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Human interferon-γ has three domains associated with its antiviral function: a neutralizing epitope typing scheme for human interferon-γ

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SUMMARY

An antiviral activity-neutralizing monoclonal antibody (mAb), MIF3037, was developed by the immunization of BALB/c mice with recombinant human interferon-γ (rhuIFN-γ). Its neutralizing activity suggests that its epitope may be at or adjacent to a functional domain on the huIFN-γ. MIF3037 was compared with representative mAb of previously identified epitope-specific groups in a competitive binding assay. In an attempt to determine if there are other functional epitopes recognized by mAb developed with different preparations of huIFN-γ or different hybridoma screening methods, 14 additional mAb contributed by five other laboratories were similarly analysed. Based on their ability to bind to huIFN-γ, all the neutralizing mAb except MIF3037 may be classified into three previously defined groups: E1, E2 and E1/E2. Monoclonal antibodies of the E1 group do not compete with those of the E2 group for huIFN-γ binding, indicating that the E1 and E2 epitopes are distinct domains on the huIFN-γ important for the antiviral function. Monoclonal antibodies of the E1/E2 group compete with some of the mAb of E1 and/or E2 groups and may bind to regions of the huIFN-γ that partially overlap the E1 and E2 epitopes. MIF3037 demonstrated no competitive binding inhibition with mAb of the previously identified epitope specificity groups and, therefore, must represent a distinct functional epitope, E3. The huIFN-γ, therefore, must have at least three distinct functional domains; none of these appeared to be responsible for cell surface receptor binding. Based on this finding, the epitope typing scheme must be extended to include the E3 epitope. The epitope specificity relationships of 13 neutralizing mAb developed by five other laboratories were established which allows correlation of results obtained with these mAb by different laboratories. The location of the epitopes of four widely studied mAb, 69B, 73A, 113B and 220A12, have been deduced based on their competition with the E1 mAb which have recently been mapped.

INTRODUCTION

Human interferon-γ (huIFN-γ) is a cytokine that has multiple functions, including antiviral, anti-proliferative, cell surface antigen modulatory and immunoregulatory functions. The amino acid sequence of huIFN-γ was elucidated by direct sequencing of the natural protein[1] as well as by DNA sequencing of the cloned gene.[2,3] Localizing the active site is an important step in characterizing the molecular interaction of huIFN-γ that effects the various cellular functions. However, the exact location of the active site(s) has not been identified. A number of laboratories[4–9] have developed neutralizing monoclonal antibodies (mAb) to study and identify the functional epitopes. Based on the competitive binding activities of 21 independently isolated neutralizing mAb, Alfa and Jay[10] identified two distinct antiviral neutralizing epitopes, E1 and E2. None of the mAb, however, was able to neutralize the huIFN-γ receptor-binding activity. Different research groups have raised anti-huIFN-γ mAb to different forms of huIFN-γ, including synthetic peptides as well as natural and recombinant huIFN-γ whole molecules that had been purified by different methods for structure–function studies. There is, however, no correlation of the eptiope-binding specificities between the mAb developed in different laboratories, and it is not clear if there are other neutralizing epitopes on this pleiotrophic molecule that are not represented in the epitope typing scheme proposed by Alfa and Jay.[10] Thus far, only one functional epitope, E1, has been precisely mapped, and it is located at amino acid residues 84–94 on the linear sequence of the huIFN-γ. The fact that this functional epitope carries a nuclear localization signal-like element[11] infers a nuclear function for the huIFN-γ and/or nuclear localization function for the huIFN-γ molecule to bring the huIFN-γ/receptor complex to the nucleus for further biochemical interactions.

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Table 1. Monoclonal antibodies evaluated

mAb name	Immunizing antigen	Ig subtype	Antiviral neutralization	Sample preparation	Research group
mAbB1	huIFN-γ	IgG1	Weak neut.	Hybridoma supernatant	Le *et al.*[4]
mAbB3	huIFN-γ	IgG1	Strong neut.		
5J	huIFN-γ	Unknown	Low neut.	Purified Ig	Meager *et al.*[5]
45B3	huIFN-γ	Unknown	Low neut.		
NIB42	rhuIFN-γ	Unknown	Strong neut.		
KM48	rhuIFN-γ	IgG1	Neutralizing	$(NH_4)_2SO_4$ p.p.t. of ascites fluid	Oda *et al.*[7]
KM61	rhuIFN-γ	IgG1	Non-neut.		
KM45	rhuIFN-γ	IgG1	Non-neut.		
B133.1	rhuIFN-γ	IgG1	Neutralizing	Ascites fluid	Ziai *et al.*[8]
B133.3	rhuIFN-γ	IgG1	Neutralizing		
69B.J	rhuIFN-γ	IgG1	Neutralizing	Ascites fluid	Pestka *et al.*[6]
220A12.J	rhuIFN-γ	IgG1	Neutralizing		
73A.J	rhuIFN-γ	IgG1	Neutralizing		
113B.J	rhuIFN-γ	IgG1	Neutralizing		
MIF3009	rhuIFN-γ	IgG1	Good neut.	Purified Ig	Alfa *et al.*[10]
MIF3094	rhuIFN-γ	IgG1	Good neut.		
MIF3055	rhuIFN-γ	IgG1	Good neut.		
MIF3125	rhuIFN-γ	IgG1	Good neut.		
MIF3037	rhuIFN-γ	IgM	Neutralizing	Ascites fluid	Unpublished

