased from Merck (Darmstadt, West Germany). Fetal calf serum was obtained from IBF (Villeneuve, La Garenne, France), and RPMI-1640, EMEM, L-glutamine, penicillin, streptomycin, and complete Freund's adjuvant were supplied by Gibco (Grand Island, NY, and Paisley, Scotland). Hypoxanthine, aminopterin, thymidine, 2-mercaptoethanol, Tris (free base), MTT, and isopropyl alcohol were purchased from Sigma Chemical Co. (St. Louis, MO). CNBr and formic acid were obtained from Aldrich Chemical Co. (Milwaukee, WI). Acetonitrile was purchased from Fisher (Fairlawn, NJ), and trifluoroacetic acid was obtained from Baker (Phillipsburg, NJ). Acrylamide, *N,N′*-methylenebis(acrylamide), sodium dodecyl sulfate, and nitrocellulose were supplied by Bio-Rad Laboratories (St. Louis, MO). Brij-35, sodium decyl sulfate, and sodium heptanesulfonate were obtained from Eastman Kodak Co. (Rochester, NY). Constant boiling HCl (sequenal grade) was supplied by Pierce Chemical Co. (Rockford, IL). Falcon microtiter plates were purchased from Becton Dickinson labware (Oxnard, CA). The dot–blot apparatus was supplied by Bio-Rad (St. Louis, MO). The Microsorb C_{18} column employed for amino acid analysis was purchased from Rainin Associates (Woburn, MA). The C_8 reversed-phase column employed for resolution of CNBr fragments was obtained from Beckman (Fullerton, CA).

Enzyme-Linked Immunosorbent Assay (ELISA). Microtiter plates were coated for 1 h at room temperature with 200 ng of protein or polypeptide dissolved in 0.05 mL of TBS. Binding affinities were established by varying the amount of antigen from 0.1 to 1000 ng. After the wells were washed 5 times with 200 μL per well of 0.02% Tween 20 dissolved in TBS, they were coated with either 1% bovine serum albumin or 1% bovine γ globulin and washed again with TBS–0.2% Tween 20. The wells were incubated with a solution of purified monoclonal antibody or hybridoma supernatant at room temperature for 1 h. After five washes with TBS–0.2% Tween 20 the wells were coated with horseradish peroxidase conjugated goat anti-mouse immunoglobulin at room temperature for 1 h. After five additional washes as described above, the color was developed by the addition of hydrogen peroxide and 2,2′-azinobis[3-ethylbenzothiazolinesulfonate]. Quantitation was achieved by diluting the contents of each well with 0.7 mL of H_2O and recording the absorbance observed at 414 nm on a Bausch and Lomb Spectronic 2000 spectrophotometer.

Dot–Blot Analysis. Sample (0.05 mL) dissolved in TBS was incubated for 1 h in the well of a dot–blot apparatus containing nitrocellulose sheets. After the wells were washed with 0.2 mL of TBS, the nitrocellulose sheet was removed from the apparatus, blocked with 1% bovine serum albumin, and washed with TBS. The nitrocellulose sheet was immersed in a solution containing the antibody for 1 h. After five washes with TBS, the sheet was incubated with a solution of horseradish peroxidase conjugated goat anti-mouse immunoglobulin for 1 h. A positive reaction was detected by visual inspection of the color produced after the addition of a mixture of *o*-phenylenediamine and hydrogen peroxide. All procedures were performed at room temperature.

Antiviral Activity. Cytopathic effect–inhibition assay was employed with EMC virus (ATCC-VR129) and human foreskin cells (FS-71) as target, essentially as previously described (Lydon et al., 1985). International units were determined by comparison to a natural HuIFN γ standard. Neutralization units for hybridoma supernatants or purified antibodies were obtained by multiplying the dilution factor required to prevent the cytopathic–inhibition effect of rHuIFN γ by the amount of rHuIFN γ added (i.e., 30 IU).

