Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 1237–1241, February 1988
Medical Sciences

Use of synthetic peptides to identify an N-terminal epitope on mouse γ interferon that may be involved in function

(monoclonal antibody/radioimmunoassay/receptor)

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Communicated by George K. Davis, October 26, 1987

ABSTRACT **We previously have assigned N-terminal specificity to three hamster monoclonal antibodies (mAbs I, II, and III) produced to mouse recombinant γ interferon (IFN-γ), based on the ability of the N-terminal peptide IFN-γ-(1–39) to block binding of ^{125}I-labeled IFN-γ (^{125}I-IFN-γ) and on the ability of these antibodies to bind ^{125}I-IFN-γ-(1–39). Only mAb I blocked function and binding to the IFN-γ receptor, suggesting that it may bind to a region of the molecule involved in interaction with the receptor. To further define the epitope specificities of the antibodies, a series of N-terminal peptides were synthesized and tested for their ability to block antibody binding of ^{125}I-IFN-γ. Peptides IFN-γ-(1–39), IFN-γ-(1–20), IFN-γ-(3–20), and IFN-γ-(5–20) inhibited binding of ^{125}I-IFN-γ by mAb I in order of decreasing effectiveness, while peptide IFN-γ-(7–20) was without effect. Peptides IFN-γ-(1–39), IFN-γ-(1–20), and IFN-γ-(3–20) also inhibited binding of ^{125}I-IFN-γ by mAb II but were less effective when compared with their inhibition of mAb I. IFN-γ-(5–20) and IFN-γ-(7–20) did not inhibit binding by mAb II. Peptides IFN-γ-(1–10), IFN-γ-(10–30), and IFN-γ-(21–44) did not inhibit either mAb I or mAb II. While IFN-γ-(1–39) and IFN-γ-(10–30) inhibited binding by mAb III, neither IFN-γ-(1–20) nor any of its truncated forms were inhibitory. All three antibodies had similar K_d values for ^{125}I-IFN-γ. A prediction of the secondary structure of the molecule and the peptide inhibition data suggest that the epitope (possible receptor binding region) for mAb I involves a loop in the area containing residues 12–20, with sequences N-terminal to these residues possibly stabilizing the loop conformation. Direct evidence that the N-terminal 1–39 region of IFN-γ is important in receptor binding was the observation that IFN-γ-(1–39), but not the C-terminal IFN-γ-(95–133), competed with ^{125}I-IFN-γ for the receptor on mouse L cells. IFN-γ-(1–39) also specifically blocked IFN-γ antiviral activity at concentrations that blocked binding to the receptor. The fact that IFN-γ-(1–39) was the only peptide that blocked both IFN-γ binding to receptor and function is consistent with the antibody competition data, where it was the most effective peptide in blocking binding of ^{125}I-IFN-γ by the N-terminal-specific mAbs. The combination of peptide mapping of epitope specificities and receptor competition should further help define the structural basis for IFN-γ action.**

Mouse γ interferon (IFN-γ) is a secretory glycoprotein product of T lymphocytes involved in the regulation of many facets of the immune response. Its functions as a lymphokine include induction of an antiviral state, upregulation of Ia cell-surface molecules, control of antibody production, and priming of macrophages for tumor cell killing (1). A specific membrane receptor for IFN-γ has been determined (2). The structural basis for the interaction of IFN-γ with its receptor has not been well characterized, although we have suggested that the N-terminal region of the molecule may be important for binding, as determined by experiments that demonstrate the ability of a monoclonal antibody (mAb) specific for this region to block binding and function (3). Other laboratories have indicated the importance of the C-terminal polycationic region in the induction of at least some biological effects (4, 5). Some investigators have argued for a two-domain model in which different portions of the molecule may be responsible for its different biological functions (6). Of the four mAbs we have studied to date, three showed N-terminal specificity based on competition with an N-terminal peptide, but only one of the three was able to block IFN-γ binding to receptor and IFN-γ function in cultured cells (3). We now have synthesized a series of peptides corresponding to the N-terminal region of IFN-γ to further determine the structural basis for its action.

