

Inhibition of the Biological Activity of Human Interferon- γ by Antipeptide Antibodies

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ABSTRACT

To study the domain(s) responsible for the different biological activities of human interferon- γ (HuIFN- γ), rabbits were immunized with peptides corresponding to the five most hydrophilic amino acid sequences of the lymphokine. The resulting antisera were able to recognize both the immunizing peptide and the native protein. Antibodies to the carboxy-terminal region (amino acids 125-137) of HuIFN- γ were able to interfere with the immunomodulating, antiviral, and antiproliferative activities of the lymphokine. Inhibition of the antiproliferative and antiviral activity was also observed using antibodies raised against the amino-terminal region (amino acids 4-16) and amino acids 80-95, respectively, but to a lesser extent than that observed with antibodies to the carboxyl terminus. The capability of these antibody preparations to partially interfere with only one of the lymphokine's biological activities might be explained by a mechanism of steric hindrance. The use of polyclonal antibodies allowed us to limit the presence of epitopes responsible for recombinant (r)HuIFN- γ biological activities to the carboxy-terminal region.

INTRODUCTION

INTERFERON- γ (IFN- γ) is a glycoprotein of 143 amino acids produced by T lymphocytes in response to mitogenic or antigenic stimuli, and it expresses potent antiviral, antiproliferative, and immunomodulating functions.⁽¹⁻³⁾ So far, approaches to investigate the relationship between the structure of human IFN- γ (HuIFN- γ) and its biological activities mainly have involved the use of specific monoclonal and polyclonal antibodies, as well as modifications in the structure of the lymphokine by limited proteolytic digestion and by the recombinant DNA technique. Several investigators have noted that the carboxyl terminus is involved in the biological activity of HuIFN- γ ,^(4,5) and that the removal of more than 14 residues⁽⁶⁾ or the interaction of antibodies with this region⁽⁷⁾ results in a sharp decrease in antiviral and antiproliferative activity. However, antibodies specific to the first 20 amino-terminal residues of HuIFN- γ were also found to be inhibitory.^(8,9) Moreover, recent studies have indicated that HuIFN- γ may act by a mechanism involving more than one active site on the molecule.⁽⁹⁻¹²⁾

The identification of the functional sites on the HuIFN- γ molecule that elicit the various effector functions is important, and antibodies of a predetermined specificity may help to identify them. So far, no systematic studies of the biological activity of HuIFN- γ using antibodies against synthetic peptides corresponding to different epitopes of the lymphokine have been carried out. Leist *et al.*⁽¹³⁾ produced in rabbits antibodies to synthetic polypeptides corresponding to two hydrophilic sequences of amino acids in the HuIFN- γ molecule; however, their results were impaired by the inability of most of the antisera to recognize the native protein. In addition, the authors considered only the antiviral activity of HuIFN- γ . Since it has been shown that the most hydrophilic amino acid sequence often corresponds to an antigenic determinant in a protein,⁽¹⁴⁾ we decided to use synthetic peptides of the five most hydrophilic regions of HuIFN- γ for immunizing rabbits. The resulting antisera, which were able to recognize both the immunizing peptide and the native protein, were then studied for their ability to neutralize the antiviral, antiproliferative, and immunomodulatory activity of HuIFN- γ .

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MATERIALS AND METHODS

Peptides: The peptides (positions 4–16, 34–47, 53–68, 80–95, 125–137, of the HuIFN- γ gene product) corresponding to the five most hydrophilic regions of the lymphokine according to the method of Hopp and Wood⁽¹⁴⁾ (see Fig. 1) were obtained from Cambridge Research Biochemicals (Northwich, Cheshire, UK): *i.e.*, IFN- γ peptide (IGP) 1 (amino acids 4–16), YVKE-AENLKKYFN; IGP 2 (amino acids 34–47), KNWKEES-DRKIMQS; IGP 3 (amino acids 53–68), YFKLFKNFKDDQ-SIQK; IGP 4 (amino acids 80–95), KFFNSNKKKRDDFEKL; IGP 5 (amino acids 125–137), KTGKRKRSQMLFQ. Cysteine was added to the amino terminus of aliquots of each peptide to facilitate conjugation to a carrier molecule. Peptides were coupled to keyhole limpet hemocyanin (KLH) using *m*-Maleimido-benzoyl-*N*-hydroxysuccinimide esters (MBS) as coupling agent.⁽¹⁵⁾