Antiproliferative Activity. Cells were grown as adherent monolayers in microtiter plates. For quantitation of cell

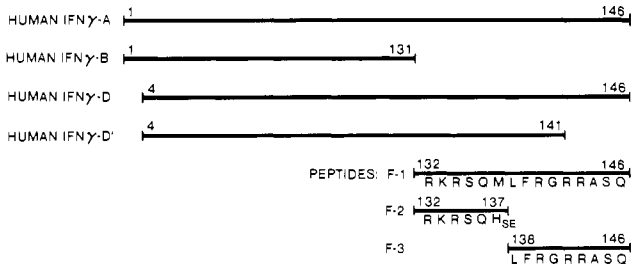

FIGURE 1: Schematic illustration of rHuIFN γ variants and related oligopeptides. Peptide F-1 contains the carboxyl-terminal 15 amino acids of rHuIFN γ-A. Peptides F-2 and F-3 were derived from peptide F-1 by CNBr cleavage, as described under Experimental Procedures. Methionine at position 137 is converted to homoserine during the CNBr cleavage reaction.

number, MTT was added and absorbance was measured with a micro-ELISA autoreader at 570 nm, as described by Mosmann (1983). For determination of antibody neutralization units, Hep-2 cells were grown as described above in the presence of rHuIFN γ and serial dilutions of hybridoma supernatant or purified antibody. Cell growth was quantitated by staining with 0.05% Janus Green B, which was extracted with ethylene glycol monomethyl ether and quantitated by absorbance measurements at 450 and 630 nm with a micro-ELISA autoreader. Neutralization units were calculated as described above for antiviral activity.

Immunoblotting. This procedure was performed essentially as described by Towbin et al. (1979).

Amino Acid Analysis. The sample dissolved in 0.5–1.0 mL of water was evaporated, dissolved in 0.5 mL of constant-boiling HCl, and sealed in vacuo. After a 22-h hydrolysis at 110 °C, the HCl was removed by evaporation. The sample was dissolved in 0.5 mL of water and evaporated 3 times. Separation of amino acids was performed on a Microsorb C_{18} HPLC column, as described by Radjai and Hatch (1980). After postcolumn derivatization with *o*-phthalaldehyde, peaks were detected by fluorescence in an Aminco Fluoro Monitor. Quantitation was achieved by comparison to a standard mixture of known amino acids.

Protein Concentration. The method of Lowry (1951) or Bradford (1976) was employed with bovine serum albumin as standard.

RESULTS

rHuIFN γ Variants and Related Oligopeptides. The forms of purified rHuIFN γ and oligopeptides shown schematically in Figure 1 were employed for identification of epitopes and classification of antibodies. Initial screening of hybridoma supernatants that produced antibodies directed to rHuIFN γ was performed with rHuIFN γ-A, which is the full-length 146 amino acid species predicted from the cDNA sequence (Gray & Goeddel, 1983). rHuIFN γ-B, a truncated derivative lacking the carboxyl-terminal fifteen amino acids, represents a variant designed for identification of monoclonal antibodies directed to the carboxyl terminus. Two other genetically engineered forms were also useful for epitope identification: rHuIFN γ-D, in which the first three amino-terminal amino acid residues have been deleted, and rHuIFN γ-D′, which, in addition to the latter deletion, is also deficient in the 5 carboxyl-terminal amino acids. Peptide F-1 contains the 15 carboxyl-terminal amino acid residues of rHuIFN γ-A. Two fragments prepared by CNBr cleavage of the parental oligopeptide were derived from the first six amino acid residues (peptide F-2) and the last nine amino acid residues (peptide F-3).

Table I: Antiviral and Antiproliferative Activities of rHuIFN γ Variants and Peptide F-1[a]

	antiviral activity (ng/mL)[b]	antiproliferative activity (ng/mL)[c]
rHuIFN γ-A	7.0	3.5
rHuIFN γ-B	25.0	200.0
peptide F-1	>5000.0[d]	>5000.0[d]
rHuIFN γ-A+ peptide F-1[e]	7.0	3.5

[a] rHuIFN γ variants are defined in Figure 1. Assay methodology is described under Experimental Procedures. [b] Concentration required to inhibit in vitro infectivity of EMC virus by 50%. [c] Concentration required to inhibit in vitro proliferation of Hep-2 cells by 10%. [d] No activity was observed at the highest concentration tested (5000 ng/mL). [e] Peptide F-1 was tested at a concentration of 8600 ng/mL (i.e., 5000 pmol/mL).