MATERIALS AND METHODS

Synthetic Peptides. Eight peptides were synthesized in our laboratory. IFN-γ-(1–10), IFN-γ-(1–20), IFN-γ-(1–39), IFN-γ-(3–20), IFN-γ-(5–20), IFN-γ-(7–20), IFN-γ-(10–30), and IFN-γ-(21–44) were made by the solid-phase technique of Merrifield (7) utilizing symmetric anhydride coupling chemistry except for the amino acids arginine, asparagine, and glutamine, for which 1-hydroxybenzotriazole esters were used as the active intermediates. Purified C-terminal peptide IFN-γ-(95–133) was obtained from Peninsula Laboratories (Belmont, CA). Its physicochemical and immunochemical properties have been described (3). A cysteine residue was added at the N-terminal position of IFN-γ-(1–10), (IFN-γ-(10–30), and IFN-γ-(21–44) to facilitate purification by thiol-affinity chromatography (8). Peptides were synthesized on an Applied Biosystems (model 430A) automated peptide synthesizer with standard software programs. Each amino acid was double-coupled, resulting in stepwise yields of 99.6% or greater reaction efficiency as indicated by the ninhydrin test. Peptides were cleaved from the resin by standard hydrogen fluoride cleavage, except for the tryptophan-containing IFN-γ-(1–39) and IFN-γ-(21–44) peptides, which were cleaved from the resin by the low/high hydrogen fluoride method (9). Peptides were lyophilized, redissolved in 5% acetic acid, desalted on a Sephadex G-10 column (2.5 × 90 cm), and finally relyophilized and stored at −20°C.

Purification of Peptides. Peptides were analyzed by reversed-phase HPLC using as mobile phase a mixture of 0.1% trifluoroacetic acid with acetonitrile, while the stationary phase was either octyl (C_8)-silica or octadecyl (C_{18})-silica. IFN-γ-(1–39) was determined to be 54% pure by integration of HPLC profiles, with an additional 33% of the peptide

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Abbreviations: IFN-γ, mouse recombinant γ interferon; mAb, monoclonal antibody; EGF, epidermal growth factor.

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alkylated or oxidized at a single tryptophan residue as determined by UV spectroscopy. IFN-γ-(1–10) showed a single peak on HPLC after the desalting procedure and, thus, did not require thiol-affinity purification. IFN-γ-(10–30) and IFN-γ-(21–44) required partial purification by thiol-affinity chromatography (8) prior to final purification by HPLC. HPLC-purified IFN-γ-(1–20), IFN-γ-(3–20), IFN-γ-(5–20), IFN-γ-(7–20) and IFN-γ-(10–30) yielded single characteristic peaks upon reinjection on HPLC, whereas IFN-γ-(21–44) showed two peaks. These two peaks demonstrated identical amino acid composition and probably differed only in the oxidation state of their α-cysteines, since only the peak eluted earlier gave a positive reaction for sulfhydryl groups with Ellman's reagent (10). Amino acid analyses in general deviated less than 5% from the expected values; in some cases, partial N-terminal sequencing was used to confirm the homogeneity of these peptides.

mAbs. mAbs were produced and purified as described (3). The resulting antibodies had been named 5.102.12, 5.74.1, and 4.6.27 (3) but are referred to here by the designations mAb I, mAb II, and mAb III, respectively.

Recombinant IFN-γ. Two lots of murine recombinant IFN-γ were obtained from Schering with specific activities of 2×10^6 and 5×10^5 units per mg of protein, respectively. The lot with the higher specific activity was radiolabeled for use in binding studies, while the other lot was used in blocking experiments.

Radioiodinations. IFN-γ was labeled with ^{125}I (^{125}I-IFN-γ) by using chloramine-T as described (3). The specific activity of ^{125}I-IFN-γ was generally 40–50 μCi/μg of protein (1 Ci = 37 GBq). After labeling, IFN-γ typically retained 95% of its antibody binding ability and 100% of its antiviral activity.