Production and Screening of Rabbit Hyperimmune Sera: Two rabbits were immunized with each of the five peptides as follows: 100 μ g of conjugated peptide in Freund's complete adjuvant were injected subcutaneously into each rabbit. Aliquots of 100 μ g of peptide in Freund's incomplete adjuvant were then injected into each rabbit at 1-week intervals for 11 weeks. Antisera produced in rabbits were tested against the five peptides and recombinant HuIFN- γ (rHuIFN- γ) (Hoffman-La Roche, Basel, Switzerland) by solid-phase radioimmunoassay (RIA) as described previously.⁽¹⁶⁾ In brief, wells of 96-well flexible polyethylene terephthalate glycol (PETG) microtiter plates (Costar Corp., Cambridge, MA) were coated with 50 ng/well of each peptide, or with 250 ng/well of rHuIFN- γ diluted in 50 μ l of phosphate-buffered saline (PBS) containing calcium and magnesium (pH 7.2) and allowed to dry overnight at 37°C. To minimize nonspecific protein absorption, the microtiter wells were then filled with 200 μ l of PBS containing

5% bovine serum albumin (BSA) (wt/vol) and incubated for 1 h at 37°C. The plates were next washed with PBS containing 1% BSA (wt/vol) (assay buffer). Rabbit sera were dispensed in duplicate (in 50- μ l volumes at a dilution of 1:200 in assay buffer) into wells coated with each of the five peptides and rHuIFN- γ . The plates were incubated for 1 h at 37°C. After two washes with assay buffer, 25 μ l of ¹²⁵I-labeled Protein A (Amersham, Amersham, UK) containing 50,000 counts per minute (cpm) were added to each well. The plates were incubated for 1 h at 37°C and washed three times with assay buffer. The bound cpm were detected by cutting the wells from the plates and counting them in a Gamma Counter (Clinigamma 1272; Wallac Oy, Turku, Finland).

Purification of Antibodies and Production of F(Ab)₂ Fragments: Antibodies were purified using Protein A-Sepharose (Pharmacia, Uppsala, Sweden). Aliquots of each rabbit antibody preparation were hydrolyzed with pepsin to produce F(Ab)₂ fragments as described,⁽¹⁷⁾ with minor modifications. In brief, antibodies at a concentration of 10 mg/ml in sodium acetate buffer at pH 4.5 were incubated with pepsin at a final concentration of 100 μ g/ml. Following a 24-h incubation at 37°C, the mixture was neutralized by adding solid Tris salt to reach pH 8.0, and then chromatographed on Sephadex G150. The resulting F(Ab)₂ preparations were concentrated by Centricon 30 (Amicon, W.R. Grace & Co., Danvers, MA) and dialyzed against PBS overnight at 4°C.

Induction of HLA-DR Antigens and Inhibition Experiments: The human monocytic cell line U937 was cultured at 37°C in RPMI-1640 medium (GIBCO, Paisley, UK), supplemented with 20% fetal calf serum (GIBCO) in an atmosphere of humidified air containing 5% CO₂. Recombinant HuIFN- γ at a concentration ranging from 1 to 200 U/ml was incubated for 2 h at room temperature in the presence or absence of F(Ab)₂ fragments of purified rabbit anti-peptide antibodies or F(Ab)₂ frag-