We report here the identification of a third functional epitope, E3, on the huIFN-γ that demonstrated no cross-reactivity with any of the previously classified mAb and extended the huIFN-γ-neutralizing mAb classification scheme to include four immunoreactivity groups. Thirteen additional mAb contributed by five other laboratories were analysed and classified according to our epitope classification scheme to correlate results published by different laboratories. The locations of the epitopes of four of these mAb were also deduced, on the basis of their classification in the E1 group which has been mapped.

MATERIALS AND METHODS

mAb

The production and characterization of the anti-rhuIFN-γ mAb MIF3009, MIF3094, MIF3055 and MIF3125 were as previously described.[9,10] These mAb were prepared as ascites fluids by injecting hybridoma cells into BALB/c mice that had been primed 7 days earlier with an intraperitoneal injection of 0·5 ml pristane. The mAb were affinity purified from the ascites fluid on Protein A–Sepharose as outlined by the manufacturer (Pharmacia, Baie d'Urfe, Canada). MIF3037, which is an IgM, was similarly produced in BALB/c mice.

Fourteen mAb were generously provided by Dr J. Le (Department of Microbiology, New York University School of Medicine, New York, NY); Dr A. Meager (National Institute for Biological Standards and Control, London, U.K.); Dr S. Oda (Tokyo Research Laboratories, Tokyo, Japan); Dr S. Pestka (Department of Molecular Genetics and Microbiology, Robert Wood Johnson Medical School, Piscataway, NJ); Dr G. Trinchieri (Wistar Institute of Anatomy, Philadelphia, PA). The name, source and references of published descriptions of each of these mAb are summarized in Table 1. These mAb were stored at $-20°$ until used.

ELISA

The ability of the mAb to bind huIFN-γ was tested using a sandwich ELISA.[9] ELISA plates were coated with purified rabbit anti-huIFN-γ immunoglobulins at 0·5 μg/well. The plates were incubated overnight at 4° and the residual binding sites in the wells were blocked by incubation with 3% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Recombinant huIFN-γ was expressed in *Escherichia coli* as described by Jay *et al.*[12,13] at $> 10^7$ U/mg of cell extract. HuIFN-γ diluted with 1% BSA in PBS was added to the ELISA plates and incubated at 37° for 2 hr and then washed with PBS. The mAb were serially diluted 1/2 in BSA–PBS [1% (w/v) BSA in PBS] and then 50 μl was transferred to the appropriate well in the ELISA tray and incubated at 37° for 1 hr. The wells were washed three times with 100 μl PBS containing 0·02% Tween 20 (T20–PBS). Peroxidase-conjugated goat anti-mouse IgG, IgM, IgA (Cederlane Laboratories, Hornby, Canada) was diluted 1/3000 with T20–PBS containing 1% BSA and 50 μl was dispensed into each well. The plate was incubated at 37° for 1 hr and then washed five times with T20–PBS. Colour development was achieved by incubation with freshly prepared 2,2′-azino-bis (3-ethylbenzthiazolinesulphonic acid) (ABTS; Sigma Chemical Co., St Louis, MO) at 0·015% (w/v) in 100 mM citrate buffer, pH 8, containing 0·03% (v/v) H_2O_2. The reaction was allowed to proceed at room temperature for 15 min in the dark and then 50 μl of 10% (w/v) SDS was added to stop the reaction. Absorbance values were determined using an ELISA reader equipped with a 405 ηm filter. The end-point was taken as the reciprocal of the dilution in the last well that produced an

absorbance value of ≥0·150 which was three times greater than the background values (0·05).