RIA. Assays were performed at room temperature in 96-well microtiter plates (Falcon, 3912) as described (11). Plates were coated with protein A (Sigma) by treatment with 30 μl of a 100 μg/ml solution for 1 hr. After washing three times with 100-μl volumes of phosphate-buffered saline (PBS), 30 μl of antibody was incubated in the wells for 1 hr to allow binding to protein A. Wells were washed four times with 100-μl volumes of PBS containing 1% bovine serum albumin (BSA) and 0.05% Tween 20 (Sigma), after which 20 μl of ^{125}I-IFN-γ was added and incubated in the antibody-coated wells for 1 hr. For competitive RIA, 20 μl of peptide were added 20 min prior to the ^{125}I-IFN-γ. After washing five times with 100-μl volumes of PBS/BSA/Tween 20, the bottoms of the wells were cut out, placed into test tubes, and assayed in a γ counter (Rock Gamma II, 1270, LKB).

Functional Assays. Antibodies were preincubated with various concentrations of IFN-γ at 37°C for 20 min. Residual antiviral activity was determined as described (12). Briefly, antibody-treated IFN-γ was incubated with mouse L cells for 16–18 hr at 37°C, after which inhibition of virus replication was determined in a plaque-reduction assay with vesicular stomatitis virus. One unit of IFN-γ caused a 50% reduction in plaque formation.

Receptor Bindings. For ^{125}I-IFN-γ bindings to mouse L cells, the cells were plated into a 96-well microtiter plate at a concentration of 6×10^4 cells per well and allowed to adhere and grow to confluency over 24 hr at 37°C (3). Medium was removed, and 25 μl of various concentrations of cold competitors in HMEM buffer (Hepes/minimal essential medium, pH 7.4) containing 5% fetal bovine serum was added to the wells and incubated for 30 min at 24°C, followed by addition of 25 μl of ^{125}I-IFN-γ (final concentration, 4 nM; 17 μCi/μg of protein) in HMEM buffer. The cells were then incubated for 1.5 hr at 24°C, followed by removal of unbound ligands. The cells were washed five times with 100-μl volumes of cold HMEM buffer, and NaOH (50 μl of 1 M) was added to each well to solubilize the cells. The liquid was then absorbed by cotton-tipped applicators and assayed in an LKB counter.

Epidermal growth factor (EGF) bindings were carried out as described above for IFN-γ by using BALB/c mouse 3T3 cells. Purified mouse submaxillary gland EGF was obtained from Toyobo Biochemicals, Osaka, Japan. EGF was labeled with ^{125}I as described for IFN-γ and was used at a final concentration of 7 nM (22 μCi/μg).

RESULTS AND DISCUSSION

Fusion of Sp2/0-Ag14 mouse myeloma cells with spleen cells from two hamsters immunized with IFN-γ yielded four hybridomas that produced antibodies reactive with the immunogen. The fusion and screening results for these hybridomas have been described (3). Three of the mAbs (I, II, and III) demonstrated N-terminal specificity, as evidenced by their ability to bind ^{125}I-IFN-γ-(1–39) but not the C-terminal peptide ^{125}I-IFN-γ-(95–133). In addition, IFN-γ-(1–39), but not IFN-γ-(95–133), was effective in blocking the binding of ^{125}I-IFN-γ by these antibodies in a competitive RIA. The antibodies were also tested for their abilities to block the biological functions of IFN-γ. Only mAb I was effective in neutralizing the antiviral property of IFN-γ: the average units of IFN-γ neutralized per μg of mAbs I, II, and III were 1160, 6, and 6, respectively. (Various concentrations of the mAbs had been preincubated with 3-, 6-, or 10-unit aliquots of IFN-γ before titration of antiviral activity in a standard assay.) Thus, we were interested in determining why only