The relative antiviral and antiproliferative activities of rHuIFN γ variants and peptide F-1 are presented in Table I. A comparison of the specific activities of rHuIFN γ-A and rHuIFN γ-B indicated that removal of the carboxyl-terminal 15 amino acids resulted in ca. 3- and 60-fold increases in the amount of rHuIFN γ required for antiviral and antiproliferative effects, respectively. However, peptide F-1 did not display either antiviral or antiproliferative activities at concentrations up to 5000 ng/mL. In addition, when peptide F-1 was added at concentrations up to 8600 ng/mL (i.e., 5000 pmol/mL) to either the antiviral or antiproliferative assays simultaneously with rHuIFN γ-A, it was unable to antagonize either of these activities (Table I).

Characterization of Hybridoma Supernatants. Thirty-three of eighty-three hybridomas were observed to produce IgG that recognized rHuIFN γ-A. Antibodies secreted by clones were classified by patterns of binding to four antigens: rHuIFN γ-A, rHuIFN γ-B, a CNBr digest of rHuIFN γ-A, and peptide F-1. Four groups of monoclonal antibodies were identified. Six out of 18 clones produced antibodies that recognized all antigens except the carboxyl-terminal peptide F-1. These antibodies were assumed to recognize epitopes contained in domains that did not contain a significant contribution from the carboxyl terminus. A second group (5/18 clones) was distinct from the first group since antibodies in this class did not bind to a CNBr digest of rHuIFN γ-A. This group also appeared to be directed to regions other than the carboxyl terminus since these antibodies bound to rHuIFN γ-B and did not recognize peptide F-1. A third group of antibodies (5/18 clones) was observed to bind to only rHuIFN γ-A and hence could not be further characterized with respect to potential location of epitopes. Two clones were identified that were distinct from any of the above since they produced antibodies that did not recognize rHuIFN γ-B but did bind to peptide F-1. Thus, these antibodies appeared to be directed to an epitope residing, at least in part, in the carboxyl-terminal 15 amino acids of rHuIFN γ-A. One of the latter two clones, 47N3-6 was chosen for further characterization. A clone from group I, 30N47-1, was employed as a control for epitope identification.

Characterization of Purified Monoclonal Antibodies. (1) Effects on Biological Activity. Selected properties of the monoclonal antibodies produced from clones 47N3-6 and 30N47-1 are summarized in Table II. The immunoglobulin subclasses of antibodies 47N3-6 and 30N47-1 were determined to be IgG1/K and IgG2A/K, respectively. Antibody 47N3-6 completely inhibited the antiviral activity of rHuIFN γ-A against EMC virus and also inhibited the antiproliferative activity of rHuIFN γ-A measured on Hep-2 cells. In dis-

Table II: Properties of Two Monoclonal Antibodies Raised to rHuIFN γ-A[a]

	antibody 30N47-1	antibody 47N3-6
immunoglobulin class	IgG2a/K	IgG1/K
binding specificities[b]		
rHuIFN γ-A	+	+
rHuIFN γ-B	+	−
CNBr digest[c]	+	+
peptide F-1	−	+
neutralization[d]	−	+
neutralization titer[e]	ND[f]	6.4×10^3

[a] Experiments were performed with hybridoma supernatants prepared as described under Experimental Procedures. [b] Determined by ELISA performed as described under Experimental Procedures. A positive (+) sign indicates binding, and a negative (−) sign indicates no detectable binding; i.e., the optical density increase compared to binding in the absence of antigen was not significant. [c] CNBr digestion was performed on rHuIFN γ-A. [d] Serial dilutions of the antibodies dissolved in RPMI-1640 supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin (100 units/mL), and streptomycin (100 μg/mL) were incubated with rHuIFN γ (100 units/mL) in 96-well microtiter plates at 37 °C for 1 h. A positive (+) sign indicates neutralization of antiviral activity, and a negative (−) sign indicates lack of significant neutralization. [e] Expressed as neutralization units per milliliter. [f] Not detectable.

tinction, antibody 30N47-1 was nonneutralizing in both assays performed under similar conditions.

