ANTIVIRAL NEUTRALIZATION EPITOPES

OF THE HUMAN INTERFERON GAMMA

Presented by:

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A Thesis
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I DEDICATE THIS THESIS TO MY FAMILY

FROM WHOM I DERIVE MY STRENGTH AND LOVE

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LIST OF ABBREVIATIONS

aa:	amino acids
ADCC:	antibody-dependent cellular cytotoxicity
Ag:	antigen
BCIP:	5-bromo-4-chloro-3-indolyl phosphate
bp:	base pair
BSA:	bovine serum albumin
CPE:	cytopathic effect
cpm:	counts per minute
ddH_2O:	double distilled water
DNA:	deoxyribonucleic acid
ExoIII:	Exonuclease III
E1:	neutralizing epitope 1
E2:	neutralizing epitope 2
E. coli:	Escherichia coli
ELISA:	Enzyme linked immunosorbent assay
EMC:	encephalomyocarditis
ER:	(EU_{50} of test)/(EU_{50} of control)
EU:	ELISA units
EU_{50}:	ELISA units of test MAb required to inhibit the binding of 10 ng ^{125}I-MAb probe by 50%
Fig.:	figure
g:	gram
hr:	hour
HLA:	human leukocyte antigen
HuIFN:	human interferon
IFN:	interferon
IFN-α:	interferon-alpha (α)
IFN-β:	interferon-beta (β)
IFN-γ:	interferon-gamma (γ)
Ig:	immunoglobulin
Iodagen:	1,3,4,6-tetrachloro-3α, 6α-diphenylglycouril
IPTG:	isopropylthiogalactoside
MAb:	monoclonal antibody
MHC:	major histocompatibility complex
min:	minute
MuIFN-γ:	murine interferon-gamma
NAV:	neutralization of antiviral titre
NBT:	nitroblue tetrazolium chloride
NK:	natural killer cells
OD:	optical density
PBS:	phosphate buffered saline
rHuIFN-γ:	recombinant human interferon-gamma

RNA:	ribonucleic acid
RNase A:	ribonuclease A
SDS:	sodium dodecyl sulfate
SDS-PAGE:	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sELISA:	sandwich ELISA
sRIA:	competition sandwich radioimmunoassay
TCA:	trichloroacetic acid
Tween 20:	polyoxyethylene-sorbitan monolaurate
UV:	ultraviolet
X-gal:	5-bromo-4-chloro-3-indolyl-β-D-galactosidase
5':	five prime
3':	three prime
^{125}I:	iodine-125
^{0}C:	degrees centigrade
ug:	microgram
ml:	milliliter
ul:	microliter
M:	molar
mM:	millimolar
mm:	millimeter
um:	micrometer

ABSTRACT

Seventeen monoclonal antibodies (MAbs) directed against the human interferon gamma (HuIFN-γ) representing all the different neutralizing MAbs from each of six laboratories were analyzed by immuno cross-competition to identify the different functional epitopes for the antiviral activity of the HuIFN-γ. Based on their ability to bind to HuIFN-γ, all the neutralizing MAbs may be classified into 3 groups: E1, E2 and E1/E2. MAbs of the E1 group do not compete with those of the E2 group for HuIFN-γ binding, indicating that these are two distinct antiviral functional epitopes on the HuIFN-γ. MAbs of the E1/E2 group compete with MAbs of E1 and /or E2 groups and may bind to regions of the HuIFN-γ that partially overlap E1 and/or E2. No other antiviral-neutralizing epitope was identified among the neutralizing MAbs obtained from five other laboratories. Thus, all HuIFN-γ-neutralizing MAbs may be classified into the three immuno-reactive groups representing at least two distinct functional epitopes. This epitope classification scheme is essential for the correlation of results reported by different laboratories, particularly when the location of the epitope of each MAb is unknown.

In a separate study, an attempt was made to develop a recombinant DNA approach to map MAb epitopes. A lambda gt11 recombinant DNA library expressing the HuIFN-γ polypeptide with various extent of deletion (0% - 100%) from the N-terminus was constructed. The strategy is to use the MAb to be mapped to screen and select the clones producing the epitope. The library was screened with MAb MIF3152 and 30 positive plaques were isolated and purified. The recombinant DNA was purified and analyzed with restriction enzymes. Seven clones produced inserts, but the size of the inserts did not agree

with the size predicted. It is not clear in the present study if these positive clones contain IFN-γ gene. Further studies are needed.

CHAPTER I

INTRODUCTION

In 1957, Isaacs and Lindenmann (1) discovered a protein, designated interferon (IFN), that could confer resistance upon cells against infections from a variety of viruses. Since then, multiple biologic effects of IFN were demonstrated (Table 1) (2). These include immune modulation (3) and cell proliferation inhibition properties (4-5) as well as a direct effect on tumor growth in animals (6-7). These activities of the interferons have suggested their potential clinical applications in the treatment of viral infections and malignancies and have generated great excitement and vigorous research in many academic and industrial laboratories.

Mammalian interferons are grouped into two types based on physical and chemical properties such as acid stability. Type I interferon is divided into the leukocyte and fibroblast species, based on the major producer cell type. Type II interferon is acid labile and is produced by lymphocytes, Type II is thus referred to as immune interferon. Three species are now defined by their nucleotide sequences and serological cross-reactivities. They are presently referred to as α, β and γ interferon respectively (8). It has been shown that there are many subspecies or molecular forms of interferon-alpha (IFN-α) and interferon-beta (IFN-β), but it appears that there is only one single species of interferon-gamma (IFN-γ) (Table 2) (9).

Among the three classes of human interferons (HuIFNs), IFN-γ has been shown to be the most potent in terms of its immunomodulatory and antiproliferative activities (10). IFN-γ differs from other IFNs in various physicochemical characteristics such as molecular weight, degree of glycosylation, and isoelectric point (Table 2) (11-13). Another difference between IFN-γ and other interferons is the fact that the spectrum of cells which produce IFN-γ is more narrow, being limited to T-lymphocytes and natural killer cells (14-15).

TABLE 1. Biologic Activities of IFN-γ

antiviral
antitumor
antimicrobial
MHC antigen expression
cell differentiation
cell growth inhibition
enhancement of ADCC
enhancement of NK-activity
macrophage activation
modulation of B cell response
inhibition of T suppressor cells
enhancement of T cell cytotoxicity

MHC, major histocompatibility; ADCC, antibody-dependent cellular cytotoxicity; NK, natural killer cells.

Table 2. Classification and general properties of human interferons

Class	IFN-α	IFN-β	IFN-γ
Type	I	I	II
Primary natural source	Leukocytes	Fibroblasts	T cells
Number of species	>15	1	1
Molecular weight	16,500-25,000	20,000	17,000
pH 2 stability	+	+	-
N-linked glycosylation	Some species	+	+
Isoelectric point	5.5-6.6	6.8-7.8	8.6-8.7

Production of IFN-γ occurs only when these cells are activated, a process which is triggered by exogenous agents (e.g. bacteria, viruses, and antigens) (16), and regulated by a large set of endogenous factors. Cytokines (17-18) other than IFN-γ constitute an important group of factors that are able to regulate IFN-γ production: the other interferons, the interleukins, the lymphotoxins and others.

For many years biochemical and biological studies, and to an even greater extent, clinical evaluation of the human interferon-γ (HuIFN-γ) have been hampered by the inability to obtain sufficient amounts of purified IFN. However, this situation has changed recently due to the availability of monoclonal antibodies to IFN-γ for purification (19-20) and the successful cloning of human IFN-γ complementary DNA and its expression as a functional molecule in bacteria (21).

Interferons exert their antiviral effect by rendering cells incapable of supporting virus multiplication. Interferons are measured generally by their antiviral activity in cell culture. Routine laboratory assays for IFNs are generally based on their ability to inhibit the lysis of cultured cells by viruses (16).

1. Structure of IFN-γ

There is evidence that the cDNA for the HuIFN-γ was derived from a single copy gene located on chromosome 12 (21). The amino acid sequence of IFN-γ was elucidated by direct sequencing of the natural protein (22) as well as DNA sequencing of the cloned gene (23). It has a very limited structural homology with IFN-α and -β (21).

Natural IFN-γ is a glycoprotein. The gene for IFN-γ in human codes for a protein of 166 amino acid residues (21). But it has been shown that post-translational processing

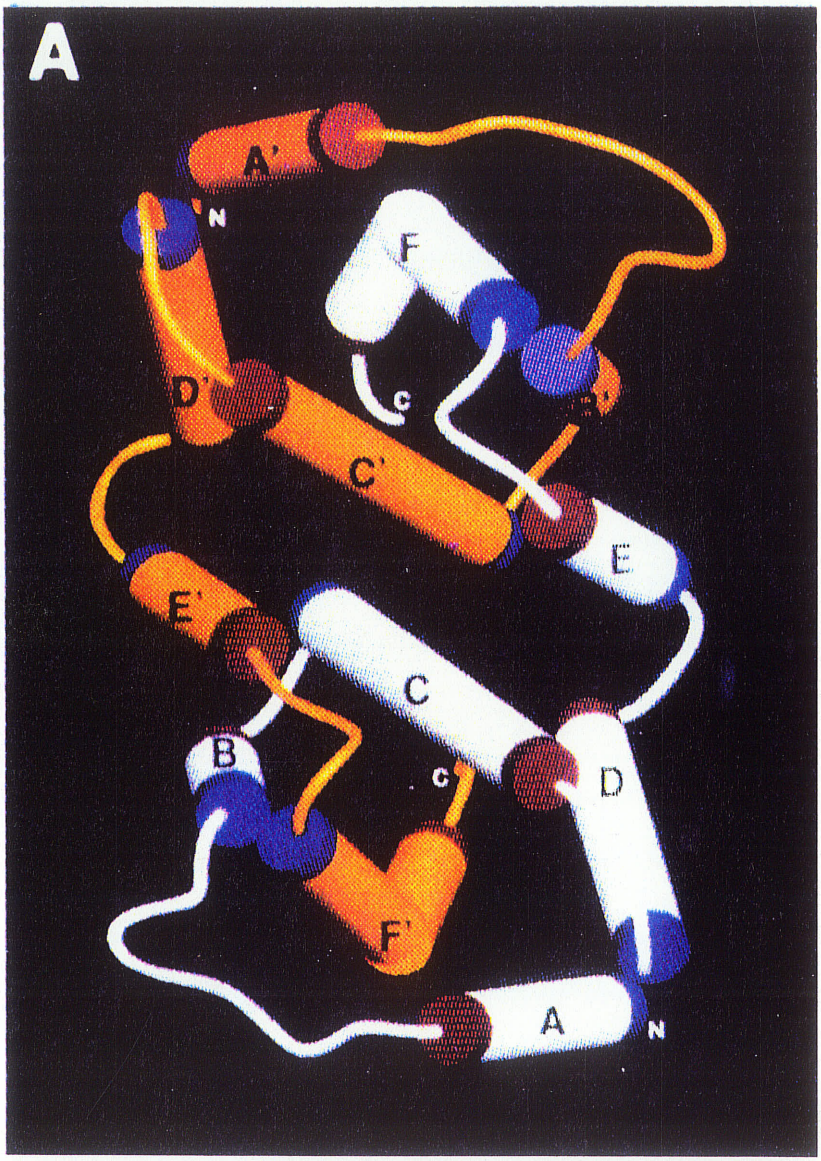
removes 23 amino-terminal residues (22). C-terminal processing has also been shown to occur in IFN-γ isolated from phytohemagglutinin-stimulated human peripheral blood lymphocytes resulting in a heterogenous population of molecules with different C termini (22). It is not known whether similar processing occurs in vivo. Glycosylation at Asn 48 and Asn 120 has been directly demonstrated (22), and purification of IFN-γ from human peripheral blood lymphocytes has demonstrated that IFN-γ occurs naturally in three forms with molecular weights of 25,000, 20,000, and 15,500 (24). These species differ in glycosylation as well as C-terminal processing but are equally active in vitro (24-25). Under physiological conditions, IFN-γ forms non-covalent dimers with an apparent molecular weight of 40,000-50,000 (25).

Secondary structural studies by far-ultraviolet circular dichroic spectra estimated an α-helix content of 40 to 60% and a low percentage of β sheet in this protein (26-28). This was confirmed only recently with the available X-ray crystal structure (Fig. 1) of recombinant human interferon-γ D' (in which the five COOH-terminal residues are deleted) determined with the use of multiple-isomorphous-replacement techniques (29). It demonstrated that HuIFN-γ, which is dimeric in solution, crystallizes with two dimers related by a noncrystallographic twofold axis in the asymmetric unit. The protein is primarily α helical, with six helices in each subunit that comprise 62 percent of the structure, the helices range in length from 9 to 21 residues; there is no β sheet.

Although recombinant human IFN-γ (rHuIFN-γ) derived from Escherichia coli (E. coli) differs from the natural protein in that it has an N-terminal methionine residue, is not glycosylated and not heterogeneous at the C-terminus, it is similar in overall tertiary conformation to glycosylated Chinese hamster ovary cell-derived human IFN-γ (30). So the

Figure 1. Three-Dimensional Structure of Recombinant Human Interferon-γ

Schematic drawing of the recombinant human IFN-γ D' dimer. The α helices are represented as cylinders, and the nonhelical regions are shown as tubes. The NH_2- and COOH-terminal ends of the helices are colored blue and red, respectively. (Taken from: Ealick et al 1991 Three-dimensional structure of recombinant human interferon-γ. Science 252:698)


A
A'
N
F
C
D'
C'
E
E'
C
B
C
D
F'
A
N

dimeric structure and high α-helical content noted for recombinant human IFN-γ are probably characteristic of native human IFN-γ. Taken together the fact that unglycosylated rHuIFN-γ has potent biological activity that is qualitatively identical to that of natural IFN-γ (IFN-γ) (21) and the removal of up to 13 amino acid residues from the C-terminus of rHuIFN-γ does not affect either self-association or conformation (31), it suggests that E. coli-derived IFN-γ may be used to assess the structure-activity relationship of IFN-γ (32).

2. Mechanism of actions

Despite intensive study in the mechanism of IFN action, the integration of all the information into a coherent picture has not been achieved. It is clear that IFNs are not themselves the active molecule of those biological activities, but act as the extracellular messenger to induce these activities.