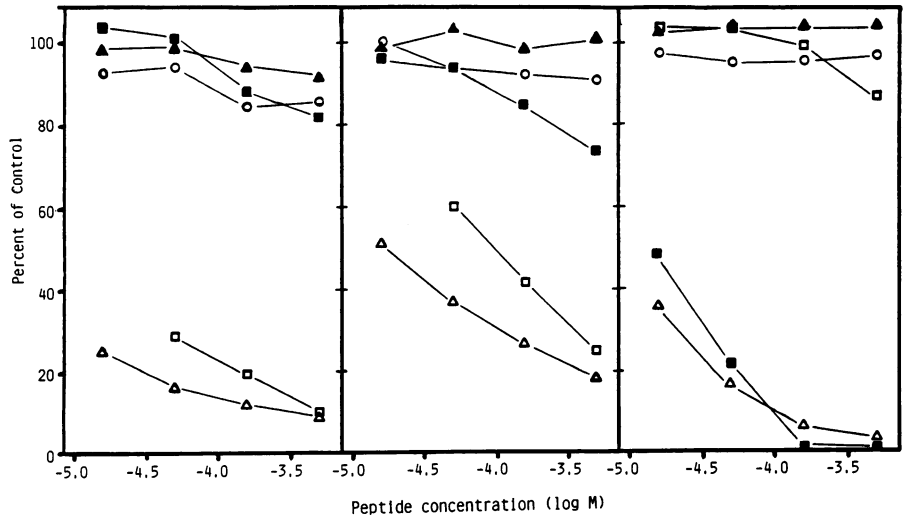

FIG. 1. Competitive RIA with synthetic peptides and anti-IFN-γ mAb. The abilities of the synthetic peptides to block binding of ^{125}I-IFN-γ were determined in a competitive RIA. Binding by mAb I (4 μg/ml) (*Left*), mAb II (7 μg/ml) (*Center*), and mAb III (3 μg/ml) (*Right*) is expressed as percent of control. ^{125}I-IFN-γ was used to give a final concentration of 8 nM. Control binding of ^{125}I-IFN-γ in the absence of any peptide competitor was 146,299 ± 7,249 cpm for mAb I, 143,519 ± 1,547 cpm for mAb II, and 128,563 ± 9,288 cpm for mAb III. Experiments were performed in triplicate, and coefficients of variation were generally 10% or less. ○, IFN-γ-(1–10); □, IFN-γ-(1–20); ■, IFN-γ-(10–30); △, IFN-γ-(1–39); and ▲, IFN-γ-(21–44).

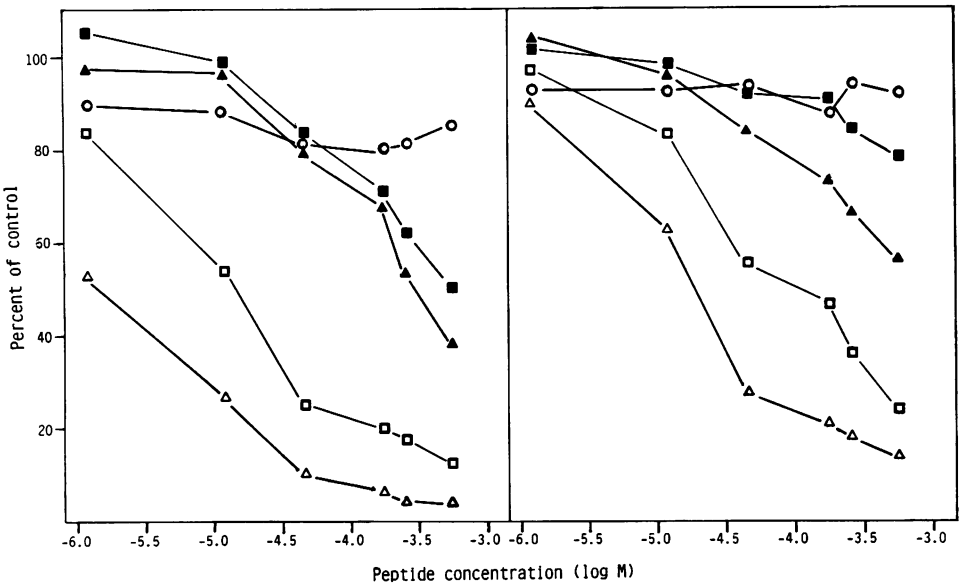

FIG. 2. Competitive RIA with IFN-γ-(1–20) and its N-terminal truncated peptides. Competitive RIAs using mAb I (*Left*) and mAb II (*Right*) were performed with ^{125}I-IFN-γ as described in Fig. 1, except that ^{125}I-IFN-γ was used to give a final concentration of 10 nM. □, IFN-γ-(1–20); ▲, IFN-γ-(3–20); ■, IFN-γ-(5–20); ○, IFN-γ-(7–20); and △, IFN-γ-(1–39).

one of the three N-terminal-specific mAbs significantly blocked IFN-γ function (3).