Antiviral neutralization

The antiviral neutralization (NAV) assay was carried out as described by Alfa *et al.*[10] Briefly, the test mAb was serially diluted in a 96-well culture plate that contained monolayer cultures of A_{549} (human lung carcinoma) cells at 4×10^4 cells/well. A constant amount of huIFN-γ, 4 working units/ml (10 U/ml), was added. After incubation at 37° for 18 hr, each culture was challenged with encephalomyocarditis (EMC) virus at a multiplicity of 5 $TCID_{50}$/cell for 24 hr. The monolayers were fixed and stained with 0·25% crystal violet, 20 mM Tris–HCl, pH 7·5, 0·9% (w/v) NaCl in 20% (v/v) methanol and the excess dye was washed out with tap water. Methanol (100 μl) was added to each well to extract the dye that was adsorbed by the viable cells. The absorbance was determined with an ELISA reader equipped with a 590 ηm filter. The NAV titre was defined as the reciprocal of the dilution that could neutralize 1 reference unit of rhuIFN-γ. Thus, the NAV titre was obtained by taking the reciprocal of the dilution at 50% protection against the virus challenge and multiplying by the concentration of the huIFN-γ in the assay (10 U/ml). The activity of huIFN-γ was standardized against the US National Institutes of Health Reference huIFN-γ, Gg23-901-530.

^{125}I-labelling of mAb

Purified immunoglobulin was labelled with carrier-free Na ^{125}I using the method of Fraker and Speck.[14] Briefly, glass test tubes were coated with Iodogen (Pierce Chemical Co., Rockford, IL) by dispensing 50 μl of 2% Iodogen in chloroform (w/v) into each tube and allowing the chloroform to evaporate completely. The purified immunoglobulin (50 μg in 50 μl PBS) and 0·5 mCi of carier-free Na ^{125}I were added to the Iodogen-coated tube. The reaction was allowed to proceed for 15 min and then the reaction mixture was transferred to a separate tube. The Iodogen-coated reaction tube was rinsed with 50 μl PBS which was then pooled with the reaction mixture. The free Na ^{125}I was separated from bound [^{125}I]immunoglobulin by column chromatography on Sephadex G-100 fine in a 0·4 × 25 cm column. Labelled immunoglobulin prepared in this manner had ≥95% precipitability in 10% TCA.

Competition sandwich radioimmunoassay (sRIA)

The competition sRIA of Alfa and Jay[10] was modified because the mAb obtained from the various contributors were in quantities that precluded purification. Therefore, all quantitative evaluations were based on EU rather than mg of mAb. Briefly, 96-well ELISA trays were coated with rabbit anti-rhuIFN-γ immunoglobulin by incubating 50 μl of the immunoglobulin (100 μg/ml carbonate buffer) at 4° overnight. The residual binding sites in the wells were blocked by adding 100 μl of 3% BSA–PBS/well and incubating at 37° for 2 hr. Human IFN-γ (8×10^4 IU/50 μl of 1% BSA–PBS) was added to each well and incubated at 37° for 2 hr, and then washed with T20–PBS. Each test mAb was serially diluted 1/5 in 1% BSA–PBS in separate 96-well trays and then a constant amount of [^{125}I]mAb (200,000–400,000 c.p.m.) was added to each well. A 50 μl aliquot of each dilution was transferred to the appropriate wells of the huIFN-γ-coated tray. The competition mixture was incubated overnight at 4° and then washed five times with PBS. The wells were allowed to air dry and individual wells were cut out with a hot-wire. The radioactivity in each well was determined using an LKB gamma-counter. All experiments were performed in duplicate. The concentration ratio of coating antigen (huIFN-γ) and [^{125}I]mAb (probe) were optimized to produce 50% inhibition of binding when an equimolar amount of the homologous mAb was added. Monoclonal antibodies MIF3009, MIF3055, MIF3094 and MIF3125 were used as the radiolabelled probes in the competition analysis. All mAb provided by other laboratories were evaluated for their ability to compete with each of the four representative mAb produced in this laboratory. The end-point was determined as the number of EU of mAb required to inhibit the binding of the radiolabelled probe by 50%.

RESULTS

Nineteen mAb from six independent research laboratories were studied (Table 1). The relative concentration of each of the mAb was determined based on their reactivities with excess huIFN-γ in a standard ELISA and expressed as ELISA U/ml (EU/ml). One EU was defined as the reciprocal of the maximum dilution that produced a positive reaction of 0·15 A_{405} (three times background at ≤0·05 A_{405}) in a standard sELISA. All mAb were found to have no reactivity with extracts of the *E. coli* cell that harbour the same expression vector which lacked the huIFN-γ coding sequence. The NAV titre was also determined and expressed as the reciprocal of the dilution that could neutralize 1 U of huIFN-γ (NAV/ml) (Table 2).