2.1 Receptor interaction

At the cellular level, the HuIFN-γ exerts its effects through the interaction with a specific cell surface receptor which is different from that of the IFN-α and IFN-β (33). IFN-γ does not bind to the IFN-α/β cell surface receptor and the IFN-α/β do not compete with IFN-γ for cell surface binding (33-34). HuIFN-γ does not bind specifically to mouse, hamster, or bovine cells (35-37) and mouse IFN-γ does not bind to human or hamster cells (38). The species specificity of IFN-γ resides in the interaction of IFN-γ with its receptor (10).

To date, only one high affinity receptor for the HuIFN-γ has been identified by association kinetics and has since been cloned, the gene is located on chromosome 6 (39).

Binding of IFN-γ to its receptor is followed by receptor-mediated endocytosis and ligand degradation (40). Whether binding of IFN-γ to its receptor is sufficient to transfer its signal to the cell or whether IFN-γ also need to be internalized for biological activity, is not fully established (41). Clearly, the presence of a high-affinity membrane receptor alone is not sufficient to render cells sensitive for IFN-γ since mouse cells transfected with the human IFN-γ receptor gene can bind human IFN-γ but fail to respond to it (42). And it was shown, the HuIFN-γ receptor is located on chromosome 6, however chromosome 21 is also required to impart antiviral response (43).

2.2 Biochemical Actions

Receptor-bound IFN-γ initiates a series of metabolic modifications, leading to the expression of a large series of genes, de novo synthesis of RNA and synthesis of polypeptides. In addition to the other IFNs, IFN-γ induces the production of at least 12 distinct proteins (44).

Two IFN-induced, double-stranded RNA-dependent enzymes have been identified that may play important roles in the regulation of viral and cellular macromolecular synthesis and degradation. They are the (2'-5')-oligoadenylate synthetase (45) and the P1/eIF-2α protein kinase (46). The majority of IFN-induced proteins have not yet been characterized.

The mechanism of antiproliferative activity has been studied in detail in Daudi cells, a Burkitt's lymphoma cell line particularly sensitive to IFN; a variety of effects on DNA synthesis have been observed (47). Down-regulation of oncogene expression has been speculated to be another mechanism by which IFN-γ may exert antiproliferative efficacy. Einert et al (48) described the relationship between reduction of oncogene expression (c-myc)

by interferon-γ and arrest in the G_0/G_1 cell cycle, indicating inhibition of proliferative activity.

3. Structure-activity Studies

To date, relatively little is known about the structure and function relationships of this pleiotropic cytokine. The exact location of the active site(s) has not been identified. Localizing the active site is an important step in characterizing the molecular interaction of HuIFN-γ with its cell receptor. The construction of variants IFN to allow better control in its therapeutic applications is a long term possibility.

3.1 Monoclonal Antibody Approach

One of the most useful methods to study the functional sites of a macromolecule is the analysis of its interaction with neutralizing monoclonal antibodies (MAbs). The working hypothesis is that if the MAb binds the functional site on the HuIFN-γ molecule, then the corresponding effector function will be neutralized. The closer the MAb binds to the active site, the greater will be its neutralizing ability (49). However, functional effects observed with different MAbs may or may not be the result of antibody binding to the same domain on the molecule if there were multiple neutralizing epitopes. So in order to identify all the functional epitopes, a relatively large number of neutralizing MAbs is required. An effective means of correlate the epitopes recognized by different MAbs is to coat the plate with antigen and then study the competition between labelled and unlabelled MAbs. The degree of cross-reactivities reflects the relatedness of the respective epitopes recognized by these MAbs.

Also it is noted that in rare incidents, the neutralization is due to a conformational

change, or failure to effect a conformational change required for its function, as a result of the MAb binding to some other part of the molecule. Thus, in those cases, the location of the epitope may not have any relationship to the functional site. Fortunately, these are exceptional cases, and therefore, monoclonal antibody analysis remains to be the most frequently used methods.

3.1a Development of MAbs

The hybridoma technique developed by Kohler and Milstein (50) has made it a routine technique to produce monoclonal antibodies in many laboratories. The most important step in the process is the design of an appropriate strategy for screening hybridomas for specific antibody production. Solid phase radio-immunoassay, enzyme immunoassay (EIA), radio-immunoprecipitation and direct enzyme linked immunosorbent assay (ELISA) are methods that have been used to detect MAb production to HuIFN-γ. All of these screening methods require highly purified HuIFN-γ.

The production of anti-HuIFN-γ MAbs has been hampered because the purification of biologically active HuIFN-γ was difficult and sufficient amounts of purified antigens for screening purposes were not generally available.

Functional assays, such as antiviral activity neutralizing assays or blocking of human leukocyte antigen (HLA) class II induction for the production of anti-IFN-MAbs are tedious, time consuming and relatively insensitive, thereby limiting the number of hybridomas that can be screened. Relatively small numbers of MAbs against HuIFN-γ have been generated by a few laboratories:

Based on the neutralization of the antiviral activity of HuIFN-γ in a cytopathic effect

assay, the first neutralizing MAb GIF-1 was generated by Rubin et al (51). It showed antiviral neutralizing activity to natural IFN-γ, but not to recombinant IFN-γ. Another five MAbs against natural IFN-γ were prepared by Wang et al (52) using the same screening method. All five antibodies have high IFN-γ binding activity but exhibited differential IFN-γ neutralizing activities.

Using solid phase radio-immunoassay to detect MAb production to HuIFN-γ, Le et al (20) produced two MAbs B1, B3. B3 showed potent neutralizing activity against both natural and recombinant IFN-γ. B1 showed neutralizing activity only when very high concentration were employed. Stefanos et al (53) produced three MAbs against recombinant IFN-γ. Hybridomas were screened by a soluble phase radioimmunoassay using pure ^{125}I-labelled cloned IFN-γ as antigen. Two of them (S1-1, S1-2) were able to neutralize specifically the antiviral activity of natural and recombinant IFN-γ. Kelder et al (54) produced 72 MAbs to HuIFN-γ by screening the culture fluids with a solid phase immunoassay. Only 26 of the 72 MAbs are high affinity antibodies. Of the five neutralizing antibodies, four were of the high affinity type.

Liang et al (55) produced seven hybridomas (BG1-7) which secreted MAbs against recombinant IFN-γ. The hybridomas were screened by ELISA. Four of the seven MAbs (BG1-4) neutralized the antiviral activity of both natural and recombinant IFN-γ. Oda et al (56) raised three MAbs (KM45, KM48 and KM61) against recombinant HuIFN-γ as detected by binding ELISA. All antibodies bound to rHuIFN-γ although KM61 showed weak binding. Among these MAbs, only KM48 has an ability to neutralize the antiviral activity of rHuIFN-γ.

Alternatively, using radio-immunoprecipitation technique to detect MAb production

to HuIFN-γ, five MAbs were generated by Meager et al (57). Four of them showed antiviral neutralizing activity.

Van der Meide et al (58) established two stable hybridoma cell lines secreting specific antibodies against HuIFN-γ as detected by enzyme immunoassay. Both MAbs (designated as MD-1 and MD-2) neutralize the antiviral activity of natural and recombinant HuIFN-γ, although MD-1 is far more effective than MD-2.

Ziai et al (59) generated two MAbs M133.1, B133.3 against rHuIFN-γ. Hybridoma culture supernatants were screened for their ability to block the HLA class II enhancing effect of rIFN-γ on melanoma cells. Both MAbs inhibited the antiproliferative and antiviral actions of rIFN-γ and the increase in the expression of cell surface human lymphocyte antigen class I and II antigens.

Oleszak et al (60) developed a monoclonal antibody specific for human interferon gamma. Hybridoma culture supernatants were screened for neutralization of antiviral activity of HuIFN-γ by the method determining the inhibition of nucleic acid synthesis assay. This MAb was of IgM subclass.

Favre et al (61) produced five MAbs specific for human recombinant IFN-γ. The supernatants of the hybridomas were tested in the direct ELISA, in immunoprecipitation, in the antiviral assay and in the HLA class I induction assay. MAbs B22, B27 and 35 inhibit the antiviral activity of rHuIFN-γ, whereas MAbs 32 and 3-6 had no effect.

In contrast, relatively large number of MAbs against the rHuIFN-γ were produced in our laboratory:

Alfa et al (62) have reported the design of a sandwich ELISA (sELISA) that does not bind the HuIFN-γ directly to the plate and does not depend on the availability of purified

HuIFN-γ. It was able to efficiently detect neutralizing MAbs as well as non-neutralizing ones directed against the rHuIFN-γ. Based on this screening method, a panel of more than 130 MAbs directed against the rHuIFN-γ has been produced (M. J. Alfa and F. T. Jay unpublished data). All of the 130 MAbs have been characterized on the basis of neutralization of the antiviral activity, immunoprotein blot and immunoglobulin isotype. Of the 130 MAbs, 21 antiviral neutralizing MAbs were selected for further studies (63).

3.1b Characterization of MAbs

The panel of 21 neutralizing MAb generated in our laboratory has allowed a comprehensive search for the antigenic epitopes of the rHuIFN-γ that are involved in the antiviral effector function (63). These neutralizing MAbs were selected on the basis that each one was produced by independently isolated hybridomas resulting from separate spleenocyte-myeloma cell fusion events. This selection criterion ensured maximum diversity of neutralizing MAb whereas maintaining a panel that was practically manageable for such a study.

To determine the relatedness of the epitopes recognized by each of the MAb in the panel, a competition sandwich radioimmunoassay (sRIA) was developed to quantitatively determine the efficiency of each MAb to compete with each of the other MAbs in the panel for their respective epitopes on the rHuIFN-γ. The ^{125}I-labelled MAbs were allowed to compete with varying amounts of unlabelled MAb for binding to rHuIFN-γ under antigen-limiting conditions, and the 50% inhibition endpoints were determined for each of the 21 MAbs. The competition of each heterologous MAb relative to the competition of the homologous MAb was determined. By grouping the competition patterns of the 21 MAbs,

it was apparent that at least two epitopes (E1 and E2) were important to the antiviral function of rHuIFN-γ (Table 3). E1 and E2 are two distinct neutralizing epitopes. The MAbs of the E1 group do not compete with those MAbs of the E2 group. Five of the MAbs in the E1 group, but none in the E2 group, were able to react with the SDS-denatured HuIFN-γ in western blot suggesting that those MAbs in E1 group are likely to be recognizing linear epitopes. There were three MAbs that appear to recognize epitopes that overlap or interfere with the reactivity of both E1 and E2. It suggests that E1 and E2 may be spatially adjacent to each other in the tertiary structure of the rHuIFN-γ.

Consistent with the above result, in the murine IFN-γ, Schreiber et al (64) has also identified two neutralizing epitopes that have differential effects on the non-specific tumoricidal activity in macrophage.

Studies by other investigators have also indicated that there may be multiple neutralizing epitopes on the HuIFN-γ:

Liang et al (55) found that competition between labelled and unlabelled neutralizing MAbs for IFN-γ showed that BG 1 was competed by both BG 3 and BG 4, but not by BG 2; BG 2 was competed by BG 3 but not by BG 1 nor by BG 4. It suggests that these MAbs recognize two different epitopes, one reacted with BG 1 & BG 4, while the other reacted with BG 2. BG 3 either binds to a region overlapping with the other two epitopes or reacts with both epitopes.

Van der Meide et al (58) also demonstrated that neutralizing MAbs MD-1 and MD-2 recognize different epitopes and do not compete with each other in binding to HuIFN-γ as concluded from competition assays.

Stefanos et al (53) reported that although the antibodies produced by clones S1-1 and

TABLE 3. FUNCTIONAL EPITOPES OF HUMAN IFN-γ

Competition Profiles of 21 MAb to rHuIFN-γ

UNLABELLED MONOCLONAL ANTIBODIES	RADIOLABELLED MONOCLONAL ANTIBODIES																				
	3061	3015	3009	3081	3075	3169	3074	3152	3091	3102	3094	3059	3070	3098	3055	3045	3043	3069	3054	3052	3125
3061	1.00	2.24	2.19	1.74	17.86	0.93	1.62	1.21	0.10	0.17	0.07	-	-	-	-	-	-	-	-	-	-
3015	0.81	1.00	1.00	0.60	0.78	0.59	1.41	0.43	0.03	0.08	0.05	-	-	-	-	-	-	-	-	-	-
3009	0.81	1.00	1.00	0.48	0.79	0.63	1.55	0.33	0.04	0.09	0.05	-	-	-	-	-	-	-	-	-	-
3081	0.87	1.91	1.66	1.00	1.00	1.45	2.34	0.85	0.04	0.09	0.03	-	-	-	-	-	-	-	-	-	-
3075	0.96	1.48	1.91	1.51	1.00	1.32	1.12	1.26	0.05	0.16	0.08	0.66	-	-	-	-	-	-	-	-	-
3169	1.26	3.02	2.95	1.72	2.00	1.00	3.55	1.00	0.04	0.10	0.05	2.95	-	-	-	-	-	-	-	-	-
3074	0.91	1.48	1.66	1.38	1.00	1.15	1.00	0.71	0.05	0.12	0.07	0.42	-	-	-	-	-	-	-	-	-
3152	0.85	3.98	4.62	2.39	3.02	1.70	4.27	1.00	0.05	0.01	0.06	3.31	-	-	-	-	-	-	-	-	-
3091	2.14	-	-	-	-	-	8.32	15.98	1.00	1.99	0.41	0.32	-	-	-	-	-	-	-	-	-
3102	8.32	-	-	-	-	-	-	7.64	0.74	1.00	0.72	3.31	1.93	1.86	-	-	-	-	1.17	0.46	-
3094	-	-	-	-	-	-	-	30.46	2.97	4.46	1.00	1.59	1.22	74.18	-	-	-	-	-	19.95	-
3059	-	-	-	-	-	-	-	-	-	67.10	21.54	1.00	3.06	9.41	-	-	-	1.95	12.90	4.82	-
3070	-	-	-	-	-	-	-	-	31.15	67.10	7.36	1.05	1.00	9.26	1.95	1.32	0.85	0.65	3.77	3.16	187.70
3098	-	-	-	-	-	-	-	-	-	-	1.10	0.31	0.41	1.00	1.00	1.05	0.79	0.46	0.66	0.59	87.80
3055	-	-	-	-	-	-	-	-	-	-	0.61	0.63	3.85	0.69	1.00	1.48	1.12	0.82	0.66	0.54	34.90
3045	-	-	-	-	-	-	-	-	-	-	6.51	0.26	0.39	1.18	1.62	1.00	1.00	0.48	0.67	0.71	67.80
3043	-	-	-	-	-	-	-	-	-	-	4.14	0.29	0.37	1.38	1.74	1.10	1.00	0.42	0.90	0.81	105.60
3069	-	-	-	-	-	-	-	-	-	-	12.02	1.05	1.88	6.17	4.27	1.38	0.89	1.00	3.24	1.10	303.00
3054	-	-	-	-	-	-	-	-	-	-	30.43	0.45	0.64	1.62	1.70	1.35	2.63	0.58	1.00	0.59	303.00
3052	-	-	-	-	-	-	-	-	-	-	32.86	0.42	0.42	1.66	1.48	1.23	2.14	0.52	1.00	1.00	71.80
3125	-	-	-	-	-	-	-	-	-	-	47.50	0.21	0.20	0.02	0.20	0.79	0.58	0.31	0.02	0.01	1.00
3097	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3067	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EPITOPES	E1									E1/E2			E2								E2'

(Taken from: Alfa M and Jay FT. 1988. Distinct domains of recombinant human IFN-γ responsible for anti-viral effector function. J Immunol 141:2472)

S1-2 were both able to neutralize specifically the antiviral activity of natural and recombinant HuIFN-γ, they appeared to recognize different epitopes on the HuIFN-γ molecule resulting from an epitope blocking test.