(2) *Epitope Identification.* An indirect ELISA was employed to establish the binding of antibody 47N3-6 to rHuIFN γ variants and peptide F-1. As shown in Figure 2A,B, a plot of the absorbance at 414 nm, which is proportional to the amount of bound antibody, versus the total amount of antigen indicated that the apparent affinities for rHuIFN γ-A, rHuIFN γ-D, and rHuIFN γ-D′ were comparable. In distinction, antibody 47N3-6 failed to bind to rHuIFN γ-B even at antigen levels as high as 10000 pmol. These data strongly suggested that the epitope on rHuIFN γ-A recognized by this antibody resided, at least in part, in the carboxyl-terminal fifteen amino acid residues. In order to provide more direct evidence for this interpretation, the binding of 47N3-6 to peptide F-1 was also evaluated. The titration curve shown in Figure 2A clearly supported binding to this peptide, although the apparent affinity for peptide F-1 was ca. 100-fold lower than that observed with the parent molecule. Nonneutralizing antibody 30N47-1 demonstrated an antigen binding pattern distinct from that observed for antibody 47N3-6 (Figure 2C). Binding was observed to occur with all three rHuIFN γ variants over approximately the same range of 0.1–1.0 pmol of antigen. As also noted for antibody 47N3-6, antibody 30N47-1 appeared to recognize rHuIFN γ-D somewhat better than rHuIFN γ-A (data not shown). In distinction to the data observed for antibody 47N3-6, significant binding to rHuIFN γ-B was observed. However, this antibody did not recognize peptide F-1 even when the latter was tested at 800 pmol. These results support that the binding pattern observed for antibody 47N3-6 reflects specific interactions with antigenic sites and is not a consequence of nonspecific protein–protein interactions.

Competition experiments were also performed to confirm the binding of antibody 47N3-6 to peptide F-1. As shown in Table III, the addition of peptide F-1 reduced antibody binding to rHuIFN γ-A to <10% of that observed in the absence of peptide. In order to reduce the possibility of nonspecific competition by the high concentration of peptide employed, a binding of antibody 47N3-6 to an octapeptide consisting of the seven carboxyl-terminal amino acid residues of rHuIFN γ-B containing a cysteine at the amino terminus (i.e., CPAAKTGK) was determined. No binding of antibody 47N3-6 to this peptide coated directly onto microtiter plates