Previously study has allowed the classification of the mAb into distinct (non-cross-reacting) groups E1 and E2 as well as an E1/E2 group that cross-reacts with either or both E1 and E2.[10] An mAb, MIF3037, directed against the huIFN-γ was recently developed and found to be of the IgM type. Its dose-dependent binding to captured huIFN-γ in an sELISA is shown in Fig. 1. A typical dose–response binding of the MIF3037 to the ELISA

Table 2. Characterization of mAb

mAb name	NAV/ml	EU/ml	NAV/EU
mAbB1	$5·67 \times 10^2$	$7·46 \times 10^0$	76·0
mAbB3	$4·06 \times 10^4$	$7·01 \times 10^2$	57·9
5J	$1·30 \times 10^4$	$2·21 \times 10^3$	5·9
4SB3	$1·53 \times 10^4$	$7·50 \times 10^4$	0·2
NIB42	$2·05 \times 10^6$	$1·42 \times 10^5$	14·4
KM48	$1·26 \times 10^6$	$1·71 \times 10^5$	7·3
KM61	$2·04 \times 10^3$	$1·02 \times 10^4$	0·2
KM45	$< 1·64 \times 10^1$	$1·10 \times 10^5$	—
B133.1	$2·10 \times 10^9$	$1·45 \times 10^7$	144·8
B133.3	$1·79 \times 10^7$	$8·91 \times 10^6$	2·0
69B.J	$4·10 \times 10^6$	$2·30 \times 10^5$	17·8
220A12.J	$4·60 \times 10^5$	$1·21 \times 10^5$	3·8
73A.J	$1·65 \times 10^7$	$1·50 \times 10^5$	110·0
113B.J	$5·54 \times 10^4$	$3·43 \times 10^3$	16·1
MIF3009	$4·19 \times 10^7$	$1·02 \times 10^6$	41·1
MIF3094	$3·28 \times 10^4$	$6·40 \times 10^4$	0·51
MIF3055	$1·05 \times 10^7$	$6·4 \times 10^5$	16·4
MIF3037	$1·23 \times 10^4$	$1·18 \times 10^4$	1·04
MIF3125	$1·31 \times 10^7$	$1·60 \times 10^4$	81·9

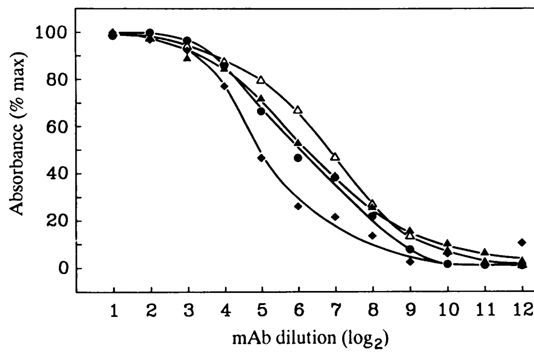


Figure 1. Human IFN-γ binding activities of mAb in a sELISA. Human IFN-γ antigen for the sELISA was captured onto the ELISA plate with rabbit anti-huIFN-γ immunoglobulin which has been adsorbed to the plastic plate. Monoclonal antibodies MIF3009 (▲), MIF3055 (●), MIF3094 (◆) or MIF3037 (△) were serial twofold diluted into ELISA plates and 50 μl was transferred to the corresponding wells in the huIFN-γ plate. The amount of mAb bound to huIFN-γ in the plate was detected by peroxidase-conjugated goat anti-mouse Ig(A, G and M). Results are expressed as percentage of maximum for each mAb.

plate was observed. Similar huIFN-γ-binding curves for MIF3009 of the E1 epitope group, MIF3055 of the E2 group and MIF3094 of the E1/E2 group are also demonstrated (Fig. 1). The huIFN-γ neutralization activity was determined in an assay that measures the residual antiviral activity against an EMC virus challenge resulting from the addition of a varying concentration of the test mAb with a fixed concentration of huIFN-γ (4 working units/ml). The extent of protection is reflected in the amount of crystal violet adsorbed by the remaining viable cells at the end of the challenge period. Typical dose–response neutralization of the huIFN-γ by MIF3009, MIF3055 and MIF3094 was observed (Fig. 2). A similar result