Ziai et al (59) concluded that the anti-rHuIFN-γ MAbs B133.1 and 133.3 bind to two distinct determinants on the rHuIFN-γ molecule, as determined by binding competition experiments in a solid phase radioimmunoassay. Both MAbs have strong antiviral neutralizing ability.

Ichimori et al (65) reported that a synthetic peptide corresponding to amino acid residues 4 to 21 of HuIFN-γ was able to inhibit the neutralizing activity of one of their MAb, but not that of another also concluded that there was another active site on the HuIFN-γ molecule.

Five MAbs (A7, B24, I14, L12, and M2) developed by Wang et al (52) had high IFN-γ binding activity. MAb A7 was most effective in neutralizing the antiviral activity induced by IFN-γ, whereas MAb I14, L12, and M2 exhibited intermediate neutralizing activities. MAb B24, on the other hand, had low IFN-γ-neutralizing activity. The fact that all five MAbs had high IFN-γ binding activities but exhibited differential IFN-γ neutralizing activities suggested that these MAbs were directed at different epitopes on the IFN-γ molecule.

The three neutralizing MAbs (B22, B27 and 35) developed by Favre et al (61) were tested in the competition experiments. The MAbs B22 and B27 blocked each other's binding indicating that they defined the same, or at least closely adjacent epitope. MAb 35 showed only partial cross-reactivity with the epitope detected by B22/B27 indicating that MAb 35 defined another epitope on the IFN-γ molecule.

Johnson et al (66) generated a MAb to a synthetic peptide representing the amino-terminal 20 amino acids of IFN-γ and demonstrated that this monoclonal antibody did neutralize the antiviral activity of IFN-γ. The lack of reactivity of this 20-mer with other IFN-γ neutralizing antibodies suggests that this amino-terminal region is not the only active site in the IFN-γ molecule.

Fish et al (67) introduced changes at amino acid residue position 45 and 92 of the rHuIFN-γ which located in the hydrophobic core of the protein and demonstrated that these homologues (Thr^{45})IFN-γ and (Cys^{92})IFN-γ exhibited dramatically diminished antiviral activities. Taken together with the results by Johnson et al (66), the loss of biological activity associated with residue changes in the hydrophobic core of IFN-γ supports this notion of multiple active sites.

3.2 Other Approaches

With the availability of advanced gene modification technology, it is relatively easy to synthesize IFN-γ homologues containing deletions or amino acid substitutions and to study their activities. Or, alternatively, the truncated molecule could be obtained after treatment of HuIFN-γ with different enzymatic cleavages.

Also there are several procedures that could be employed for identifying the neutralizing epitope to which the antibodies were directed, including binding to polypeptide prepared by chemical or enzymatic cleavage and cross-reactivity with homologous IFN subtypes. Alternatively, chemically synthesized short oligopeptides corresponding to sequences in the parental molecule may be employed either for the identification of epitopes or for use as immunogens to raise antibodies of predetermined sequence specificity.

Thus far, these analyses have produced conflicting results:

A MAb raised against a synthetic polypeptide corresponding to the sequence 131-146 (68) and 121-130 (69) did not inhibit the antiviral activity of IFN-γ, the MAb IBA 10, (67) (generated to a synthetic peptide representing the carboxy-terminal 19 amino acids of IFN-γ) showed no associated inhibition of antiviral or antiproliferative properties, the inhibition of the antiviral activity of IFN-γ observed (61) with the MAbs 35 and B27 which bind to the IFN-γ lacking 15 amino acids (aa) at the C-terminus; whereas polyclonal (66) or monoclonal antibodies (65) directed against the sequence 1-20 are inhibitory indicating the biological activity is associated with an epitope determined by the sequences 1-20. This was confirmed by the result that truncated IFN-γ molecule lacking 10 amino-terminal residues obtained by V-8 protease digestion has reduced antiviral activity (70). In addition, another MAb specific for the peptide 22-130 was also found to be inhibitory (65).

Studies of human recombinant IFN-γ analogs suggested that the amino acid residues 25 and 78 play an important role in the activity, whereas residue 45 is implicated in the maintenance of secondary structure of the protein (32). In addition, truncated molecules deleted of 15 aa (71) or 21 aa (72), and 23 aa (73) at their C-terminus retain almost full antiviral activity.

Taken together these results suggest that the C-terminal part is not implicated in the biological activity and that the portion 1-20 and some residues 22-130 are associated with the active site.

In contrast other authors reported that the C-terminal portion contributes to the active part of the molecule, they found that C-terminal truncated IFN-γ molecules had a greatly reduced biological activity. These truncated molecules were obtained after treatment of

human recombinant IFN-γ with trypsin (31), clostripain (74), a protease containing fraction prepared from mechanically lysed E. coli cells (75) or by purification from natural sources (22). Also it was reported (76) that one neutralizing MAb 47N3-6 did not recognize a genetically engineered variant terminating at residue 131, but it demonstrated binding to a 15 amino acid residue oligopeptide corresponding to residues 132-146 at the carboxyl terminus of rHuIFN-γ.

Though the apparent contradiction in the role of the C-terminal portion in the activity and structure of HuIFN-γ remains unclear, it is realized that conclusions regarding functions of IFN-γ based on negative data can be misleading and have to be taken carefully. Luk, Jay and Jay (72) demonstrated that the loss of the C-terminal 21 amino acid residues of HuIFN-γ resulted in a substantial reduction in the ability of the molecule to be renatured in vitro after the treatment with chaotrophic agents, a method frequently used to extract and purify recombinant polypeptides from E. coli host. This result may account for some reports which inferred the involvement of the C-terminus in the functions of the HuIFN-γ due to their failure to detect activity upon deletions of only 11-18 residues.

The mature form of murine IFN-γ (MuIFN-γ) is 10 residues shorter than the HuIFN-γ with a nucleotide homology of 60 percent and a protein sequence homology of 40 percent (77). Interestingly, although HuIFN-γ and MuIFN-γ share only a moderate sequence homology and demonstrate high species specificity with respect to biological activity, evidence also exists in MuIFN-γ for a functional involvement of both amino and carboxyl termini. It was reported (78) that monoclonal antibodies specific for a C-terminal 35 amino acid region of MuIFN-γ were capable of inhibiting both the antiviral activity and macrophage-priming activities of IFN-γ. Furthermore, it was reported (79) that polyclonal

antibodies specific for the nine carboxy-terminal amino acids of MuIFN-γ can specifically inhibit the antiviral and immunoregulatory activities of this IFN in vitro. Langford et al (80) demonstrated that antibodies to an N-terminal synthetic peptide of MuIFN-γ neutralized the antiviral activity of MuIFNγ. Zavodny et al (81) constructed several analogs (amino-terminal deletions) of MuIFN-γ and found that when the first nine amino acids of IFN-γ were elinimated, the protein product lacked detectable antiviral activity. Recently, the amino-terminal region of MuIFN-γ has been shown to contain a binding site for the IFN-γ receptor, based on specific blockage of IFN-γ function and receptor binding by a synthetic peptide corresponding to the first 39 residues of IFN-γ and based on blockage of IFN-γ function by the amino-terminal 39 residues as well as the first 20 residues of IFN-γ (82). Schreiber et al (64) produced four MAbs to recombinant MuIFN-γ and demonstrated that these MAbs displayed two distinct epitope specificities: one displayed by H1 and H2, the other displayed by H21 and H22. The H1/H2 epitope was shown to depend on the amino-terminus of IFN-γ, whereas the H21/H22 epitope was formed by the carboxyl-terminal amino acid sequence. All four MAbs inhibited IFN-γ dependent antiviral activity. These results suggested that MuIFN-γ may have at least two distinct functional domains, one in the amino-terminal region and one in the carboxy-terminal region.

Biological activity measurements on recombinant murine-human hybrid IFN-γ variants support that neither antiviral activity nor species specificity resides exclusively in either the amino or carboxyl terminus (83). 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. The fact that the C-

terminus may be deleted without extensive loss of activity proved that the C-terminus is not essential for the functions tested.

3.3 Binding site and effector site

There is evidence to suggest that the receptor alone is not sufficient to transduce the effector signals of the HuIFN-γ. Using somatic cell hybrids, Rashidbaigi et al (39) has mapped the HuIFN-γ receptor to chromosome 6, but its expression alone was not sufficient to allow a heterologous cellular response to the HuIFN-γ. Complete functional response to HuIFN-γ required additional coding sequences from chromosome 21 (43).

In the panel of 21 neutralizing MAbs developed in this laboratory, the preliminary data indicated that none of the 21 neutralizing MAbs by themselves can inhibit HuIFN-γ binding to its receptor (M. J. Alfa and F. T. Jay unpublished data) suggesting that the neutralization of activity by the MAbs in this panel of MAbs did not depend on the inhibition of HuIFN-γ binding to the cell surface receptor. The receptor binding domain must be separate from the antiviral effector site(s).

The concept that IFN-γ has separate domains for receptor binding site and effector site is also consistent with observations by other investigators:

Schreiber et al (64) demonstrated that MAb H1 and H2 inhibited IFN-dependent antiviral activity but not IFN-γ receptor interaction.

Ziai et al (59) has found that the two MAbs that they tested did not prevent binding of the HuIFN-γ to the eukaryotic receptor but did neutralize HLA induction, antiproliferation, and the anti-viral effect of HuIFN-γ.

Fish et al (67) demonstrated that residue changes at two sites associated with β-turns

on the surface of IFN-γ resulted in homologues with reduced antiviral and antiproliferative activities. These diminished biological activities are not a direct consequence of the receptor-IFN interaction. This suggests that separate effector site(s) exist on the surface of the IFN-γ molecule, distinct from receptor binding epitopes associated with the induction of antiviral and growth inhibitory effects.

3.4 Multiple functional sites

Numerous cellular-regulatory properties have been attributed to interferon. The ability to inhibit viral growth was the first recognized biological activity of IFN and was the property for which the protein was named. Later the ability to inhibit cell multiplication, i.e., the antiproliferative activity, was detected as were immuno-modulatory activities. However, the ratio of antiviral to antiproliferative activity was not constant from one purified fraction to another (84), or from one IFN type to another (85). This observation was extended to other activities as well: stimulation of cytotoxic activities of lymphocytes and macrophages, and of natural killer cell activity (86-87); an increase in expression of some tumor-associated antigens (88-89). In the case of natural killer cell stimulation, IFN-αJ was found to be deficient in this activity while exhibiting other activities (88, 90). These and other observations indicated that many of the effects of the IFNs can be dissociated (10). It is likely, therefore, that IFN activities are highly regulated in vivo. A mechanism likely exists to select a function or a range of functions to accommodate a particular challenge.

Ziai et al (59) produced two neutralizing MAbs. Both MAbs inhibited the increase in the expression of cell surface human lymphocyte antigen class I and II antigens and the antiproliferative and antiviral actions of rIFN-γ. On the other hand, neither MAb affected

the binding of rIFN-γ to melanoma cells and its ability to reduce the expression of a high molecular weight-melanoma associated antigen. These data indicate that the functional domains of IFN-γ responsible for antiviral activity, increased human lymphocyte antigen expression and antiproliferative effects on human melanoma cells may be distinct from that (those) involved in reduced expression of the high molecular weight-melanoma associated antigen and in IFN-γ binding to cell receptors.

Schreiber et al (64) demonstrated that MAb H1 and H2 selectively inhibited IFN-γ-dependent antiviral activity but not IFN-γ-dependent macrophage activation factor (MAF) activity.

These findings suggested that the pathways leading to the cellular responses must separate at the level of HuIFN-γ-receptor interaction and not at some later cellular processes. Since it is known that there is only one high affinity receptor molecule for the HuIFN-γ (39), the hypothesis is that there are multiple effector sites on the surface of IFN-γ that allow differential functional response to the same HuIFN-γ receptor binding.

CHAPTER II

AN EPITOPE TYPING SCHEME FOR HuIFN-γ MAbs

INTRODUCTION

Although Alfa et al (63) have studied a relatively large panel (21 clones) of independently isolated neutralizing MAbs and identified only two distinct antiviral neutralizing epitopes, E1 and E2, it was not clear if the anti-HuIFN-γ MAbs from other laboratories raised to different forms of HuIFN-γ, including natural and recombinant IFN-γ that had been purified by different methods, using different methods for hybridomas screening, was able to detect other functional epitopes, such as that for receptor binding. Furthermore, in the absence of information on whether the MAbs used in different studies are binding similar neutralizing epitopes, it is difficult to correlate the findings from different research groups using untyped MAbs. Thus, a means of classifying each neutralizing MAb into the various epitope specificities is essential.

The objective of this study is to attempt to classify all the available anti-HuIFN-γ MAbs according to our epitope assignment scheme.

MATERIALS AND METHODS

Extraction and purification of rHuIFN-γ

The synthesis and cloning of the entire coding sequence of HuIFN-γ (91) and its efficient expression as authentic polypeptide by plasmid vector, pJP_1R_3 in Escherichia coli (92) have been described previously. A modified plasmid vector, $pJP_{14}R_9$, was used to express authentic HuIFN-γ for this study. The E. coli strain expressing HuIFN-γ is designated by the plasmid it carries, $pJP_{14}R_9$-IFN, while the control strain that carries the same plasmid without the gene for HuIFN-γ is designated $pJP_{14}R_9$.