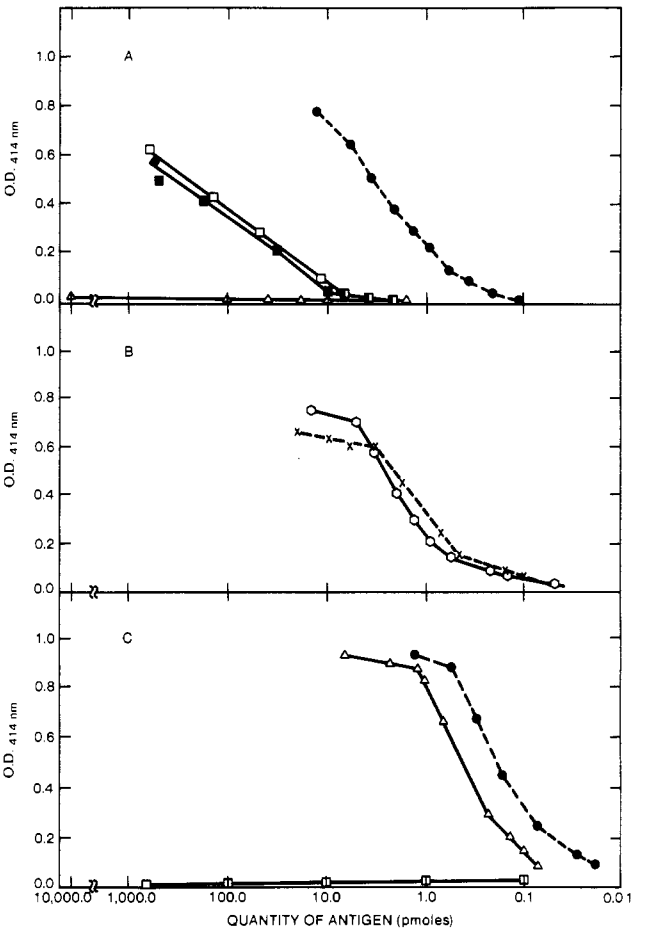

FIGURE 2: ELISA titration of two monoclonal antibodies raised to rHuIFN γ-A. (A) Antibody 47N3-6. Closed circles, rHuIFN γ-A; open squares, peptide F-1; closed squares, CNBr digest of peptide F-1; open triangles, rHuIFN γ-B. (B) Antibody 47N3-6. Open hexagons, rHuIFN γ-D; crosses, rHuIFN γ-D′. (C) Antibody 30N47-1. Closed circles, rHuIFN γ-A; open triangles, rHuIFN γ-B; open squares, peptide F-1.

Table III: Effect of Peptide F-1 on the Affinity of rHuIFN γ-A for Antibody 47N3-6[a]

antigen	competitors added to 47N3-6	ELISA (%)
rHuIFN γ-A	none	100
peptide F-1	none	60
peptide CPAAKTGK	none	0
rHuIFN γ-A	peptide F-1	<10[b]
rHuIFN γ-A	peptide CPAAKTGK	100

[a] Antigen was coated onto microtiter plates, and an ELISA was performed as described under Experimental Procedures. For direct coating of antigen onto the plates, 500 pmol of rHuIFN γ-A or 80 nmol of peptide were applied per well. In competition studies antibody 47N3-6 was added in the presence of 80 nmol of peptide per well. The absorbance obtained at 414 nm when rHuIFN γ-A was coated onto the plates and no competing peptide was present was arbitrarily set at 100%. [b] Represents the upper limit of sensitivity for detection of a significant optical density increase compared to a control well coated with bovine serum albumin.

was observed, nor was this peptide able to compete with rHuIFN γ-A for binding to antibody 47N3-6 (Table III). In contrast, under the same conditions antibody 47N3-6 was able to recognize peptide F-1 at a level approximately 60% of that observed for rHuIFN γ-A.

We have attempted to define more precisely the epitope that was recognized by antibody 47N3-6 by chemical cleavage of peptide F-1. The presence of a methionine residue at the sixth position from the amino terminus permitted CNBr cleavage into a hexamer terminating in homoserine or homoserine

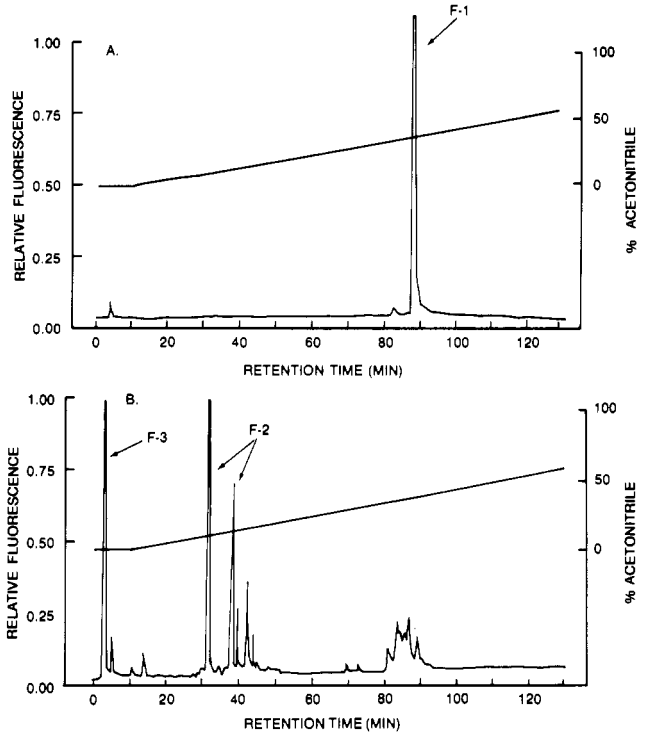

FIGURE 3: High-performance liquid chromatography of peptide F-1 and its CNBr digest. (A) Following a 10-min isocrat with 0.1 M perchloric acid, pH 2.5, a linear gradient was established between 0% and 45.5% acetonitrile for 2 h at a flow rate of 1.0 mL/min at room temperature. The column effluent was mixed with *o*-phthalaldehyde dissolved in 0.2 M sodium borate, pH 9.75, containing 0.09% brij-35 and 0.2% 2-mercaptoethanol. Fluorescence was monitored with an Aminco Fluoro Monitor as a function of retention time. (B) Peptide F-1 (1.0 mg/mL) in 70% formic acid was reacted with CNBr (15 mg/mL) at room temperature for 24 h in a sealed-glass tube. A 0.2-mL aliquot of the digest was chromatographed as in (A).