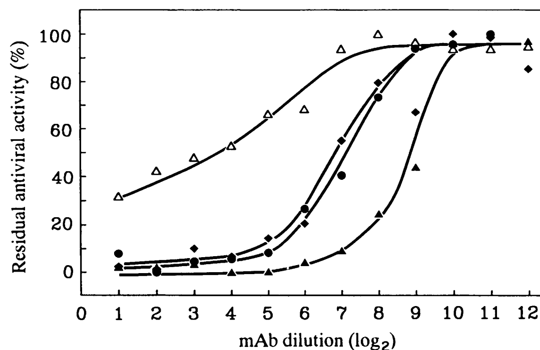


Figure 2. Antiviral neutralization activities of mAb. Monoclonal antibodies MIF3009 (▲), MIF3055 (●), MIF3094 (◆) or MIF3037 (△) were serial twofold diluted into microtitre cultures of A549 human lung carcinoma cells. Human IFN-γ was then added to each well to a final concentration of 4 working units/ml. After incubation for 18 hr at 37°, the cultures were challenged with 5 $TCID_{50}$ of EMC virus for 24 hr. Viable cells were then detected by staining with crystal violet and determined spectrophotometrically at 590 nm. Average absorbance of cultures lacking the huIFN-γ treatment (<0·1 A_{590}) served as the background in each assay. Results are expressed as a percentage of the antiviral activity remaining compared to that of similarly treated and EMC virus challenged cultures in the absence of any mAb (100%).

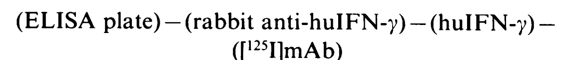
Table 3. Epitope assignment of anti-huIFN mAb

mAb name	E1 MIF3009*	E1/E2 MIF3094*	E2 MIF3055*	MIF3125*
69B.J	0·003	0·001	—	—
113B.J	0·153	0·011	—	—
220A12.J	0·292	0·010	—	—
73A.J	0·483	0·634	—	—
MIF3009	**1·000**	1·367	—	—
NIB42	1·553	0·037	3·160	—
B133.3	51·231	1·093	19·239	—
MIF3094	—	**1·000**	—	—
mAbB1	—	0·007	—	—
MIF3055	—	2·237	**1·000**	12·800
B133.1	—	0·468	5·962	5·574
KM48	—	0·339	1·317	2·888
MIF3125	—	—	0·548	**1·000**
5J	—	—	0·275	0·301
mAbB3	—	—	0·686	0·473
4SB3	—	—	1·230	0·351
KM61	—	—	38·152	—
MIF3037	—	—	—	—
KM45	—	—	—	—

* ^{125}I-labelled mAb probes; (—) denotes no competition detectable at CR ⩾ 100; maximum CR obtained for MIF3037 in competition with ^{125}I-labelled MIF3009, 3055 and 3094 was 32,988, 5874 and 2233, respectively.

was obtained using MIF3037, although the NAV titre was lower and 100% neutralization was not reached at the maximum mAb concentration attainable in these experiments (Fig. 2). The result, however, clearly demonstrated that MIF3037 is a neutralizing mAb.

In order to determine if the epitope recognized by MIF3037 is distinct from previously identified epitopes,[9,10] MIF3037 was analysed in a competition analysis using a modified sRIA. Three mAb, MIF3009, 3055 and 3094 representing the three previously characterized epitope specificity groups, E1, E2 and E1/E2, respectively, were selected for the present study. Additionally, mAb MIF3125, that was distinct from E1 but appeared to be partially related to E2, was also included in this study. Fourteen other mAb contributed by five other laboratories (Table 1) were similarly tested in an attempt to establish their epitope specificity relationship and to determine if additional functional epitopes are recognized by these mAb. Each of the four mAb, MIF3009, 3094, 3055 and 3125, serving as references for each of the previously identified groups, was purified and radiolabelled with ^{125}I to a specific activity of approximately 10 mCi/mg and approximately 2 mol of ^{125}I/mol of immunoglobulin. Previous analysis indicated that ^{125}I-labelling of these mAb to this level did not affect the specificity or binding capacity of these mAb to the huIFN-γ.[10] Each of the unlabelled mAb was tested by sRIA against each of the four radiolabelled mAb probes. The immunocomplex is shown as follows:


(ELISA plate) – (rabbit anti-huIFN-γ) – (huIFN-γ) – ([^{125}I]mAb)

Since the amount of huIFN-γ is constant and limiting, the presence of an unlabelled mAb that can compete with the

[125I]mAb will proportionally reduce the binding of the [125I]mAb to the immobilized huIFN-γ. The amount of test mAb, expressed as ELISA units, required to inhibit the binding of the [125I]mAb probe by 50% (EU_{50}), was taken as the end-point. Unlabelled homologous mAb (same mAb as the probe) was included as a reference control in each set of competition experiments and the EU required to inhibit the binding of the 125I-labelled probe by 50% was determined. Theoretically, an equivalent amount of unlabelled mAb would inhibit 50% of the binding of similar amount of the homologous [125I]mAb. In order to control for any variability and to allow comparison of the binding efficiency of different mAb, the EU_{50} of each mAb was normalized to the homologous mAb control which was taken as 1·00. The results of these competition assays are presented in Table 3 as competition ratios of: $CR = (EU_{50}$ of test)/(EU_{50} of homologous control).