To determine the structural basis for this differential effect on function, we performed more detailed mapping of the epitope specificities of mAbs I, II, and III, using a series of overlapping N-terminal peptides in a competitive RIA. The peptides tested in competitive binding against ^{125}I-IFN-γ were IFN-γ-(1–10), IFN-γ-(1–20), IFN-γ-(10–30), IFN-γ-(21–44), and IFN-γ-(1–39). The results are presented in Fig. 1. As described (3), IFN-γ-(1–39) blocked binding of ^{125}I-IFN-γ to all three antibodies. IFN-γ-(1–20) blocked binding of mAbs I and II at concentrations similar to those of IFN-γ-(1–39) but was less effective. Peptides IFN-γ-(1–39) and IFN-γ-(1–20) were more effective in inhibiting mAb I binding of ^{125}I-IFN-γ than in inhibiting mAb II binding, suggesting similar but different epitope specificities for the two antibodies. Since mAbs I and II were used at concentrations that bound ^{125}I-IFN-γ similarly, the different inhibition patterns are thought to be due to qualitative differences between the antibodies. mAb III clearly differs in epitope specificity from mAbs I and II, since it was not blocked by IFN-γ-(1–20) but was inhibited by IFN-γ-(10–30). Peptides IFN-γ-(1–10) and IFN-γ-(21–44) did not block binding of any of the antibodies. Inhibitions observed with IFN-γ-(1–20), IFN-γ-(1–39), and IFN-γ-(10–30) were specific, since these peptides failed to inhibit binding of ^{125}I-IFN-γ by C-terminal-specific polyclonal antibodies (3) and mAbs (data not shown).

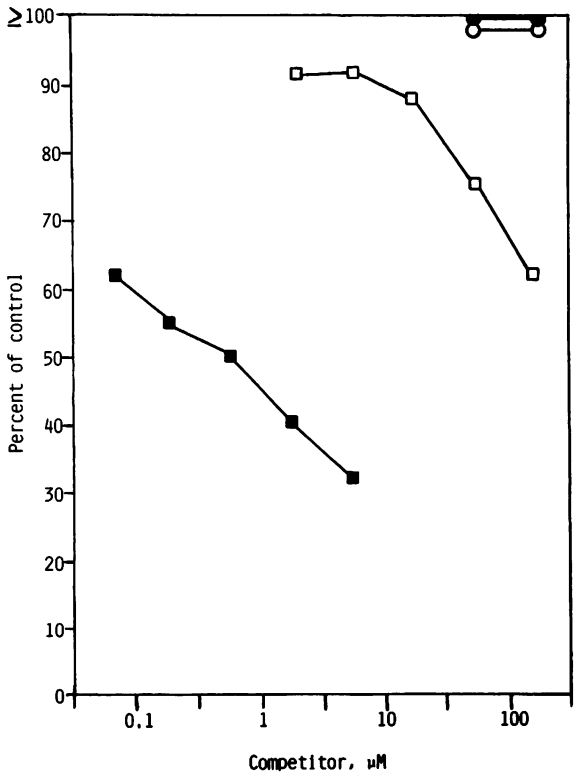

FIG. 3. Competitive binding of synthetic peptides and IFN-γ against ^{125}I-IFN-γ (final concentration, 4 nM) for receptors on mouse L cells. Experiments were performed in triplicate, and coefficients of variation were less than 10%. The data presented are representative of five experiments. ■, IFN-γ; □, IFN-γ-(1–39); ○, IFN-γ-(1–20); and ●, IFN-γ-(95–133).