lactone (peptide F-2) and a nonamer (peptide F-3) (Figure 1). As shown in Figure 3A, the majority of F-1 migrated as a single component on reversed-phase HPLC with elution occurring at about 45% acetonitrile. After CNBr digestion of peptide F-1 three new peptides were produced eluting at ca. 0%, 15%, and 20% acetonitrile, in addition to residual undigested peptide (Figure 3B). The latter represented 2% of the composition of the CNBr digest. Amino acid analysis established that the peaks with retention times of 3.95, 32.4, and 38.8 min corresponded to peptide F-3 and the homoserine and homoserine lactone forms of peptide F-2, respectively. As shown in Figure 2A, the CNBr digest mixture of peptide F-1 appeared to be recognized over the same range of antigen concentration as the undigested oligopeptide. Thus, CNBr cleavage did not significantly perturb either the conformational integrity or the primary structure of the antigenic determinant recognized by antibody 47N3-6.

In order to determine which segment of peptide F-1 retained the antigenic determinant, a dot–blot analysis was performed. Peptide F-3, peptide F-2 (both homoserine and homoserine lactone forms), and a mixture of equal amounts of each peptide were bound to nitrocellulose, and the binding of antibody 47N3-6 was determined. Antibody 47N3-6 was found to bind to peptide F-2 as well as to the mixture. However, it recognized peptide F-3 only to a minor extent, representing no greater than 5–10% of the reactivity with peptide F-2. A synthetic peptide containing the sequence of amino acids that comprise the carboxyl-terminal nine amino acids (residues 138–146) of peptide F-3 was also not recognized by antibody 47N3-6. These results provided direct support for the conclusion that the antigenic determinant resided, at least in part, between residues 132 and 137 in rHuIFN γ-A.

Discussion

We present here strong evidence that at least a portion of the carboxyl-terminal 15 amino acids of rHuIFN γ is required for full expression of biological activity. The fact that neutralizing antibody 47N3-6 recognized an epitope residing, at least in part, in residues 132–137 suggested an involvement of this carboxyl-terminal sequence in the expression of biological activity. The nature of the participation of this domain cannot be determined from the available data. Possibilities include direct interaction with the rHuIFN γ receptor, involvement in the triggering of the biological response, or secondary effects on the conformational integrity of these regions. In the studies reported by Arakawa et al. (1986) the lack of effect of removal of the carboxyl terminus of rHuIFN γ by tryptic cleavage on either the circular dichroism spectrum of rHuIFN γ or its elution profile from gel filtration indicated that gross conformational changes or alterations in the state of association did not occur. Thus, these data provide additional support for a more direct involvement of the carboxyl terminus in receptor interaction.

Although a number of previous reports exist on the preparation of neutralizing monoclonal antibodies directed to HuIFN γ (Novick et al., 1983; Le et al., 1984a,b, 1985; Meager et al., 1984; Wang et al., 1984; Stephanos et al., 1985; Tanaka et al., 1985; Van der Meide et al., 1985; Thurman et al., 1985), the epitope to which any of these antibodies was directed was not identified. The raising of monoclonal antibodies directed to the carboxyl terminus of HuIFN γ by employing the carboxyl-terminal peptide as immunogen has been previously reported (Ichimori et al., 1985; Tanaka et al., 1985). However, it is clear that the monoclonal antibodies raised in the study of Ichimori et al. (1985) must be distinct from antibody 47N3-6 reported here since none of these antibodies were observed to inhibit antiviral activity. The fact that in our study antibody 47N3-6 neutralized both the antiviral and antiproliferative activities of rHuIFN γ-A suggested, but did not definitively establish, that at least a portion of the carboxyl terminus might be critical for biological activity. Steric hindrance of the interaction of rHuIFN γ-A by antibody binding might also account for the data. Therefore, it was important to demonstrate significantly decreased antiviral and antiproliferative activities for rHuIFN γ-B compared to the corresponding values for rHuIFN γ-A.