All homologous mAb controls have CR ratios of 1·00. mAb that bind the epitope better than the probe have a $CR < 1{\cdot}00$, whereas those that bind weaker than the probe have a $CR > 1{\cdot}00$. For example, B133.3 binds epitope E1/E2 approximately equal to MIF3094 since the CR is 1·093, whereas it binds E1 much weaker than MIF3009 because the CR is 51·23. Where the $CR > 100$, the mAb was considered non-competitive with the [125I]mAb probe and therefore did not bind to the same epitope. This is shown as '—' in Table 3 to enhance comprehension of the table. The CR of each of the 14 mAb contributed by other laboratories form a cross-reactivity pattern against the four radiolabelled probes. Each of the 14 neutralizing mAb contributed by other investigators is, thus, classified within the three immune reactivity (epitope) groups as defined by MIF3009, MIF3094 and MIF3055. MIF3125, with a CR of 0·584 against [125I]MIF3055, also clearly falls into the E2 group and not a separate group of its own. The mAb KM45 did not compete with any of the probes (Table 3), and is consistent with the observation that it is non-neutralizing. However, MIF3037, which is a neutralizing mAb, did not compete with any of the 125I-labelled mAb probes, even at an excess of 2233, 5874 and 32,988 times that of MIF3094, MIF3055 and MIF3009, respectively. MIF3037, therefore, must bind to a completely separate epitope.

DISCUSSION

Several research groups have developed huIFN-γ neutralizing mAb and used these mAb to probe the structure–function relationships of the huIFN-γ. The epitope specificity relationships of the mAb used by different investigators, however, are unknown and whether results obtained with different mAb represent effects from binding an immunoglobulin molecule to the same or different domains on the huIFN-γ molecule is uncertain. Based on the immune cross-reactivities of 21 neutralizing mAb derived from independent splenocyte–myeloma cell fusion events, Alfa and Jay identified two distinct antiviral neutralizing epitopes, E1 and E2.[10] It was also unclear if different methods of huIFN-γ immunization and approaches to screening hybridomas used by different investigators may identify neutralizing epitopes that were not represented in the panel of mAb developed by Alfa and Jay.[9] Indeed, none of the mAb in that panel was able to inhibit [125I]huIFN-γ binding to its cell-surface receptor. In a continued effort to identify the different functional epitopes on the huIFN-γ to locate the different functional domains, we developed further mAb. The characteristics of MIF3037 are summarized in Tables 1 and 2.

The amount of mAb in the samples contributed by different laboratories ranged from $7{\cdot}5 \times 10^1$ EU/ml to $1{\cdot}5 \times 10^7$ EU/ml. The mAb quantitation was expressed in ELISA units, which is a measure of the amount of mAb that can bind to an excess of huIFN-γ. The NAV/EU (neutralization of antiviral activity/ELISA unit) ratio is a measure of the neutralizing capability of each mAb relative to its binding capability. The NAV/EU ratio therefore provides a measure of the relative neutralizing effect of the mAb upon binding to its epitope. Thus, the result reflects the functional involvement of that epitope and is less affected by the differences in the binding capacity of the individual mAb. The larger the ratio, the more effective is the neutralizing ability.

Initial characterization of the 14 mAb from different investigators in the current study confirmed the characteristics of each of these mAb as previously reported by the original authors (Tables 1 and 2). Le *et al.*[4] described mAbB1 as a weakly neutralizing mAb compared to mAbB3. However, the relative neutralizing capacity was found to be similar (Table 2), demonstrating the need for a relative quantitative measure that is less affected by the binding affinity of the mAb. Oda *et al.*[7] reported that KM48 was neutralizing but KM61 and KM45 were non-neutralizing. The neutralization activity of KM45 was below the limit of detection in the present study and was confirmed as non-neutralizing. However, low neutralizing activity (0·2 NAV/EU) was detected with KM61, although it was 36-fold less than KM48 (7·3 NAV/EU) (Table 2). The difference in the sensitivity of the assay used may account for Oda describing KM61 as non-neutralizing. Meager *et al.*[5] used natural huIFN-γ as the immunogen to produce mAb 5J and 4SB3 and reported that these mAb had low neutralizing activity against the rhuIFN-γ (5·9 NAV/EU and 0·2 NAV/EU, respectively); whereas, mAb NIB42, which was produced using rhuIFN-γ as the immunogen, had good neutralizing ability against rhuIFN-γ (14·4 NAV/EU). The mAb provided by Pestka *et al.*[6] and Ziai *et al.*[8] all had detectable antiviral neutralization titres. The current study generally confirmed the relative neutralizing activity among these mAb as previously reported. Previously unreported mAb MIF3037 also has neutralizing activity (1·04 NAV/EU).