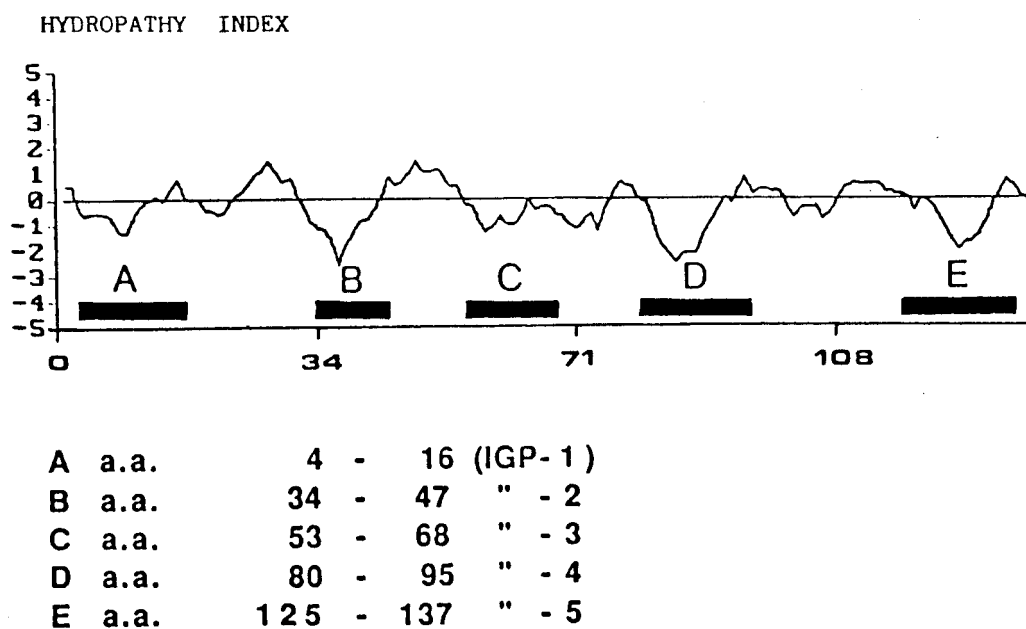


Fig. 1. Hydropathy profile prepared according to the method of Hopp and Woods.⁽¹⁴⁾ Black boxes indicate positions of synthetic peptides.

ments of purified unrelated hyperimmune rabbit antibodies. The mixtures were then added to 2×10^5 U937 cells and HLA-DR induction evaluated after 24 h in a 5% CO₂ incubator at 37°C. The different F(Ab)₂ fragment preparations at a final concentration of 1, 10, and 100 μ g/ml, and the inducer were present in the cell culture during the whole time of culture. As a negative control, U937 cells were cultured in the medium alone.

Antiviral Assay and Neutralization: Antiviral activity was detected by a biological neutralization assay of the cytopathic effect of vesicular stomatitis virus on human WISH cells as described previously.⁽¹⁸⁾ Neutralization experiments were carried out to determine whether the rabbit anti-peptide antibodies blocked the rHuIFN- γ antiviral activity. In brief, 200 U of rHuIFN- γ were incubated with various concentrations of purified rabbit anti-peptide antibodies or their F(Ab)₂ fragments. After 2 h of incubation at room temperature, the mixtures were assayed for their antiviral activity.

Antiproliferative Activity and Neutralization: The antiproliferative activity of rHuIFN- γ was evaluated as described earlier.⁽⁵⁾ Hep-2 cells (a cell line from an epidermoid carcinoma derived from a human larynx) were grown in Eagle's minimal essential medium containing 5% fetal calf serum and antibiotics, in a 5% CO₂ incubator at 37°C. Dilutions of rHuIFN- γ were preincubated for 2 h at room temperature in the presence or absence of different concentrations (ranging from 25 to 200 μ g/ml) of each rabbit anti-peptide antibody or of F(Ab)₂ fragment preparations. The mixtures were then added to cells seeded 24 h before at a density of 10^4 cells per 2-cm² well. The cells were counted at day 7, just before the control non-rHuIFN- γ treated cells reached confluency. Monolayers were washed twice with PBS, trypsinized, suspended in Isotonic II (Coulter Electronics, Inc., Hialeah, FL), and counted using an automated cell counter (Coulter Counter, model D2N, Coulter Electronics, Inc.).