In order to further examine the epitope specificities of mAbs I and II, IFN-γ-(1–39), IFN-γ-(1–20), and the truncated IFN-γ-(1–20) peptides IFN-γ-(3–20), IFN-γ-(5–20), and IFN-γ-(7–20) were compared for their relative abilities to block antibody binding of ^{125}I-IFN-γ. Peptides IFN-γ-(1–39) and IFN-γ-(1–20) inhibited binding of ^{125}I-IFN-γ by mAbs I and II as described above (Fig. 2). Of particular interest were the differential inhibitions observed with IFN-γ-(1–20) and its truncated peptides. Peptides IFN-γ-(1–20),

Table 1. Competitive binding between IFN-γ-(1–39), IFN-γ, and EGF against ^{125}I-labeled EGF (^{125}I-EGF) for membrane receptors

Competitor	Concentration, μM	Receptor binding, % of control	
		^{125}I-IFN-γ*	^{125}I-EGF*
IFN-γ-(1–39)	56	76	120
	167	62	140
IFN-γ	2	40	98
	6	32	101
EGF†	44	112	10
	131	106	10

*^{125}I-IFN-γ (final concentration, 4 nM) bindings were carried out on mouse L cells. ^{125}I-EGF (final concentration, 7 nM) bindings were carried out on mouse 3T3 cells. Experiments were performed in triplicate, and coefficients of variation were less than 10%. IFN-γ-(1–39) blockage of ^{125}I-IFN-γ binding was significant at both concentrations presented with $P < 0.001$.

†EGF was used at concentrations of 74 and 222 μM against ^{125}I-IFN-γ.

Table 2. Inhibition of antiviral activity of IFN-γ by synthetic peptides

IFN-γ, units/ml	Concentration of synthetic peptide required to block IFN-γ antiviral activity, μM		
	IFN-γ-(1–39)	IFN-γ-(95–133)	IFN-γ-(1–20)
15	22 ± 8	>167	125 ± 59
20	63 ± 30	>167	>167
100	167 ± 0	>167	>167

Synthetic peptides were added to mouse L cell cultures 30 min prior to IFN-γ addition. Experiments were carried out in duplicate, and values show the means and range of the concentrations of peptides that inhibited the antiviral activity of the indicated concentrations of IFN-γ against vesicular stomatitis virus in a standard assay.

IFN-γ-(3–20), and IFN-γ-(5–20) inhibited binding of ^{125}I-IFN-γ by mAb I with decreasing effectiveness, while IFN-γ-(7–20) was without effect. Peptides IFN-γ-(1–20) and IFN-γ-(3–20) also inhibited binding of ^{125}I-IFN-γ by mAb II but were less effective when compared with their inhibition of ^{125}I-IFN-γ binding by mAb I. IFN-γ-(5–20) and IFN-γ-(7–20) did not inhibit binding by mAb II. Thus, the IFN-γ-(1–20) truncated peptides further demonstrate the differences in epitope specificity of mAbs I and II.

It is possible that mAb I blocked function because of a higher binding affinity for IFN-γ than that of mAb II. Therefore, mAbs I and II as well as mAb III were compared for their relative binding affinities (K_d) for IFN-γ. The three antibodies bound ^{125}I-IFN-γ with similar affinities, yielding K_d values of $10^{-7.7}$, $10^{-7.7}$, and $10^{-7.8}$ for mAbs I, II, and III, respectively (data not shown). We conclude that inhibition of IFN-γ function by mAb I was not due to a higher binding affinity but rather to its distinct epitope specificity.