It is notable that previous data on the role of the carboxyl terminus in the biological activity of rHuIFN γ has been somewhat controversial. Thus, for example, although a 1000-fold loss in antiviral activity was reported after removal of the carboxyl terminus of rHuIFN γ by tryptic cleavage (Arakawa et al., 1986), it was not established in these studies that a second, lower molecular weight component (M_r 14400) reported to be present in the tryptic digest did not interfere with the determination of antiviral activity, as, e.g., by competition with native rHuIFN γ for binding to the receptor. These results were in disagreement with the report of Rose et al. (1983), who identified by gas chromatographic/mass spectrometric analysis a truncated derivative of rHuIFN γ that terminated at position 131 but yet retained full antiviral activity. These data are difficult to interpret, however, since neither a quantitative evaluation of antiviral activity nor a description of the antiviral assay employed were provided. Similarly, although the failure of the carboxyl-terminal-directed monoclonal antibodies of Ichimori et al. (1985) to neutralize antiviral activity appeared to provide support for the lack of importance of this region, it was not demonstrated

in these studies that the antibodies remained bound to HuIFN γ during performance of the antiviral assay. Dissociation of the antibody–antigen complex or, alternatively, attachment of these antibodies to a segment of the carboxyl terminus different than the polypeptide sequence identified in our study could explain these negative results. Thus, the data we report here represent the first study on the role of the carboxyl terminus of HuIFN γ utilizing two independent approaches (i.e., immunochemical mapping and genetic engineering) and have extended previous observations by localizing a critical amino acid sequence.

Peptide F-1, which is composed of residues 132–146, did not appear to be sufficient for either biological activity or receptor binding since it neither exhibited antiviral or antiproliferative activity nor did it antagonize the expression of these activities. These observations imply either that a significant conformational difference existed in comparison to the conformation of the same peptide segment present in the intact protein or, alternatively, that the peptide represents only a portion of a critical domain. The fact that antibody 47N3-6 exhibited significantly lower affinity for peptide F-1 than for rHuIFN γ-A provided additional support for either of these interpretations. However, it has not been excluded that peptide F-1 may be degraded during the incubation periods required for measurement of biological activity.

Rabbit polyclonal antibodies raised to an oligopeptide composed of the 20 amino-terminal amino acids of rHuIFN γ have been reported to neutralize in vitro antiviral activity (Johnson et al., 1982). Polyclonal antibodies directed to the first 59 amino-terminal amino acids of rHuIFN γ also neutralize antiviral activity (Leist et al., 1985). These data suggest that a critical functional domain may also reside at the amino terminus. It is consistent with the observation that the amino-terminal 45 amino acids of natural HuIFN γ were reported to compete with the parent molecule for binding to the receptor (Orchansky et al., 1985). Thus, HuIFN γ may possess two idiotopes residing in the amino and carboxyl termini, respectively, as has been proposed for recombinant human IFN α (Streuli et al., 1981). Biological activity measurements on recombinant hybrid murine human IFN γ variants support that neither antiviral activity nor species specificity resides exclusively in either the amino or carboxyl terminus (Burton et al., 1985). Interestingly, although HuIFN γ and murine IFN γ share only a moderate amino acid sequence homology (ca. 50%) and demonstrate high species specificity with respect to biological activity, evidence also exists in murine IFN γ for a functional involvement of both amino and carboxyl termini (Langford et al., 1983; Le et al., 1985; Schreiber et al., 1985; Russell et al., 1986).

The available data do not permit a determination as to whether binding to the cell surface receptor or triggering of the biological response preferentially resides in either the amino- or carboxyl-terminal domain of rHuIFN γ or whether these regions must interact for expression of these functions. Further proof for the existence of a double-idiotope model must await expansion of the panel of antibodies directed to rHuIFN γ as well as structure–function analysis through genetically engineered variants/mutations. The recent attainment of crystals of rHuIFN γ-D that diffract to 2.85-Å resolution (Vijay-Kumar et al., 1987) has provided the basis for a crystallographic determination of whether the amino and carboxyl termini are juxtaposed.

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