RESULTS

Antisera

The reactivity of the rabbit anti-peptide antibodies used in this study is shown in Table 1. All rabbits immunized with five different IFN- γ -relating peptides developed antibodies that reacted specifically with their own immunizing peptide and with rHuIFN- γ , whereas they did not cross-react with unrelated peptides, as assessed by solid-phase RIA. The specific reactivity of rabbit anti-peptide antisera to rHuIFN- γ was confirmed by Western blot and liquid competition RIA experiments (data not shown). To perform all the subsequent experiments, we chose from each group of immunized rabbit antisera, the one with the highest anti-IFN- γ antibody titer (rabbit #4 for rabbits immunized with the IGP-2 peptide).

Inhibition of HLA-DR antigen induction

Purified rabbit anti-peptide antibodies were digested with pepsin to generate F(Ab)₂ in order to avoid unspecific binding of rabbit immunoglobulins to Fc receptor sites on the rHuIFN- γ -treated U937 cells. The reactivity pattern of anti-peptide antisera was not affected by enzymatic digestion, as detected by solid-phase RIA. U937 cells increased HLA-DR antigen expression when incubated in a medium containing rHuIFN- γ at a concentration ranging from 1 to 200 U/ml. The effect of rHuIFN- γ on U937 cells, observed after 24 h of culture, was already maximum at a concentration of 10 U/ml. When the five rabbit anti-peptide F(Ab)₂ fragment preparations were present in U937 cell cultures stimulated with rHuIFN- γ , only the anti-IGP-5 antibodies were able to inhibit the expected increase in HLA-DR antigens (Table 2). The inhibition was found to be dose dependent, and most greatly effective with rabbit anti-IGP-5 F(Ab)₂ fragments at a concentration of 100 μ g/ml. No inhibitory effect was observed with all the other anti-peptide

TABLE 1. REACTIVITY OF RABBIT ANTIPEPTIDE ANTIBODIES^a

Rabbit	Immunogen	Reactivity against					rHuIFN- γ
		IGP-1	IGP-2	IGP-3	IGP-4	IGP-5	
1	IGP1	5,400	<200	<200	<200	<200	5,400
2	IGP1	33,600	<200	<200	<200	<200	1,800
3	IGP2	<200	11,800	<200	<200	<200	11,800
4	IGP2	<200	5,400	<200	<200	<200	11,800
5	IGP3	<200	<200	5,400	<200	<200	5,400
6	IGP3	<200	<200	11,800	<200	<200	1,800
7	IGP4	<200	<200	<200	33,600	<200	5,400
8	IGP4	<200	<200	<200	33,600	<200	1,800
9	IGP5	<200	<200	<200	<200	5,400	1,800
10	IGP5	<200	<200	<200	<200	33,600	5,400

^aExpressed as antibody titer.

TABLE 2. EFFECT OF F(Ab)₂ FRAGMENTS OF RABBIT ANTIPEPTIDE ANTIBODIES ON THE EXPRESSION OF HLA-DR ANTIGENS ON U937 CELLS STIMULATED WITH rHuIFN- γ ^a

Addition to culture	HLA-DR-positive cells (%)	Inhibition (%)
—	76.3	—
Antibodies to		
IGP-1	74.5	2.4
IGP-2	76.2	0.2
IGP-3	75.3	1.4
IGP-4	77.4	0
IGP-5	2.8	96.4
Unrelated rabbit antibodies	79.8	0

^a rHuIFN- γ was incubated alone or with F(Ab)₂ fragments of rabbit antibodies for 2 h at room temperature. The mixtures were then added to U937 cells and HLA-DR expression evaluated after 24 h of incubation. The final concentrations of rHuIFN- γ and F(Ab)₂ fragments were 10 U/ml and 100 μ g/ml, respectively.

F(Ab)₂ fragment preparations or with the unrelated rabbit F(Ab)₂ fragment preparation used as a control.

Inhibition of rHuIFN- γ antiviral activity

Purified anti-peptide antibodies raised by IGP-4, and -5, showed the capability of interfering with the antiviral activity of rHuIFN- γ , while no neutralization was observed with antisera to IGP-1, -2, and -3, or with hyperimmune unrelated sera. However, while IGP-5 antibodies were able to inhibit the antiviral activity of rHuIFN- γ completely, IGP-4 antibodies were only able to inhibit the lymphokine's activity partially at the highest concentrations used (data not shown).