Based on their competitive binding pattern, all of the mAb, except KM45 and MIF3037, could be grouped into three immuno-competitive groups, E1, E2 and E1/E2 (Table 3). The fact that KM45 has no detectable neutralizing activity is consistent with the criterion that these groupings are based on neutralizing epitopes, and non-neutralizing mAb do not compete with neutralizing ones.[10] MAb 5J, mAbB3, and 4SB3 are assigned to the E2 group and none of them showed cross-reactivity with the overlap region (MIF3094) or the E1 epitope. This observation is similar to MIF3054 or MIF3052 where > 30 fold excess of these mAb is required to compete with MIF3094.[10] KM61 also competed only with MIF3055 (E2) (Table 3) but this competition was very weak, requiring 38-fold excess to produce 50% inhibition of MIF3055. MAbB1 competed only with MIF3094, and not with MIF3009 and 3055, and is assigned to the E1/E2 group. The mAb NIB42 and B133.3, which are classified in the E1/E2 group, competed with mAb of both E1 and E2 groups (Table 3). This is similar to mAb MIF3102 and 3059 previously described by Alfa and Jay.[10] All four mAb, 69B.J, 220A12.J, 73A.J and 113B.J produced by Pestka *et al.*[6]

and subcloned, or clone purified, in this laboratory, have reactivities consistent with those of the E1 group which also compete with MIF3094 of the E1/E2 group, but exhibit no competition with MIF3055 of the E2 group. Thus, no additional immuno-competitive group was identified among the previously published neutralizing mAb contributed by five other laboratories, but their epitope specificity relationships are clearly identified in this study. MIF3037, which is a neutralizing mAb (Fig. 2), did not demonstrate any competition with any mAb probes of these three epitope specificity groups. The results, therefore, indicate that MIF3037 must bind to an epitope completely separated from those of the other three groups. This new epitope is named E3.

The present study confirmed that MIF3125 had no cross-reactivity with MIF3009 of the E1 group and was only inhibited by MIF3094 and 3055 of the E1/E2 and E2 respective groups (Table 3). In a previous study,[10] because the mAb of the E2 group competed with MIF3125 only very weakly (at 35–300-fold molar excess), it was suggested that the epitope of MIF3125 may be related but not necessarily identical to that region bound by the E2 mAb and it was provisionally assigned as E2′, pending further studies. In the present study, however, the comparison of the efficiency of competition was based on ELISA units, which represents the amount of mAb bound rather than the total mAb input, to minimize the effects due to differences in affinity. The competition pattern of different mAb with MIF3125 (E2′) was not significantly different from that of MIF3055 (E2). On the basis of this result, it must be concluded that E2′ is not significantly distinct from E2 and that MIF3125 should be included in the E2 group. Thus, there are three neutralizing epitopes, E1, E2 and E3, identified on the huIFN-γ, resulting in four epitope specificity groups: E1, E1/E2, E2 and E3 for anti-huIFN-γ neutralizing mAb.

Functional neutralization by specific antibody binding can, on rare occasions, result from conformational changes that affect a distant functional site, rather than direct steric hindrance at the active site. However, with the exception of the MIF3037 for the E3 epitope, because there are large numbers of different mAb isolated in different laboratories which fall into distinct reactivity groups, it is highly unlikely that any of the E1 and E2 epitopes represents neutralization by conformational changes. Steric hindrance at the functional site is likely to be the mechanism for the neutralization. The epitopes E1 and E2, therefore, must either be the functional site, or very close to the functional site, that is necessary for the induction of the antiviral activity. This confidence cannot be fully extended to the E3 epitope at this time. However, the fact that MIF3037 did not interfere with the binding of MIF3055, which recognizes only native (functional) huIFN-γ, suggests that the conformation of the huIFN-γ molecule is maintained after the binding of MIF3037.

None of the neutralizing mAb thus far identified was able to inhibit the attachment of the huIFN-γ molecule to the cell surface receptor. Neither B133.1 or B133.3 inhibited receptor binding.[5] Indeed, our preliminary data also indicated that none of the neutralizing mAb by themselves can inhibit huIFN-γ binding to its receptor. This suggested that none of the neutralizing epitopes identified thus far is essential for the receptor binding function. We hypothesize that the receptor binding function and the signal transduction functions are separate on the huIFN-γ molecule and that the epitopes E1, E2 and E3, separately or jointly, form the effector structure(s) on the huIFN-γ molecule that is essential for signal transduction either across the membrane or other intracellular signalling mechanisms that ultimately effect the appropriate switch in gene expression that establishes the antiviral state. At the present, only epitope E1 has been precisely located on the huIFN-γ (residues 84–94) and the amino acid sequence at the E1 domain resembles the nuclear localization (targeting) signal (NLS)[11] of many nuclear proteins.[15] Based on the competitive binding results (Table 3), the respective epitopes of four neutralizing mAb, 69B.J, 113B.J 220A12.J and 73A.J, must all localize at, or very close to, the E1 domain at residues 84–94 on the huIFN-γ molecule. Thus all of these mAb probably neutralize the huIFN-γ by binding to the nuclear localizing signal-like element.