The recombinant proteins were isolated from the transfected E. coli host LE392. Individual clones were grown overnight at 37°C with shaking in 500 ml of terrific broth (1.2% w/v tryptone, 2.4% w/v yeast extract, 0.4% v/v glycerol, 0.17 M KH_2PO_4, 0.72 M K_2HPO_4) (93) containing 20 μg/ml tetracycline (Sigma Chemical Corp. St. Louis, Mo, USA). Cells were harvested by centrifugation for 10 min at 4°C in a Sorvall GSA rotor at 5000 x g and were washed twice with an equal volume of TN buffer (50 mM Tris-HCl pH 8.0, 30 mM NaCl) at 4°C. The final cell pellet was resuspended in 10 ml of TN buffer and then disrupted by passing the cells twice through a Carver press (Fred S. Carver Inc., NJ, USA) held at -20°C. The cell debris was removed by centrifugation in a Sorvall SS34 rotor at 10,000 x g for 30 min at 4°C. The supernatant was collected and stored at -20°C until needed. The specific activity of the rHuIFN-γ in the supernatant fluid was 4×10^6 units/mg (standardized against National Institutes of Health Human Reference IFN-γ Gg 23-901-530). The supernatant was used as the HuIFN-γ source in the ELISA assays and sRIA assays.

The HuIFN-γ was purified by immunoaffinity column chromatography. The affinity

column was prepared by coupling purified anti-HuIFN-γ monoclonal antibody, MIF3052 (63), to cyanogen bromide activated Sepharose 4B (Pharmacia Fine Chemicals AB, Montreal, Que., Canada) following conditions as recommended by the manufacturer. Supernatants of the cell lysates were adjusted to 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 1% Nonidet P-40 before applying onto the affinity column. The column (19 x 0.8 cm) was washed with the same buffer at pH 9.0 followed by a further wash with the same buffer at pH 9.0 without 1% Nonidet P-40. The final wash was with 50 mM diethylamine hydrochloride (Sigma), pH 10. The adsorbed rHuIFN-γ was then eluted with 50 mM diethylamine hydrochloride, pH 11.2. Fractions (1.0 ml/fraction) were immediately neutralized by collecting into tubes containing 0.10 ml of 2 M Tris-HCl (pH 7.5). Purified fractions were stable at 4^0C for up to 2 months. Fraction 2, 3, 4, and 5 were pooled together and the specific activity was 2 x 10^7 units/mg. The purified rHuIFN-γ were used as the HuIFN-γ source in the antiviral neutralization assays.

Preparation of rabbit polyclonal anti-HuIFN-γ immunoglobulin

The rabbit polyclonal anti-HuIFN-γ serum was prepared by M. Alfa et al (62). The rabbit polyclonal anti-HuIFN-γ serum was prepared by immunizing rabbits with purified denatured HuIFN-γ. Polyclonal rabbit immunoglobulins were purified using Protein A-Sepharose CL-4B according to the method of the manufacturer (Pharmacia) and the immunoglobulins were adsorbed exhaustively against $pJP_{14}R_9$ proteins that were immobilized on cyanogen bromide activated Sepharose 4B (Pharmacia). The polyclonal anti-HuIFN-γ immunoglobulin was used to coat the sRIA plate.

Monoclonal antibodies

The production of the anti-rHuIFN-γ monoclonal antibodies MIF3009, MIF3094, MIF3055, and MIF3125 was previously described by M. Alfa et al (63). These monoclonal antibodies were prepared as ascites fluids by injecting hybridoma cells into BALB/c mice (University of Manitoba Central Animal Unit) that had been primed 7 days earlier with an intraperitoneal injection of 0.5 ml pristane. The ascites fluids obtained from these mice were centrifuged at 500 x g for 10 min to remove cells and debris. The monoclonal antibodies were purified by adsorption to Protein A-Sepharose CL-4B (Pharmacia) according to the manufacturer's instructions. The column (14 x 1.5 cm) was equilibrated with 50 ml phosphate buffered saline (PBS; 0.136 M NaCl, 0.003 M KCl, 0.008 M Na_2HPO_4, 0.001 M KH_2PO_4, pH 7.2) first, two milliliter of ascites fluid was loaded onto the column. The column was washed with 100 ml PBS. Then the absorbed monoclonal antibody (20 ml) was eluted with acetic acid in saline (0.58% v/v glacial acetic acid in 0.85% w/v NaCl) and neutralized by collecting into beaker containing 10 ml 1M Tris pH 8.0. The purified immunoglobulin was dialysed against 1 litre 50 mM Tris pH 8.0 containing 0.02% SodiumAzide at 4^0C overnight and was concentrated by adding ammonium sulfate (BRL Life Technologies, Inc. Gaithersburg, MD, USA) to 50 % saturation at 4^0C, then centrifuge at 1000 x g for 15 min. The precipitate was redissolved in 2 ml solution containing 50 mM Tris-HCl pH 8.0, 30 mM NaCl. The purity of each MAb was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% reducing gel.

Fourteen monoclonal antibodies were generously provided by: Dr. Junming Le, Department of Microbiology, New York University School of Medicine, New York, NY; Dr. Antony Meager, National Institute for Biological Standards and Control, Hampstead, London,

UK; Dr. Shoji Oda, Tokyo Research Laboratories, Tokyo, Japan; Dr. Sidney Pestka, Department of Molecular Genetics and Microbiology, Robert Wood Johnson Medical School, Piscataway, New Jersey; Dr. Giorgio Trinchieri, Wistar Institute of Anatomy, Philadelphia, PA. The name, the source and references of published descriptions of each of these MAbs are summarized in Table 4. Hybridomas 69B, 220A12, 73A and 113B provided by S. Pestka were clone purified in this laboratory by M. Alfa and the sufix ".J" were added to the name solely to indicate a specific sub-clone selected for this study. All MAbs were stored at -20^{0}C until used.

Enzyme-Linked Immunosorbent Assay (ELISA)

The ability of the MAbs to bind rHuIFN-γ was tested using a direct ELISA. Five microlitre of crude $pJP_{14}R_9$-IFN lysate supernatant was diluted in 5 ml carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_2$, pH 9.0). Each well of ELISA plate (Immunolon II polyvinyl plate, Dynatech, Alexandria, VI, USA) was coated with 50 ul of the above diluted rHuIFN-γ (8.2×10^3 units/well) by incubating overnight at 4^{0}C. Residual protein binding sites were blocked by adding 100 ul of 3% (w/v) bovine serum albumin (BSA; Sigma) in PBS and incubating at 37^{0}C for 2 hours. The MAbs were serially diluted 1:2 with 1% (w/v) BSA in PBS (BSA-PBS) in a separate 96-well plate and then 50 ul was transferred to the appropriate well in the ELISA plate and incubated at 37^{0}C for one hour. The wells were washed three times with 100 ul PBS containing 0.02% polyoxyethylene-sorbitan monolaurate (PBS-Tween 20). Fifty microlitre of horse radish peroxidase-conjugated goat anti-mouse IgG, IgM, IgA (Cappel Laboratories Ltd. West Chester, PA, USA) diluted 1:1000 in 1% BSA-PBS was added to each well and incubated at 37^{0}C for one hour. The wells were washed five

times with 100 ul PBS-Tween 20. Color development was achieved by adding 50 ul/well of freshly prepared peroxidase substrate 2,2'-azinobis (3'ethylbenzthiazoline sulfonic acid) (ABTS; Sigma) at 0.015% (w/v) in citrate buffer (0.046 M trisodium citrate, 0.054 M citric acid PH 4.2) containing 0.03% (v/v) hydrogen peroxide (Fisher Scientific Company, Fair lawn, NJ, USA). The reaction was allowed to proceed at room temperature for 15 minutes in the dark and then 50 ul of 10% (w/v) sodium dodecyl sulfate (SDS) was added to each well to stop the reaction. The absorbance values were determined using an ELISA reader (Model EL308, Bio-Tek Instruments Inc., Burlington, VT, USA) equipped with a 405 nm filter. The endpoint was taken as the reciprocal of the dilution in the last well that produced an absorbance value of ≥ 0.150 which was 3 times the background value (0.05).

Anti-viral and MAb neutralization assays

In the antiviral activity assay, human lung carcinoma cells, A549, were seeded in individual wells of 96-well tissue culture plates (Corning Glass Works, Corning, NY, USA) at a concentration of 4 x 10^4 cells/well and were allowed to attach at 37°C for 4 h in humidified 5% CO_2, 95% air. Interferon preparations were serially diluted 2-fold with RPMI 1640 medium (Gibco Laboratories Ltd., Grand Island, NY, USA) in each row (12 wells) of cultures and incubated for 16 h. Encephalomyocarditis (EMC) virus in 100 ul of RPMI 1640 medium was inoculated into each well at a multiplicity of infection of 2, and the cells were further incubated for 24 h. The supernatants were removed and the cells were fixed and stained for 10 min with 0.25% (w/v) crystal violet, 20 mM Tris-HCl (pH 7.5), 0.9% (w/v) NaCl, and 20% (v/v) methanol. The excess stain was removed, and the monolayer was rinsed 3 times with water. The absorbed dye in each well was extracted with 100 ul of

methanol, and the absorbance was measured using an ELISA reader (EL308 Bio-Tek) that was equipped with a 590 nm filter. The average absorbances of the control cultures with and without the virus challenge were taken as 100% and 0% cytopathic effect, respectively. The end point of the antiviral assay was defined as the reciprocal of the highest dilution that produced 50% cytopathic effect compared with the controls. A laboratory IFN-γ standard calibrated against the National Institutes of Health standard (Gg23-901-530) was included in each assay for the conversion into U.S National Institutes of Health reference units.

In the antiviral neutralization assay, human lung carcinoma cells, A549, were seeded in individual wells of 96-well tissue culture plates at a concentration of 4×10^4 cells/well and were allowed to attach at 37^0C for 4 hours in humidified 5% CO_2, 95% air. The MAbs (100 ul) were serially diluted 2-fold with RPMI 1640 medium in each row of cultures (12 wells), then a constant amount of purified rHuIFN-γ (64-204 units/ml in 100 ul) was added to each well. The plate was incubated for 16 hours. One hundred microliter of supernatant was removed from each well before challenging with encephalomyocarditis virus. The plate was then processed as described for the IFN anti-viral assay. The purified IFN used was titrated concomitantly against a laboratory standard in each assay. The average absorbances of the control cultures with the virus challenge was taken as 100% cytopathic effect. The average absorbances of the control cultures without the virus challenge, but with same amount of input IFN-γ was taken as 0% cytopathic effect. The endpoint of the assay was taken as 50% cytopathic effect compared with the controls. The neutralizing unit was defined as the reciprocal of MAb dilution required to neutralize 1 unit/ml of the antiviral action of HuIFN-γ. The concentration of the HuIFN-γ used is described in Results for the specific experiment.

Estimation of protein concentration

Protein concentration of purified MAbs was estimated using the Amido Black (Sigma) adsorption microassay method described by Schaffner and Weissmann (94). The sample was precipitated with 10% trichloroacetic acid (TCA; Fisher) in the presence of 1% sodium dodecylsulfate, collected on a Millipore membrane (0.22 um; Millipore Corp., Bedford, MS, USA) by vacuum filtration and stained with 0.1% Amidoschwarz 10B in methanol:glacial acetic acid: distilled water (45:10:45 Vol%). The protein-dye complex was eluted by immersing filters in tubes containing 2 ml eluent solution (25 mM NaOH, 0.05 mM disodium ethylenediamine tetraacetate in 50% v/v aqueous ethanol) at room temperature for 10 min and its absorbance was determined at 630 nm on a spectrophotometer (Shimadzu UV-160; Shimadzu Corp., Kyoto, Japan). Tubes containing 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 5.0 μg of bovine serum albumin were run in parallel to provide a blank and a standard curve, respectively. The absorbance of the standard samples was plotted against input protein and the resulting standard curve was used to calculate the protein content of the unknown sample.

^{125}I-labelling of MAbs

Purified MAb were radiolabelled with ^{125}I using the method of Fraker and Speck (95). Glass test tubes were coated with 1,3,4,6-tetrachloro-3α, 6α-diphenylglycouril (Iodagen; Pierce Chemical Co., Rockford, IL, USA) by dispensing 50 ul of 2% (w/v) Iodagen in chloroform into each tube and allowing the chloroform to evaporate completely. The coated tubes were stored dessicated in the dark at 4^{0}C until use. The purified MAb (50 μg in 50 ul PBS) and 0.5 mCi of carrier-free Na^{125}I (Amersham Corp., Arlington Heights, IL, USA) mixture were added to the Iodagen coated tube. The reaction was allowed to proceed for 15

min at room temperature and then the reaction mixture was transferred to a separate tube. The reaction tube was rinsed with 50 ul of PBS that was then pooled with the reaction mixture. The free iodine was separated from labelled Immunoglobulin by column chromatography (30 x 0.7 cm) on Sephadex G-100 fine (Pharmacia). The column was equilibrated and eluted with PBS. By using a geiger counter, the first peak of radioactivity was identified which contained the labelled protein. Fractions (0.5 ml) were collected. Labelled immunoglobulin prepared in this manner had > 95% precipitability in 10% (w/v) TCA.