Since the difference in the biological activity of rabbit anti-peptide antibodies on the rHuIFN- γ antiviral and immunomodulating activity might be due to the use of the whole antibody molecule or F(Ab)₂ fragments, respectively, we decided to test the latter antibody preparation in a rHuIFN- γ antiviral assay. The results obtained with F(Ab)₂ fragments were similar to those observed with undigested antibodies. Partial inhibition of rHuIFN- γ by anti-IGP-4 F(Ab)₂ fragments occurred at concentrations of 25 and 50 μ g/ml (Fig. 2).

Inhibition of rHuIFN- γ antiproliferative activity

Treatment with rHuIFN- γ reduces the growth of Hep-2 cells by approximately 64% of the control growth level, at a concentration of 140 U/ml. Purified rabbit antibodies to IGP-5 and -1 were able to neutralize the antiproliferative activity of rHuIFN- γ with a maximum inhibitory effect achieved at a concentration of 100 μ g/ml. The neutralizing activity of the anti-IGP-5 antiserum ranged from 78 to 91%, while that of the anti-IGP-1 antiserum ranged from 57 to 60% as seen in three independent experiments. No interference with the antiproliferative activity of rHuIFN- γ was observed with rabbit antisera to peptides IGP-2, -3, and -4, or with unrelated hyperimmune

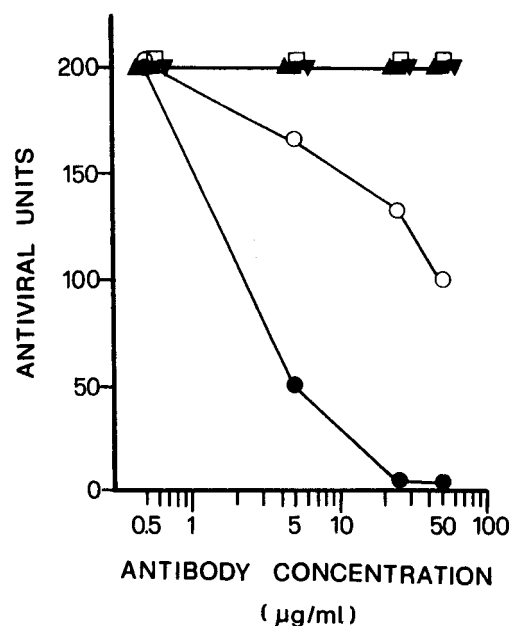


Fig. 2. Effect of F(Ab)₂ fragments of rabbit anti-peptide antibodies on rHuIFN- γ antiviral activity. Antibody to: IGP-1 (■), IGP-2 (▼), IGP-3 (▲), IGP-4 (○), IGP-5 (●), and unrelated protein (□).

rabbit immunoglobulins, even when used at a concentration of 200 μ g/ml (Fig. 3). Superimposable results were obtained when F(Ab)₂ fragment preparations were used instead of the corresponding whole antibodies (data not shown).

DISCUSSION

Antibodies to synthetic peptides representing the five most hydrophilic regions of HuIFN- γ were also able to recognize the native rHuIFN- γ molecule. The availability of such antibodies prompted us to investigate their ability to neutralize the activities of HuIFN- γ , to define the location of the functional domain(s) of the lymphokine. Neutralization of the immunomodulating, antiviral, and antiproliferative activities of rHuIFN- γ by anti-IGP-5 antibodies (which react with amino acids 125–137) is in agreement with previous studies showing that the carboxyl terminus is involved in maintaining the biological activity of HuIFN- γ .^(4–7) No inhibitory activity was observed with antibodies reacting to HuIFN- γ -related peptides IGP-2 (amino acids 34–47), and IGP-3 (amino acids 53–68). On the other hand, antibodies to IGP-1 (amino acids 4–16), and IGP-4 (amino acids 80–95) were found to interfere with the antiproliferative and the antiviral activity of rHuIFN- γ , respectively, but always to a lesser extent than the anti-IGP-5 antibodies. Since the closer the antibody binds to the active site, the greater its neutralizing ability will be,⁽¹⁹⁾ the capability of anti-IGP-1 and anti-IGP-4 antibodies to neutralize one of the biological activities of rHuIFN- γ only partially could be due to a mechanism of steric hindrance. This can easily be the case for IGP-4 antibodies since this peptide, which spans from residues 80 to 95, is