In conclusion, based on the results presented here, a new E3 functional epitope has been identified by the neutralizing mAb MIF3037. The epitope specificity typing scheme for huIFN-γ neutralizing mAb must also be expanded to include a total of four immunoreactive groups: E1, E2, E1/E2 and E3 groups. On the basis of this classification, the location of the epitopes of four of the mAb in the E1 group has been deduced and the epitope specificity relationships of all of these mAb were established. The classification of the anti-huIFN-γ neutralizing mAb according to the epitope specificity provides a means to correlate results from different investigators and allows better appreciation of results obtained with different neutralizing mAb. The classification scheme proposed here serves this purpose.

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REFERENCES

1. Rinderknecht E., O'Conner B.H. & Rodriguez H. (1984) Complete amino acid sequence of human immune interferon and determination of sites of glycosylation. *J. biol. Chem.* **259,** 6790.
2. Grey P.W., Leung D.W., Pennica D., Yelverton E., Najarian R., Simonsen C.C. *et al.* (1982) Expression of human immune interferon cDNA in *E. coli* and monkey cells. *Nature,* **295,** 503.
3. Devos R., Cheronte H., Taya Y., Degrave W., van Heuverswyn H. & Fiers W. (1982) Molecular cloning of human immune interferon cDNA and its expression in eukaryotic cells. *Nucleic Acids Res.* **10,** 2487.
4. Le J., Barrowclough B.S. & Vilcek J. (1984) Monoclonal antibodies to human immune interferon and their application for affinity chromatography. *J. immunol. Meth.* **69,** 61.
5. Meager A., Parti S., Barwich S., Spragg J. & O'Hagan K. (1984) Detection of hybridomas secreting monoclonal antibodies to human gamma interferon using a rapid screening technique and specificity of certain monoclonal antibodies to gamma intereferon. *J. Interferon Res.* **4,** 619.
6. Kelder B., Rashidbaigi A. & Pestka S. (1986) A sandwich radioimmunoassay for human interferon-γ. In: *Methods in Enzymology* (ed. S. Pestka), Vol. 119, p. 582. Academic Press, New York.
7. Oda S., Akinaga S., Kumagai A., Inoue A., Nakamizo N. & Yoshida H. (1986) Generation of a new monoclonal antibody and its application for determination and purification of biologically active human gamma-interferon. *Hybridoma,* **5,** 329.

8. ZIAI M.R., IMBERTI L., KOBAYASKI M., PERUSSIA B., TRINCHIERI G. & FERRONE S. (1986) Distinct functional domains on the recombinant human immune interferon molecule. *Cancer Res.* **46,** 6187.
9. ALFA M.J., DEMBINSKI J.J. & JAY F.T. (1987) Production of monoclonal antibodies against recombinant human interferon-gamma: screening of hybridomas without purified antigen. *Hybridoma,* **6,** 509.
10. ALFA M.J. & JAY F.T. (1988) Distinct domains of recombinant human IFN-γ responsible for antiviral effector function. *J. Immunol.* **141,** 2474.
11. ZU X. & JAY F.T. (1991) The E1 functional epitope of the human interferon-γ is a nuclear targeting signal-like element: mapping of the E1 epitope. *J. biol. Chem.* **266,** 6023.
12. JAY E., MACKNIGHT D., LUTZE-WALLACE C., HARRISON D., WISHART P., LIU W.Y. *et al.* (1984) Chemical synthesis of a biologically active gene for human immune interferon-γ. *J. biol. Chem.* **259,** 6311.
13. JAY E., ROMMENS J., POMEROY-CLONEY L., MACKNIGHT D., LUTZE-WALLACE C., WISHART P. *et al.* (1984) High-level expression of a chemically synthesized gene for human interferon-γ using a prokaryotic expression vector. *Proc. natl. Acad. Sci. U.S.A.* **81,** 2290.
14. FRACKER J.P. & SPECK J.C. (1978) Protein cell membrane iodinations with a sparingly soluble chloramide 1,3,4,6-tetrachloro 3α, 6α, diphenylglycoluri. *Biochem. biophys. Res. Commun.* **80,** 849.
15. KALDERON D., RICHARDSON W.D., MARKHAM A.F. & SMITH A.E. (1984) Sequence requirements for nuclear location of simian virus 40 large T antigen. *Nature,* **311,** 33.