Competition Sandwich Radioimmunoassay (sRIA)

The cross-reactivity of the MAb was determined by a competition sandwich radioimmunoassay (sRIA) that was adapted from the sELISA. The sRIA of Alfa and Jay (63) was modified because the MAbs obtained from other sources were in quantities that precluded their purification for ^{125}I-labelling as probes and their quantitation by weight. All quantitative evaluations were based on ELISA units (EU) rather than mg of MAb. First, each well of the ELISA plate was coated with 50 ul of rabbit anti-rHuIFN-γ immunoglobulin (80 μg/ml in carbonate buffer) by incubating at 4^{0}C overnight. The residual binding sites in the wells were blocked by adding 100 ul of 3% BSA-PBS to each well and incubating at 37^{0}C for 2 hours. Fifty microlitre of crude $pJP_{14}R_9$-IFN lysate supernatant diluted in 1% BSA-PBS was added to each well (8 x 10^4 units/well) and incubated at 37^{0}C for 2 hours. The plate was washed three times with 100 ul PBS-Tween 20. Each test MAb (80 ul) was serially diluted 1/5 in 1% BSA-PBS in separate 96 well trays and then a constant amount (32 ng/80 ul) of ^{125}I-MAb was added to each well and mixed. A 50 ul aliquot of each mixture (containing 10 ng ^{125}I-

MAb, approx = 100,000 cpm) was transferred to the appropriate wells of the HuIFN-γ coated tray. The plate was incubated overnight at 4^0C and then washed five times with 100 ul PBS-Tween 20. The wells were allowed to air dry and individual wells were cut out with a hot wire for radioactivity quantitation in a gamma counter (gamma 8000; Beckman Instruments Inc., Fullerton, CA, USA). Each competition reaction was carried out in triplicate and the average was taken. The results of each assay were corrected for non-specific binding by using a negative control that contained cell extracts of E. coli which carried the expression vector $pJP_{14}R_9$ but without the rHuIFN-γ coding sequence. The binding of ^{125}I-labelled probe in the absence of any competing MAb (positive control) was taken as 100% binding. All experiments were performed in duplicate. The endpoint was determined as the number of EU of MAb required to inhibit binding of 10 ng of the radiolabelled probe by 50%. 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 (10 ng) of the homologous MAb were added. The MAbs received from several laboratories were not in sufficient quantity to allow purification for iodination and protein quantitation. Therefore, MAbs MIF3009, MIF3055, MIF3094 and MIF3125 from this laboratory were used as the radiolabelled probes in the competition assay. All MAbs provided from other laboratories were evaluated for their ability to compete with each of the 4 representative MAbs produced in this laboratory.

RESULTS

Eighteen MAbs from six independent research laboratories were studied (Table 4).

Four MAbs derived from independent clones from separate cell fusion experiments generated in this laboratory were selected for this study. These MAbs represent the four epitope specificity groups previously identified in this laboratory (63). The culture supernatant fluids were screened by both the sELISA and the anti-viral activity neutralization assay. These four MAbs were raised against recombinant human IFN-γ. All of them were of IgG_1 class. To facilitate functional characterization and quantitative analysis, they were purified from ascitic fluids, as described in the Materials and Methods. Subsequent analysis on a 10% reducing SDS-PAGE verified that only two polypeptide bands corresponding to the H and L immunoglobulin (Ig) chains were present in each of the MAb preparations (results not shown). A previous study demonstrated that they were specific for the natural HuIFN-γ (National Institutes of Health (NIH) reference standard, Gg23-901-530) and rHuIFN-γ, but did not neutralize the antiviral activity of HuIFN-α (NIH, G023-901-527), or HuIFN-β (NIH, G023-902-527). Among these four MAbs, only MIF3009 was able to react with SDS-denatured HuIFN-γ in Western immunoblots (63).

The fourteen MAbs from other research laboratories were obtained in different forms of sample preparation (Table 4). MAb B1 and B3 provided by Le et al (20) were in the form of hybridoma culture supernatant; MAb B133.1 and B133.3 provided by Trincheiri et al (59) were ascites fluid; MAb KM48, KM46 and KM45 provided by Oda et al (56) were ammonium sulfate concentrated ascites fluid; MAb 5J, 4SB3 and N1B42 were provided by Meager et al (57) as purified immunoglobulin from ascites fluid. Hybridomas of MAb 69B.J,

Table 4. Monoclonal Antibodies Evaluated

MAb Name	Immunizing Antigen	Ig Type	Antiviral Neutralization Activity+	Sample Preparation	References
B1	HuIFN-γ	IgG_1	Weak	Hybridoma	Le et al.
B3	HuIFN-γ	IgG_1	Strong	Culture Sup.	1984 (20)
5J	HuIFN-γ	*	Low	Purified Ig	Meager et al.
4SB3	HuIFN-γ	*	Low	from	1984 (57)
N1B42	rHuIFN-γ	*	Strong	Ascites Fluid	
KM48	rHuIFN-γ	IgG_1	Neutralizing	Amm. sulfate	Oda et al.
KM61	rHuIFN-γ	IgG_1	Non-neutralizing	ppt. of	1986 (56)
KM45	rHuIFN-γ	IgG_1	Non-neutralizing	Ascites Fluid	
69B.J	rHuIFN-γ	IgG_1	Neutralizing	Ascites	Pestka et al
220A12.J	rHuIFN-γ	IgG_1	Neutralizing	Fluid	1986 (54)
73A.J	rHuIFN-γ	IgG_1	Neutralizing		
113B.J	rHuIFN-γ	IgG_1	Neutralizing		
B133.1	rHuIFN-γ	IgG_1	Neutralizing	Ascites	Trincheiri
B133.3	rHuIFN-γ	IgG_1	Neutralizing	Fluid	et al. 1986 (59)
MIF3009	rHuIFN-γ	IgG_1	Neutralizing	Purified Ig	Alfa et al.
MIF3094	rHuIFN-γ	IgG_1	Neutralizing	from	1987 (63)
MIF3055	rHuIFN-γ	IgG_1	Neutralizing	Ascites Fluid	
MIF3125	rHuIFN-γ	IgG_1	Neutralizing		

*, not reported by the author; rHuIFN-γ, recombinant molecule; HuIFN-γ, natural molecule; Sup, supernatant; Ig, immunoglobulin; Amm. sulfate, ammonium sulfate; ppt., precipitation.
+, as described in the original description by the respective author.

220A12.J, 73A.J, and 113B.J were provided by Pestka et al (54) and clone purified in this laboratory, they are in the form of ascites fluid. MAb B1, B3, 5J, and 4SB3 were raised against natural HuIFN-γ. The rest of the MAbs were against recombinant HuIFN-γ. As described in the publications by their authors, the immunoglobulin type of these MAbs were IgG_1, their antiviral neutralization activity vary from weak to strong and two MAbs (KM45 and KM61) were described as non-neutralizing (Table 4).

The relative concentration of each MAbs was evaluated by a direct ELISA, in which the IFN protein was absorbed to the surface of the polystyrene plate to capture the MAbs, and the specific interaction of antigen-antibody complex was detected by enzyme linked secondary antibody (peroxidase-conjugated goat anti-mouse IgG, IgM, IgA). The addition of the enzyme substrate (ABTS) yields a colored product upon degradation by the enzyme in the conjugate. The absorbance value of the color which is related to the amount of antibody present was determined with an ELISA reader. The relative concentration of each of the MAbs was determined based on their reactivities with HuIFN-γ. One ELISA unit was defined as the amount of antibody that produced an absorbance of 0.150 A_{405} which is 3 times the background at ≤ 0.05 A_{405} in a standard ELISA. For each of MAbs, the absorbance value was plotted against the antibody dilution, and from the curve the antibody dilution that produced an absorbance of 0.15 A_{405} (one EU) was determined. Thus, the MAb concentration of a preparation is expressed as EU/ml determined by the reciprocal of the maximum dilution of the preparation to produce an absorbance of 0.150 A_{405} in the ELISA. Typical experiments are shown in Fig. 2, in which MAb 3125, 3094, B133.3 and B3 demonstrated dose-related binding to the rHuIFN-γ. All MAbs showed dose-related binding to the recombinant human IFN-γ. No binding was observed against proteins prepared from

Fig.2 Relative concentration of MAb as demonstrated by ELISA.

ELISA plate was coated with rHuIFN-γ (8.2×10^3 units/well). Serial dilutions (1:2) of (A) MAb 3125 and 3094, or (B) B133.3 and B3 were added to the IFN-coated wells. A conjugate of goat anti-mouse IgG, IgM, IgA and peroxidase was used as the second antibody. The absorbance at 405nm was plotted against the antibody dilution. The result of the assay was expressed as EU/ml which was defined as the reciprocal of the dilution of the antibody that produced an absorbance value of 0.150 (3 times background at $\leq$ 0.05).

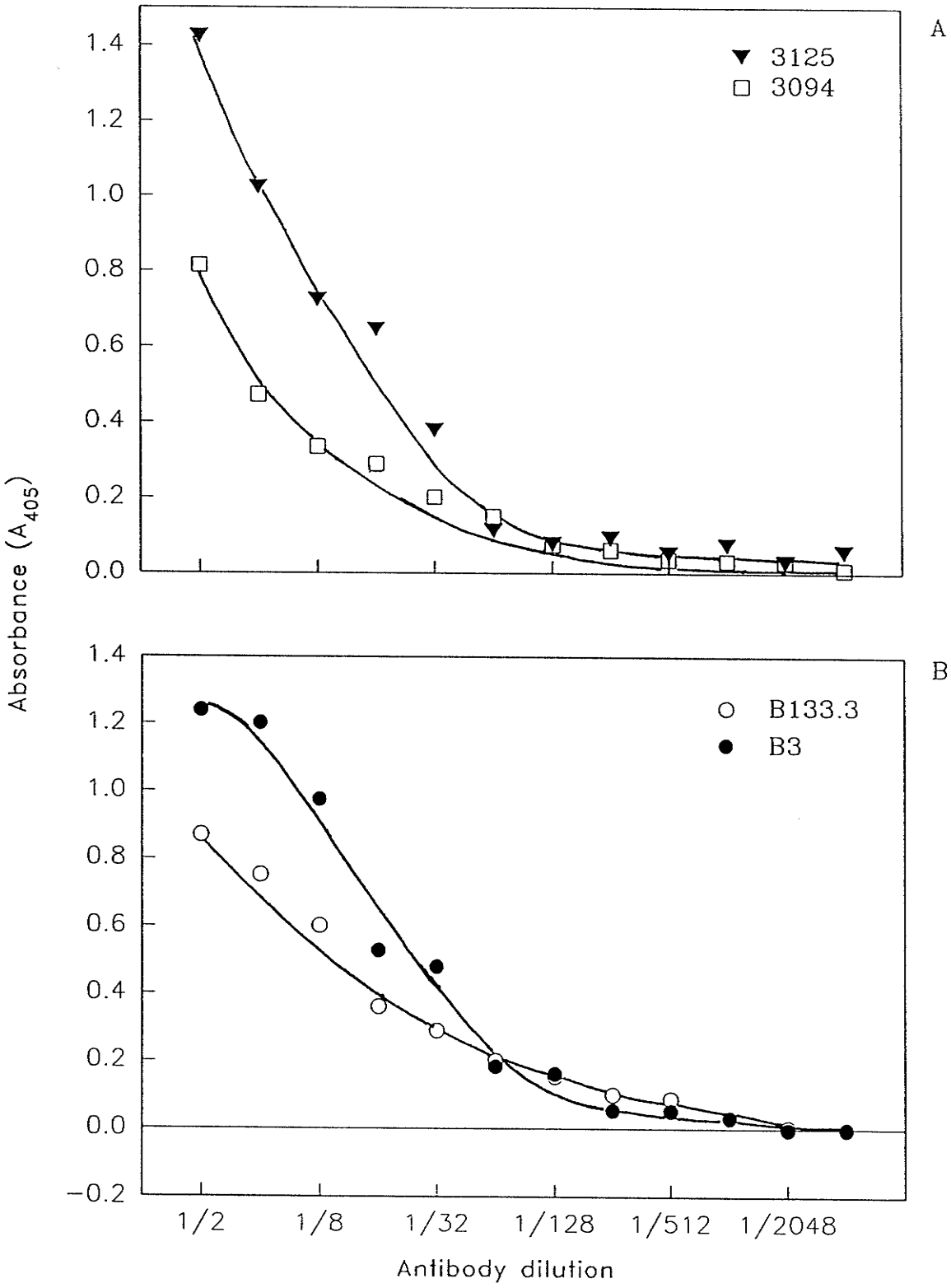

A
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
3125
3094
B
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
−0.2
B133.3
B3
1/2
1/8
1/32
1/128
1/512
1/2048
Antibody dilution
Absorbance (A_{405})

Figure 2

Fig.3 Specificity of MAb in binding ELISA

ELISA plate was coated with either $pJP_{14}R_9$-IFN (1.0 ug/well) or $pJP_{14}R_9$ (1.0 ug/well) proteins. Serial dilution of MAb 3055 then was added. A conjugate of goat anti-mouse IgG, IgM, IgA and peroxidase was used as the second antibody.

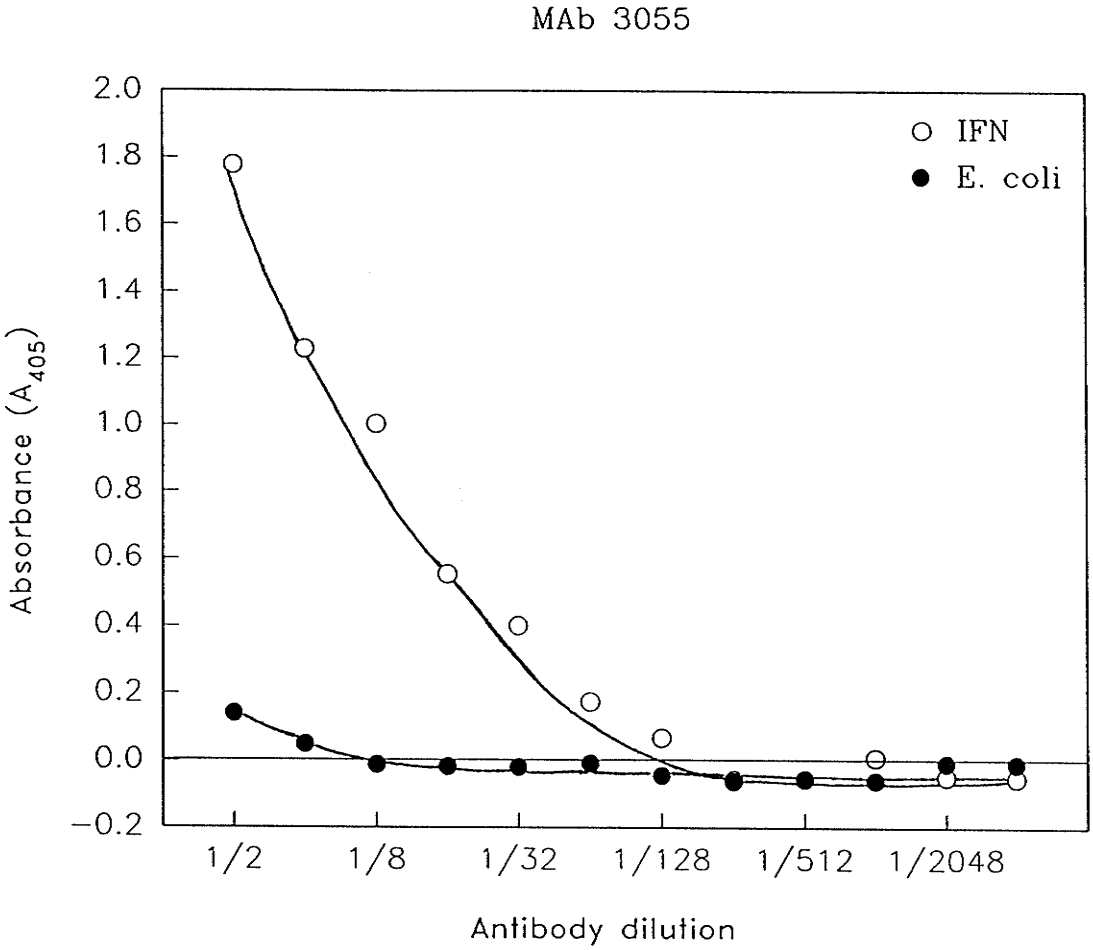

MAb 3055
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
−0.2
Absorbance (A_{405})
IFN
E. coli
1/2
1/8
1/32
1/128
1/512
1/2048
Antibody dilution

Figure 3

E. coli that had no HuIFN-γ gene by the same procedure as with the rHuIFN-γ preparation. One example is shown in Fig. 3 that MAb 3055 binds to rHuIFN-γ in a dose-related manner, but not to proteins prepared from E. coli. Similar results were observed for all MAb tested. The ralative concentration of each of the MAbs were expressed as ELISA units/ml (EU/ml) and summarized in Table 5. MAb B1 which was in the form of hybridoma culture supernatant had the lowest titer (7.46 EU/ml), MAb B133.1 had the highest titre (1.45×10^7 EU/ml) (Table 5).