To more directly assess the role of the N-terminal region of IFN-γ in function, the peptides also were examined for their ability to compete with ^{125}I-IFN-γ in binding studies for IFN-γ receptors on mouse L cells. IFN-γ-(1–39), but not the C-terminal IFN-γ-(95–133) or IFN-γ-(1–20), competed with ^{125}I-IFN-γ for receptors (Fig. 3). IFN-γ-(1–39) was approximately 1/1000th as effective as nonradioactive recombinant IFN-γ in the competition. The blockage was specific, since IFN-γ-(1–39) failed to compete with EGF binding to the EGF receptor on BALB/c 3T3 cells (Table 1). IFN-γ-(1–39) also specifically blocked IFN-γ antiviral activity at the same concentrations at which it blocked binding (Table 2). The fact the IFN-γ-(1–39) was the only peptide that blocked binding to IFN-γ receptors on mouse L cells is consistent with the antibody competitions, where it was the most effective peptide in blocking binding of ^{125}I-IFN-γ to the N-terminal-specific mAbs. IFN-γ-(1–39) did not demonstrate antiviral activity against vesicular stomatitis virus on mouse L cells at concentrations that blocked binding of ^{125}I-IFN-γ to cells.

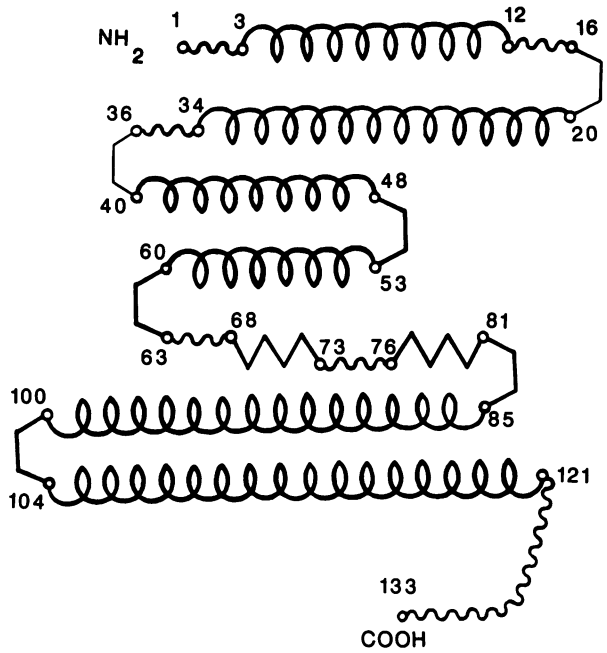

FIG. 4. Predicted secondary structure of IFN-γ (13, 14). Regions of IFN-γ predicted to attain α-helical secondary structure in the native conformation of the molecule are represented as coils (e.g., residues 3–12). The number of turns in the coil is not intended to represent the actual topography of the helix as it exists in IFN-γ. β-sheet regions are represented by zigzags (e.g., residues 68–73). β-bend regions are represented by trapezoid shapes (e.g., residues 16–20). Regions for which no stable secondary structure is predicted are represented by wavy lines (e.g., residues 121–133). Lengths of the various segments are drawn approximately to scale; however, spatial relationships between various elements are arbitrary and are not intended to imply a predicted tertiary structure.

To gain further insight into the nature of the N-terminal structure of IFN-γ and the epitope for mAb I, we examined predictive algorithms for secondary structure and for regions of the IFN-γ molecule likely to be located on its surface. Fig. 4 represents a secondary structure prediction essentially by the method of Chou and Fasman (13, 14), with assignment of the bend (rather than helix) at residues 60–63 influenced by the hydrophobicity of the sequence. In agreement with circular dichroism data on the homologous human IFN-γ molecule (15), which indicates mostly α-helical structure, this algorithm suggests a protein that is generally globular and composed mostly of helices with little β-sheet structure. However, the most interesting regions are predicted β-bends, since they are likely to be located on the surface of the folded native molecule where they would be available as antigens (16). Indeed, such surface profile predictions (16) for the sequence indicate that most of these bend structures are highly hydro-