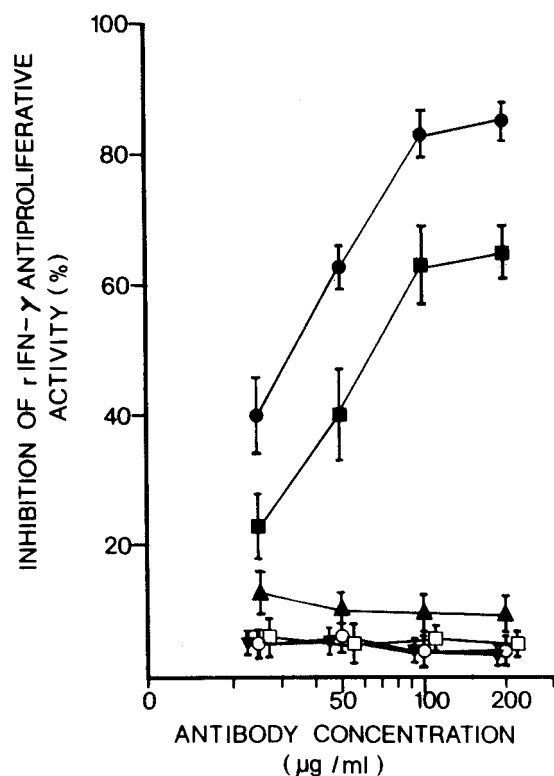


Fig. 3. Effect of rabbit antipeptide antibodies on rHuIFN- γ antiproliferative activity. Antibody to: IGP-1 (■), IGP-2 (▼), IGP-3 (▲), IGP-4 (○), IGP-5 (●), and unrelated protein (□).

located close to IGP-5. It is more difficult to explain the activity of antibodies to IGP-1, since this peptide is located at the amino terminus of HuIFN- γ . However, the recent observation that the rHuIFN- γ active molecule is a dimer of two identical monomers⁽²⁰⁾ and the determination of the three-dimensional structure of rHuIFN- γ , in which the authors observed contacts occurring between amino-terminal helix of one subunit and the carboxy-terminal helix of the other,⁽²¹⁾ support the hypothesis of a closeness of the two epitopes we defined as IGP-1 and -5.

The presence of an antipeptide antibody (anti-IGP-5) that is capable of blocking all the activities of rHuIFN- γ considered in this study, and of antipeptide antibodies (anti-IGP-1, and -4) capable of partially interfering with antiviral and antiproliferative activity, seems to point to the presence of closely related epitopes with different activities in the carboxy-terminus of rHuIFN- γ . Indeed, Alfa and Jay⁽¹²⁾ identified monoclonal antibodies capable of neutralizing the antiviral but not the antiproliferative activity of HuIFN- γ , and vice versa.

Since neither IGP-1 or -4 antibodies affect the ability of rHuIFN- γ to induce a *de novo* expression of HLA-DR antigens on the surface of U937 cells, the epitope responsible for this activity must be located in the region between residues 125–137, or very close to it. Luk *et al.*⁽²²⁾ have recently demonstrated that a loss of the carboxy-terminal 21 amino acid residues of rHuIFN- γ (rHuIFN- γ 1–122) results in a fourfold reduction in antiviral and antiproliferative activity, while deletions of 26 amino acids (rHuIFN- γ 1–117) appear to result in a complete loss of activity. Our results, along with these observa-

tions, indicate that the epitopes responsible for antiviral and antiproliferative activity might be located around the amino terminus of the IGP-5 region.

Because Luk *et al.*⁽²²⁾ did not consider the immunomodulating activity of rHuIFN- γ , the sites of amino acids involved in this activity could not be better localized.

The use of polyclonal antibodies allowed us to limit the regions of rHuIFN- γ responsible for biological activity. Studies are now in progress to produce monoclonal antibodies, to better define and characterize the neutralizing epitopes of HuIFN- γ .

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