The neutralizing antiviral activity of all MAbs were also studied (Table 5). The method was to add the mixtures containing a constant concentration of purified IFN-γ and various concentrations of MAbs to the antiviral activity assay and to determine the minimum antibody concentration that neutralized the antiviral activity of the IFN-γ and caused viral cytopathic effect (CPE). The purified IFN used was titrated concomitantly without antibody to determine the exact titer in reference unit/ml of IFN being reacted with antibody in each assay. The same endpoint (50% cytopathic effect) as in IFN titration without antibody was used. The average absorbance of the control cultures without IFN and challenged with EMC virus was taken as 100% cytopathic effect. The average absorbance of similar cultures containing IFN-γ was taken as 0% cytopathic effect. The neutralization of antiviral activity titre (NAV) was expressed as the reciprocal of the dilution of the MAb that could neutralize one reference unit/ml of IFN. Typically, four working units of purified IFN-γ was used in these assays. Typical experiments are shown in Fig. 4A, 4B, 4C, all MAbs but KM45 were able to neutralize the antiviral activity of rHuIFN-γ in a dose-dependent manner. Fig. 4D showed that IFN used was titrated against laboratory IFN standard in each assay. The neutralization of antiviral titre for each MAb are summarized in Table 5. The titres varied

Fig.4 Antiviral neutralization activity analysis of MAbs.

A549 cells were seeded in a 96-well tissue culture plate at a concentration of 4 x 10^4 cells/well. (A) MAb 3094 and 3125, (B) MAb KM48 and B3, or (C) KM45 and 69B.J were serially diluted 1:2 in each row. A constant amount of purified rHuIFN-γ (64 units/ml in 100 ul) was then added to each well. The plate was challenged with encephalomyocarditis virus at multiplicity of infection of 2. The average absorbances of the control cultures with the virus challenge was taken as 100% cytopathic effect (CPE), while similar cultures with input HuIFN-γ was taken as 0% CPE. The endpoint of the assay was taken as 50% CPE. The neutralizing unit was defined as the amount of MAb required to neutralize 1 unit/ml of the antiviral activity of HuIFN-γ. (D) The purified HuIFN-γ used was titrated concomitantly against a laboratory standard to determine the exact titre in unit/ml of IFN being reacted with antibody in each assay.

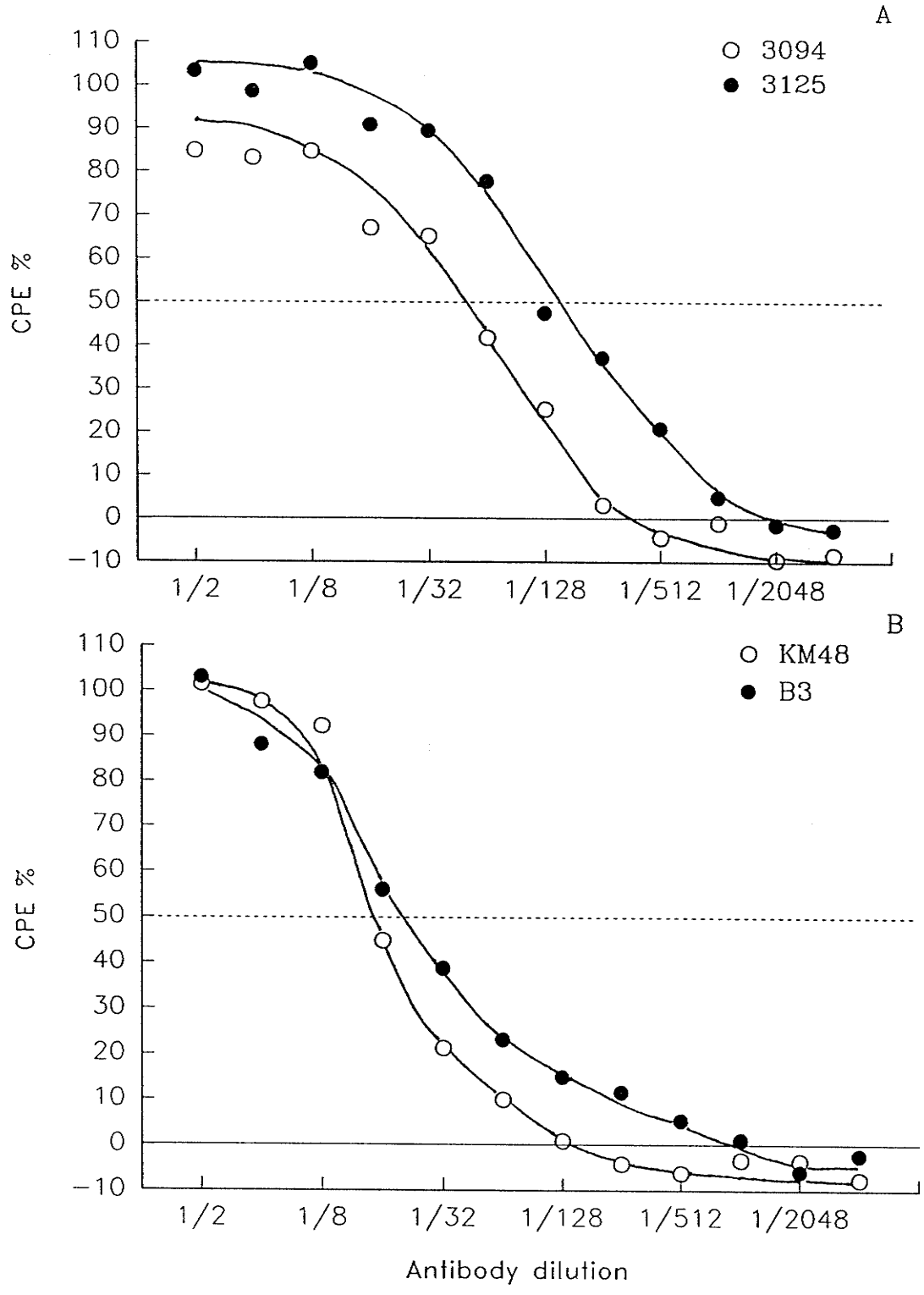

A
○ 3094
● 3125
CPE %
110
100
90
80
70
60
50
40
30
20
10
0
−10
1/2
1/8
1/32
1/128
1/512
1/2048
B
○ KM48
● B3
CPE %
Antibody dilution

Figure 4

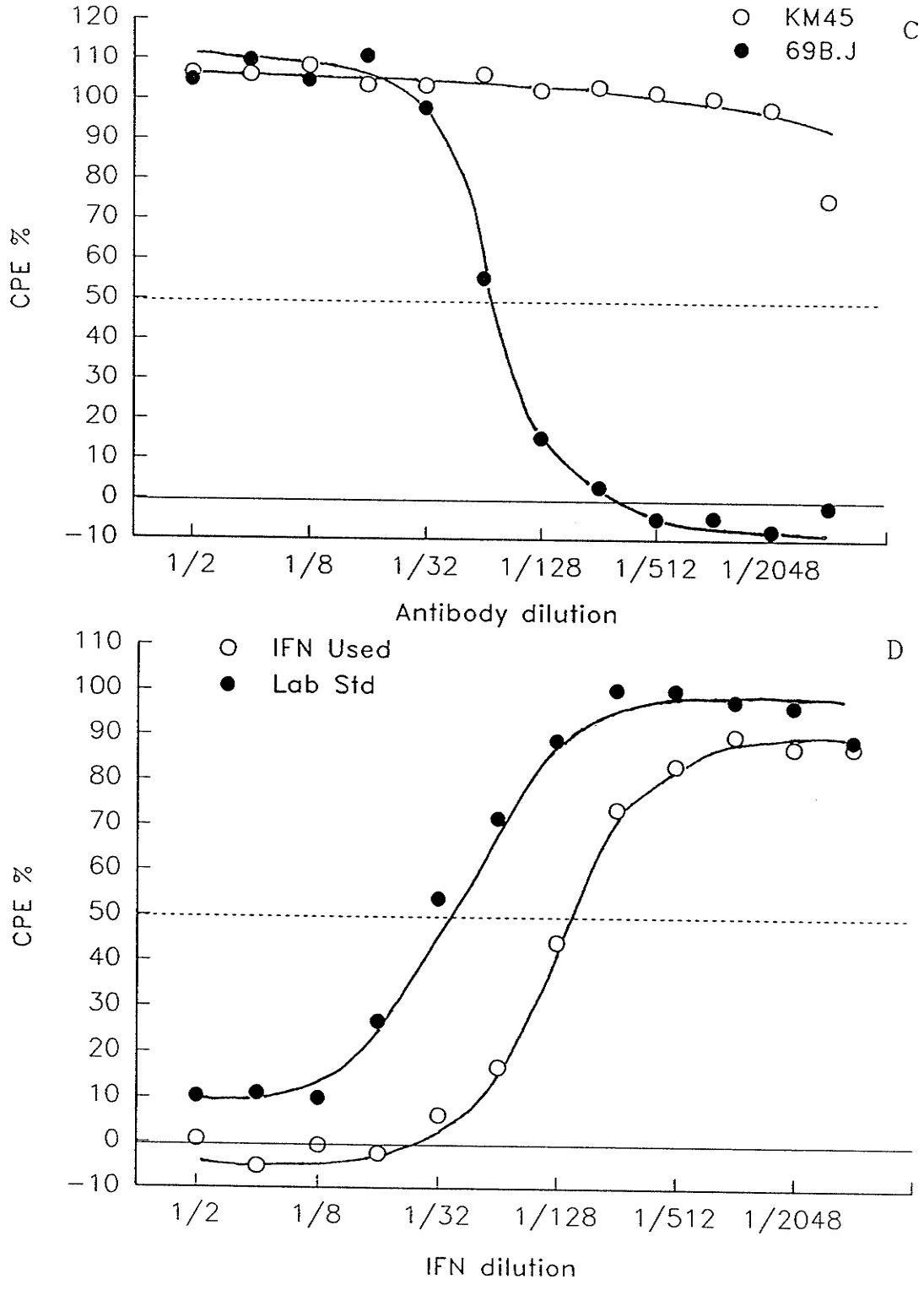

C
KM45
69B.J
CPE %
120
110
100
90
80
70
60
50
40
30
20
10
0
−10
1/2
1/8
1/32
1/128
1/512
1/2048
Antibody dilution
D
IFN Used
Lab Std
CPE %
110
100
90
80
70
60
50
40
30
20
10
0
−10
1/2
1/8
1/32
1/128
1/512
1/2048
IFN dilution

Figure 4

from 5.67 x 10^2 to 2.10 x 10^9 NAV/ml. MAB B133.1 had the highest titre, again MAb B1, in the form of hybridoma culture supernatant, had the lowest titre. The result presented in Fig. 4C showed that MAb KM45, at the highest concentration attainable (5.5 x 10^4 EU/ml), was unable to demonstrate any neutralizing activity. Taking into account that the sensitivity of the assay was 16.4 NAV/ml, any neutralizing activity must be < 1 NAV/3300EU. MAb KM45, therefore, was defined as non-neutralizing MAb.

The ratio of NAV/EU was also calculated for each MAbs (Table 5) based on the consideration that the ELISA units of a MAb reflects the binding capacity of the MAb to IFN, so that the NAV/EU ratio provides a measure of the relative neutralizing effect of the MAb upon binding to its epitope. As shown in Table 5, the ratios range from 0.2 to 144.8. MAb B133.1 has the highest relative neutralizing activity, MAb B1 had the intermediate ratio (76 NAV/EU) despite the fact that it had the lowest ELISA titre and neutralizing titre.