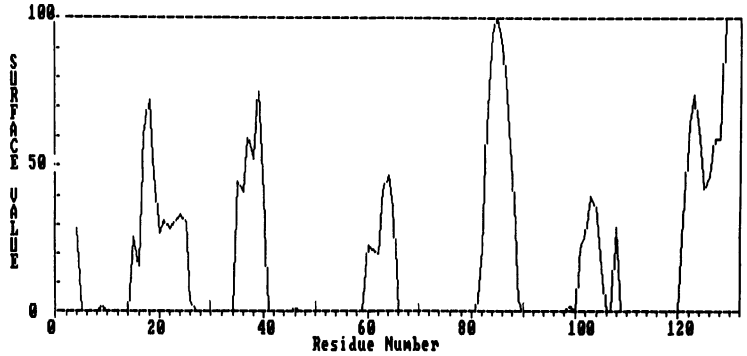

FIG. 5. Surface profile of the IFN-γ sequence. A composite surface profile of IFN-γ was developed as described by using a computer program (17). This program takes into account HPLC mobility, accessibility, and segmental mobility (*B* values) of amino acids in model proteins and peptides. Residues with high composite values are most likely to reside on the surface of a protein molecule.

philic and, therefore, are expected to be exposed to solvent on the outer surface of the molecule (Fig. 5).

We are particularly interested in the hydrophilic bend region (residues 16–19) of IFN-γ because it is contained in IFN-γ-(1–20). However, since the adjacent residues 13–15 are not predicted to fall into any regular secondary structure according to the Chou–Fasman algorithm but are highly hydrophilic, the entire region (residues 12–20) may be predicted to form a loop structure (17). Loops, which are described as Ω-shaped conformations of contiguous segments of 6–16 residues, are invariably situated on the surfaces of protein molecules. It is possible, therefore, that the epitope of mAb I involves this loop/bend region of IFN-γ with involvement of sequences N-terminal to the loop.

While IFN-γ-(1–20) inhibited antibody binding of ^{125}I-IFN-γ, neither IFN-γ-(1–10) nor IFN-γ-(10–30), which together overlap the sequence of IFN-γ-(1–20), showed this effect. A study of the IFN-γ-(1–20) sequence by synthesis of peptides shortened on the N-terminal end indicates that the first one or two amino acids are necessary for inhibition of antibody binding by this peptide. Amino acid substitutions with residues of similar structural properties and potential for binding interactions in the IFN-γ-(1–39) and IFN-γ-(1–20) sequences may help to determine further which portions of the amino acid sequence are absolutely necessary for binding to the antibodies and IFN-γ receptor. These peptides may also identify possible flanking sequences which, while not directly involved in binding, may play an important role in stabilizing the binding sequence in the proper conformation. Fibrinogen, for example, has been shown to have a specific sequence of nine residues absolutely required for blocking the interaction of the fibrinogen molecule with a strain of the *Staphylococcus* bacteria (18). Beyond these nine residues, an additional sequence of amino acids on either the N- or C-terminal side of the nonapeptide was also found to be necessary. These additional residues apparently stabilized the nonapeptide in the proper conformation for interaction with the fibrinogen receptor on the bacteria.

The fact that IFN-γ-(1–20) and IFN-γ-(1–39) inhibited binding of ^{125}I-IFN-γ by mAb I does not necessarily indicate that this antibody is specific for a continuous epitope. It is possible that the epitope is actually composed of or stabilized by discontinuous sequences and that only a part of the sequence is contained in IFN-γ-(1–20) and IFN-γ-(1–39). This is consistent with the observation that IFN-γ-(1–39) is 1/1000th as effective as IFN-γ in inhibition of binding of ^{125}I-IFN-γ by mAb I (3) and by receptor as shown above. Relevant to this is the recent determination of the structure of a complex between lysozyme and a Fab fragment from a mAb against lysozyme based on high-resolution x-ray crystallography (19). The contact regions in the ligand belong to two stretches of the lysozyme polypeptide chain, residues 18–27 and 116–129. It is possible, therefore, that distant sequences in the IFN-γ molecule identified by the predictive models may also be involved in binding. These sequences could interact with the N-terminal epitope, stabilizing its conformation, or they may themselves bind to the mAbs or IFN-γ receptor and, thus, further define the epitope and functional specificity of mAb I.

The hybridomas used in this study have been described (3) and were generated by M. P. Hayes. One lot of Schering recombinant IFN-γ was provided through the auspices of the American Cancer Society. This study was supported by National Institutes of Health Grant CA 38587.

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