Previous study of a panel of 21 neutralizing MAbs has allowed the classification of the MAbs into distinct (non-crossreacting) groups E1 and E2 as well as an E1/E2 group that cross-reacts with either or both E1 and E2 (63). In order to determine if there were other functional epitopes that were not represented in that panel of 21 independently isolated neutralizing MAbs produced in this laboratory, MAbs from 5 other laboratories were analyzed in a competition analysis using a modified competition sandwich radioimmune assay (sRIA) (63). Three MAbs, MIF3009, 3055 and 3094 representing the 3 groups, E1, E2 and E1/E2, respectively, were selected as references for the present study. Additionally, a MAb, MIF3125, that was distinct from E1, but appeared to be partially related to E2, was also included for this study. Each of these four MAbs was purified and radiolabelled with ^{125}I to a specific activity of 20 mCi/mg and approximately 2 moles of ^{125}I per mole of

TABLE 5 Characterization of MAbs

MAb Name	NAV/ml	EU/ml	NAV/EU
B1	5.67×10^{2}	7.46×10^{0}	76.0
B3	4.06×10^{4}	7.01×10^{2}	57.9
KM48	1.26×10^{6}	1.71×10^{5}	7.3
KM61	2.04×10^{3}	1.02×10^{4}	0.2
KM45	$< 1.64 \times 10^{1}$	1.10×10^{5}	-
B133.1	2.10×10^{9}	1.45×10^{7}	144.8
B133.3	1.79×10^{7}	8.91×10^{6}	2.0
5J	1.30×10^{4}	2.21×10^{3}	5.9
4SB3	1.53×10^{4}	7.50×10^{4}	0.2
N1B42	2.05×10^{6}	1.42×10^{5}	14.4
69B.J	4.10×10^{6}	2.30×10^{5}	17.8
113B.J	5.54×10^{4}	3.43×10^{3}	16.1
220A12.J	4.60×10^{5}	1.21×10^{5}	3.8
73A.J	1.65×10^{7}	1.50×10^{5}	110.0
MIF3009	4.19×10^{7}	1.02×10^{6}	41.1
MIF3094	3.28×10^{4}	6.40×10^{4}	0.51
MIF3055	1.05×10^{7}	6.40×10^{5}	16.4
MIF3125	1.31×10^{7}	1.60×10^{4}	81.9

NAV: neutralization of antiviral titre
EU: ELISA units

immunoglobulin as previously described (63). Each of the unlabelled MAbs was tested in the competition assays against each of the four radiolabelled MAbs probes. The immunocomplex is represented as follows:

(ELISA plate) - (Rabbit anti-HuIFN-γ) - (HuIFN-γ) - (^{125}I-MAb or MAb)

Since the amount of HuIFN-γ is constant and limiting, the presence of an unlabelled MAb that can compete with the ^{125}I-MAb will proportionally reduce the binding of the ^{125}I-MAb to the HuIFN-γ. Typical sRIA competition assay results are shown in Fig. 5, 6, 7, 8. In Fig. 5, MIF 3055 was the radiolabelled probe. Typical sigmoidal competition curves were observed with B133.1 and 5J, and the homologous MAb MIF3055, whereas MAb 69B.J demonstrated no competition with the radiolabelled MIF3055. The amount of test MAb, expressed as ELISA units, required to inhibit the binding of ^{125}I-MAb probe (EU_{50}) by 50%, was taken as the endpoint. The relative amount of each MAbs was quantitated in terms of ELISA unit (Table 5), which is a measure of the amount of the MAb capable of binding to the HuIFN-γ in the assay. The EU_{50} provides an indication of the amount of each MAb required to inhibit the binding of 10 ng (5 x 10^4 cpm) of the respective radiolabelled probe. The results of the sRIA were corrected for non-specific binding by using a negative control that contained cell extracts of E. coli which carried the expression vector $pJP_{14}R_9$ but without the rHuIFN-γ coding sequence. The radioactivity bound in the negative control was the same as the background count for wells without the cell extract. Unlabelled homologous MAb (same MAb as the probe) was included as an internal control and the EU required to inhibit the binding of the ^{125}I-labelled probe by 50% was determined. Theoretically, an equivalent amount (10 ng) of unlabelled MAb would inhibit 50% of the binding of 10 ng of the homologous ^{125}I-MAb. This, however, may vary slightly between assays and different

Fig.5 Immuno-competition analysis of MAbs with ^{125}I-MIF3055 in a sRIA.

ELISA plates were coated with rabbit anti-rHuIFN-γ immunoglobulin to capture HuIFN-γ as described in Materials and Methods. Saturating amounts of ^{125}I-labelled MIF3055 (270,583 cpm/10ng/well) was mixed with serial 1:5 diluted unlabelled MIF3055 (○), B133.1 (●), 5J (▽) or 69B.J (▼) and allowed to compete for binding to the rHuIFN-γ on the plate. The binding of ^{125}I-labelled probe in the absence of any competing MAb (positive control) indicates the maximum binding capacity in the well for that MAb and was taken as 100%. Results were plotted as percent of the positive control. The amount of test MAb, expressed as ELISA units, required to inhibit the binding of ^{125}I-MAb probe by 50% was taken as the endpoint.

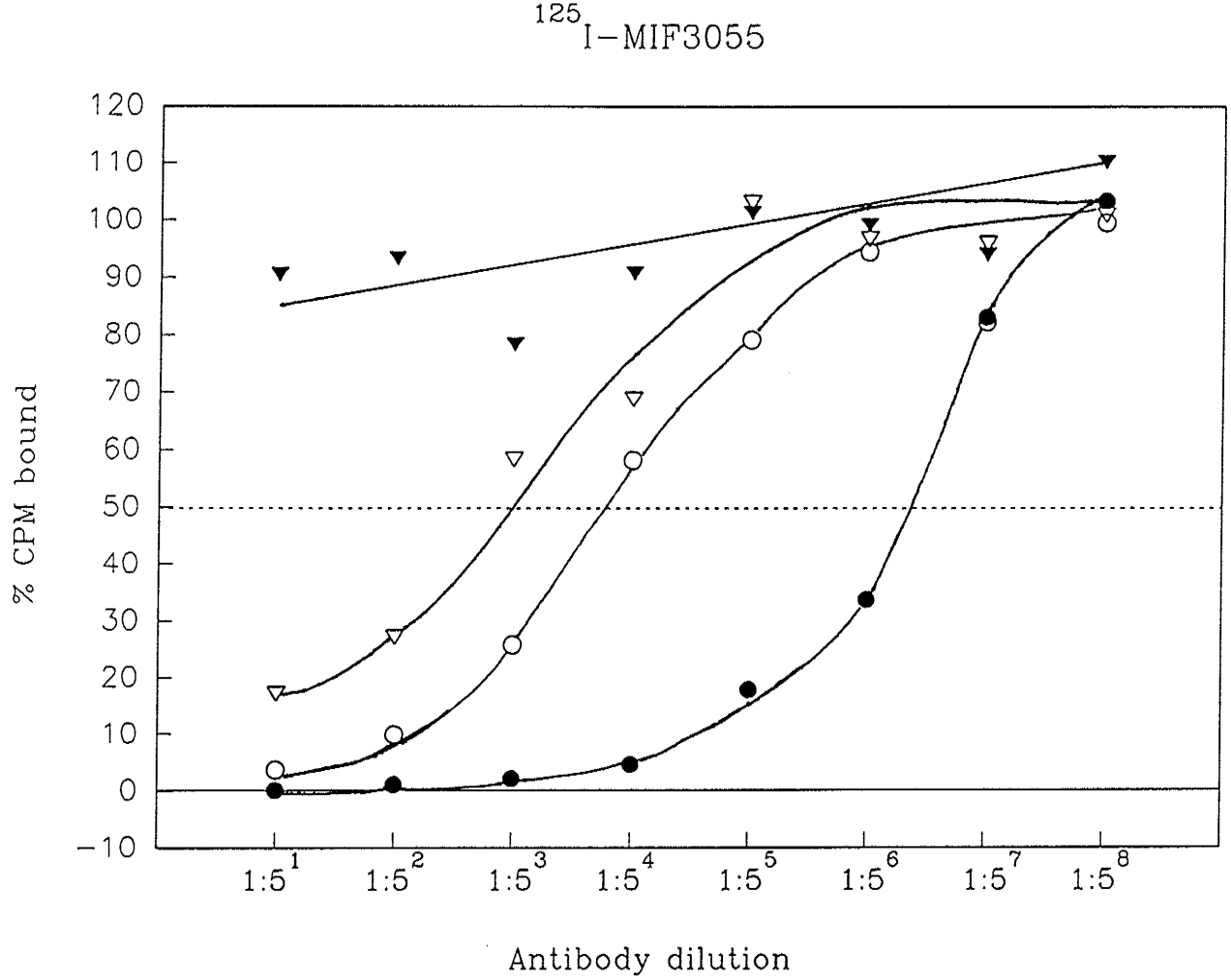

^{125}I-MIF3055
% CPM bound
120
110
100
90
80
70
60
50
40
30
20
10
0
−10
1:5^{1}
1:5^{2}
1:5^{3}
1:5^{4}
1:5^{5}
1:5^{6}
1:5^{7}
1:5^{8}
Antibody dilution

Figure 5

Fig.6 Immuno-competition analysis of MAbs with ^{125}I-MIF3009 in a sRIA.

ELISA plates were coated with rabbit anti-rHuIFN-γ immunoglobulin to capture HuIFN-γ as described in Materials and Methods. Saturating amounts of ^{125}I-labelled MIF3009 (143,867 cpm/10ng/well) was mixed with serial 1:5 diluted unlabelled MIF3009 (○), B133.3 (▽), 5J (●) or 113B.J (▼) and allowed to compete for binding to the rHuIFN-γ on the plate.

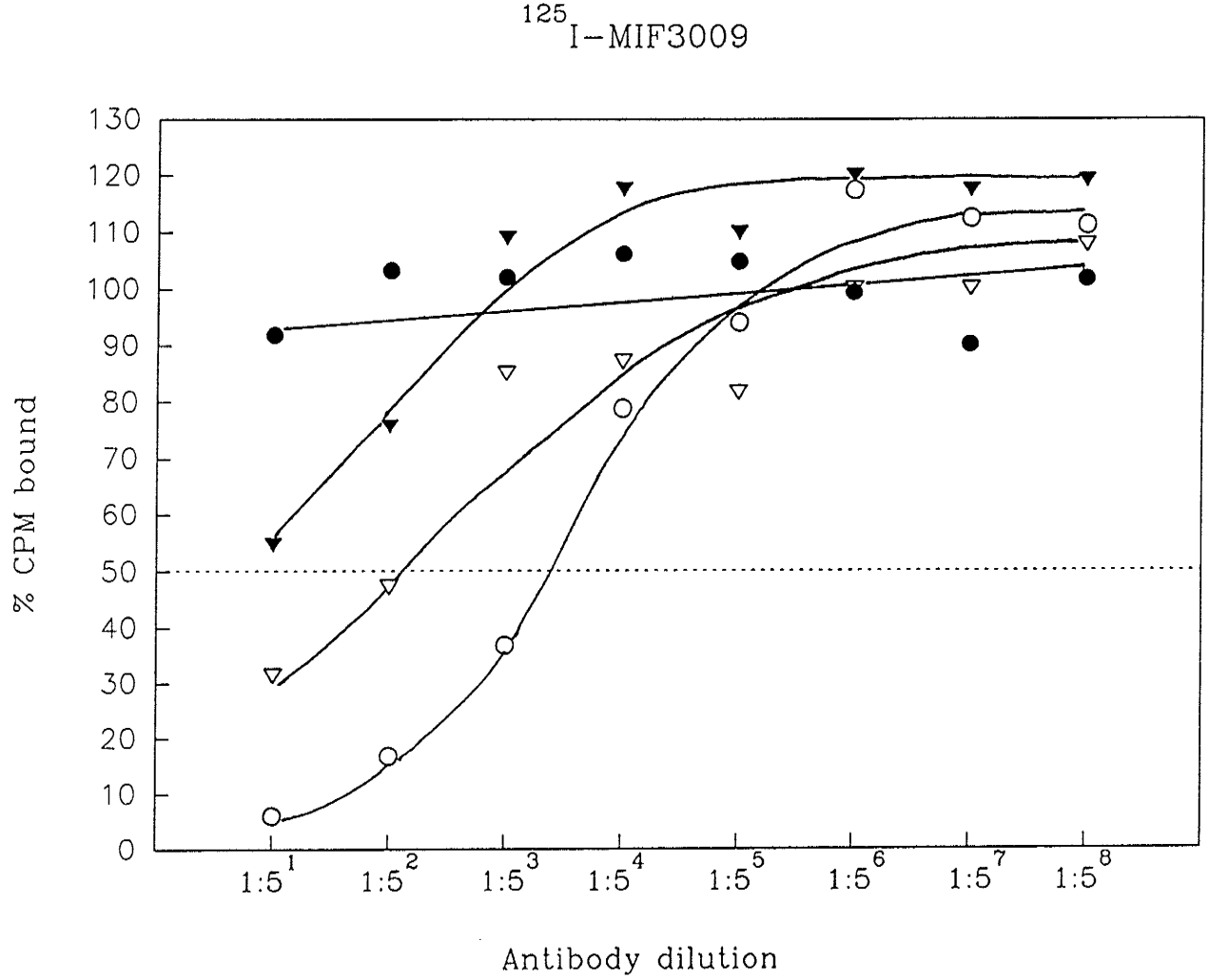

^{125}I-MIF3009
% CPM bound
130
120
110
100
90
80
70
60
50
40
30
20
10
0
1:5^{1}
1:5^{2}
1:5^{3}
1:5^{4}
1:5^{5}
1:5^{6}
1:5^{7}
1:5^{8}
Antibody dilution

Figure 6

Fig.7 Immuno-competition analysis of MAbs with ^{125}I-MIF3094 in a sRIA.

ELISA plates were coated with rabbit anti-rHuIFN-γ immunoglobulin to capture HuIFN-γ as described in Materials and Methods. Saturating amounts of ^{125}I-labelled MIF3094 (102,081 cpm/10ng/well) was mixed with serial 1:5 diluted unlabelled MIF3094 (○), B133.3 (▽), KM45 (●) or 73A.J (▼) and allowed to compete for binding to the rHuIFN-γ on the plate.

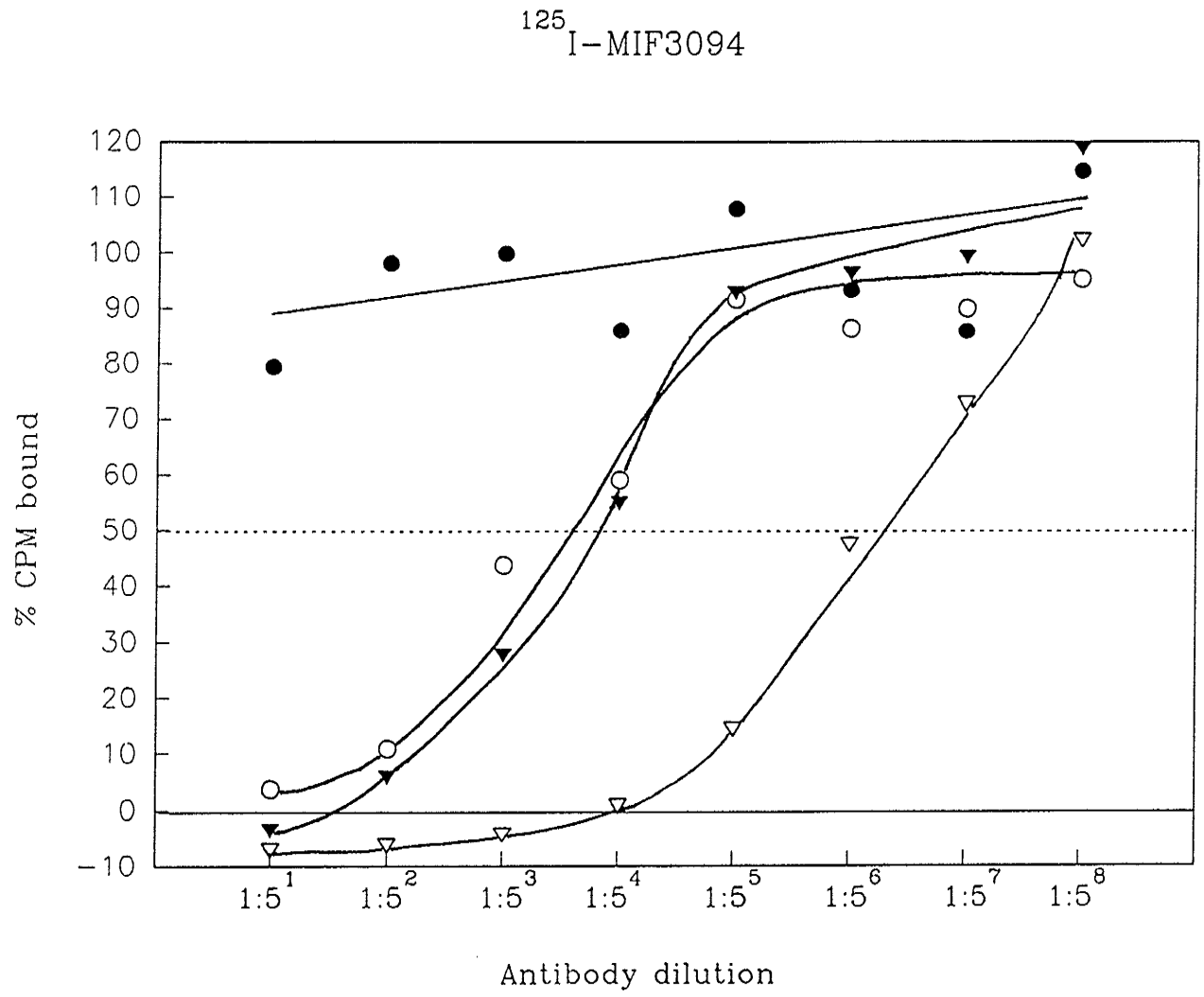

^{125}I-MIF3094
% CPM bound
120
110
100
90
80
70
60
50
40
30
20
10
0
−10
1:5^{1}
1:5^{2}
1:5^{3}
1:5^{4}
1:5^{5}
1:5^{6}
1:5^{7}
1:5^{8}
Antibody dilution

Figure 7

Fig.8 Immuno-competition analysis of MAbs with ^{125}I-MIF3125 in a sRIA.

ELISA plates were coated with rabbit anti-rHuIFN-γ immunoglobulin to capture HuIFN-γ as described in Materials and Methods. Saturating amounts of ^{125}I-labelled MIF3125 (132,119 cpm/10ng/well) was mixed with serial 1:5 diluted unlabelled MIF3125 (○), B133.3 (▽), 4SB3 (▼) or N1B42 (●) and allowed to compete for binding to the rHuIFN-γ on the plate.

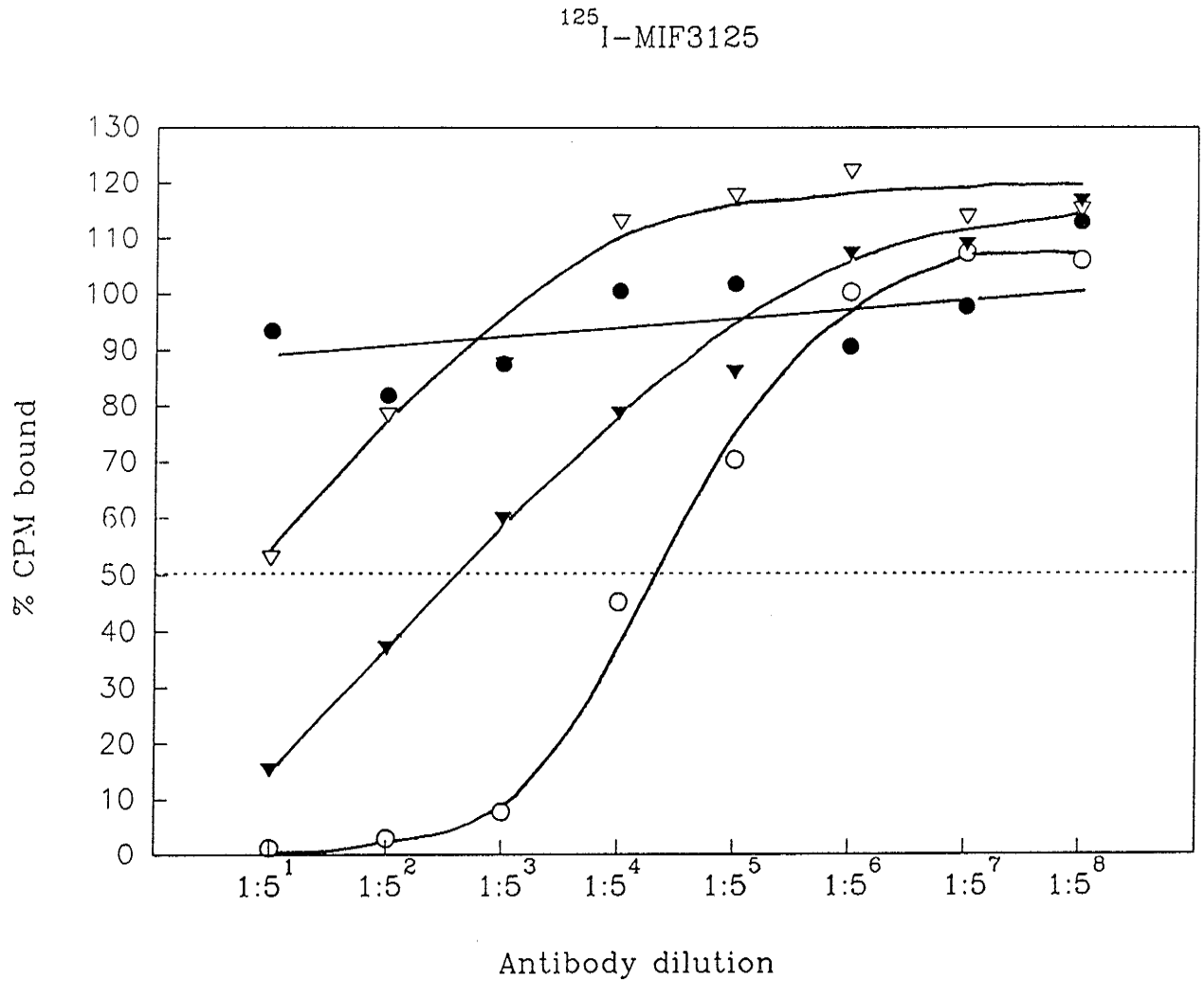

^{125}I-MIF3125
% CPM bound
130
120
110
100
90
80
70
60
50
40
30
20
10
0
1:5^{1}
1:5^{2}
1:5^{3}
1:5^{4}
1:5^{5}
1:5^{6}
1:5^{7}
1:5^{8}
Antibody dilution

Figure 8

preparations. Therefore, to control for this variability and to allow comparison of the binding efficiency of different MAbs, the results were normalized to the 50% inhibition concentration of the unlabelled homologous MAb which served as the internal control for each preparation of ^{125}I-MAb. The result was expressed as a ratio (ER) which thus indicates the ability of each heterologous MAb to compete or interfere with the binding of ^{125}I-MAb to HuIFN-γ relative to the competition produced by the unlabelled internal control MAb. The results of these competition assays are presented in Table 6 as ratios of: ER = (EU_{50} of Test)/(EU_{50} of Homologous Control). Thus, to achieve 50% inhibition of the ^{125}I-MIF3009 required 62.3 EU of MAb N1B42, 0.561 EU of 69B.J and 40.11 EU of the homologous MIF3009, yielding the ER ratios of 1.553, 0.014 respectively, when the ER ratio of the homologous MIF3009 was taken as 1.00.

All homologous internal controls have an ER of 1.00. MAbs that bind the epitope better than the probe will have an ER $<$ 1.00, whereas, those that bind weaker than the probe will have an ER $>$ 1.00. For example, B133.3 binds to HuIFN-γ (epitope E1/E2) approximately equal to MIF3094 since the ER is 1.093, whereas it binds much weaker than MIF3009 (E1) because the ER is 51.231. Where a 100-fold excess of a MAb was unable to inhibit the binding of the probe (ER $>$ 100), the MAb was considered non-competitive with the ^{125}I-MAb probe and therefore did not bind to the same epitope. This is shown as a "-" in Table 6 to enhance comprehension of the table. The ER of each of the 14 MAbs contributed by other laboratories forms a cross-reactivity pattern against the 4 radiolabelled probes. Each of the 13 neutralizing MAbs is, thus, classified within the 4 immune reactivity (epitope) groups as defined by MIF3009, MIF3094, MIF3055 and MIF3125. The non-neutralizing MAb KM45 did not compete with any of the probes (Table 6).

Table 6. Epitope Assignment of Anti-HuIFN-γ MAb

EPITOPE	E1	E1/E2	E2	E2'
MAb Name	3009	3094	3055	3125
69B.J	0.014	0.001	-	-
113B.J	0.153	0.011	-	-
220A12.J	0.292	0.010	-	-
73A.J	0.483	0.634	-	-
3009	1.000	0.050	-	-
N1B42	1.553	0.037	3.160	-
B133.3	51.231	1.093	19.239	-
3094	-	1.000	-	-
B1	-	0.012	-	-
3055	-	0.610	1.000	34.901
B133.1	-	0.468	5.962	5.574
KM48	-	0.339	1.317	2.888
3125	-	47.500	0.201	1.000
5J	-	-	0.275	0.301
B3	-	-	0.686	0.473
4SB3	-	-	1.230	0.351
KM61	-	-	38.152	-
KM45	-	-	-	-

DISCUSSION

IFN-γ is produced along with other lymphokines by mitogen or antigen stimulated lymphocytes. IFN-γ displays multiple biological properties such as antiviral activity, cell growth inhibition and immunoregulatory functions. The HuIFN-γ exerts its effects through the interaction with a specific cell surface receptor which is different from that of the IFN-α and IFN-β. To date, the mechanism(s) of action(s) of HuIFN-γ on susceptible cells remains unclear and relatively little is known about the structure and function relationships of this pleiotropic cytokine. When little is known about the structure of a molecule, functional analysis with neutralizing MAb is often a method of choice to initiate a study to identify the functional domains of a macromolecule and to understand its structure-function relationships. With the analysis with neutralizing MAbs, important information can be generated without knowing the location and sequence of the epitope and this information would be useful for further, finer but more laborious approaches such as the epitope mapping and the production of variant molecules, by site-specific mutagenesis.

The availability of monoclonal antibodies with different specificities should be useful for the investigation of structure-function relationship of HuIFN-γ. While anti-HuIFN-γ neutralizing MAb is known to be difficult to develop, a number of laboratories had been successful in the past ten years (20, 51-55, 58-59). Success, however, had been limited; not only that the functional epitopes have not been mapped, it was not even clear how many functional epitopes exists. While studies from this laboratory as well as others indicated that there are more than one separate functional epitopes on the HuIFN-γ, the epitope specificities of MAbs developed by other laboratory were unknown. Therefore, there was no basis to

compare and correlate findings from different laboratories. An epitope classification scheme to type each MAb is, therefore, essential to appreciate findings from different laboratories.

Previously, in this laboratory, twenty-one neutralizing MAbs derived from independent splenocyte-myeloma cell fusion events were developed (62) and, based on their immune cross-reactivities, 2 distinct antiviral neutralizing epitopes (E1 and E2) were identified (63). It was 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 MAbs developed by Alfa and Jay (63) in this laboratory.

The objectives of this study are: (i) to attempt to determine the number of different functional epitopes recognized by the different MAbs by some of the major laboratories in the field, and (ii) to attempt to classify these MAbs based on a epitope classification scheme that could correlate the findings from these laboratories.

In the present study, fourteen MAbs from five different investigators were obtained and their antiviral neutralization activities were analyzed. Initial characterization of the 14 MAbs from different investigators in the present study confirmed the characteristics of each of these MAbs as previously reported by the original authors (Table 4 and 5). Each of these 14 MAbs was tested in the competition assays against each of the four representative MAbs from this laboratory.

The amount of MAb in the samples contributed by different laboratories ranged from 7.5 EU/ml up to 1.5 x 10^7 EU/ml. The quantity of immunoglobulins in many of these precluded their purification for ^{125}I-labelling as probes and their quantitation by weight. 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 per

ELISA unit) ratio is therefore, 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 (63). Thus, the result reflects the functional involvement of that epitope and is less affected by the differences in the binding capacity (affinity and avidity) of the individual MAb. The latter is true if the neutralizing activity is expressed as NAV/ng. The larger the NAV/EU ratio, the more effective is the neutralizing ability. For example, although 73A.J and B133.3 have similar NAV/ml titres (1.65×10^7 and 1.79×10^7), MAb 73A.J, at 110 NAV/EU, is substantially more effective at neutralizing the antiviral effect than B133.3 at 2.0 NAV/EU (Table 5).

Le et al (20) used natural HuIFN-γ as the immunogen to produce MAb B1 and B3 and described B1 as a weakly neutralizing MAb compared to B3. However, the relative neutralizing capacity was found to be similar (76 and 57.9 NAV/EU), demonstrating the need for a relative quantitative measure that is less affected by the binding affinity of the MAb. Oda et al (56) raised MAb KM45, KM48 and KM61 against rHuIFN-γ and 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 almost 40-fold less than KM48 (7.3 NAV/EU) (Table 5). The difference in the sensitivity of the assay may account for Oda describing KM61 as non-neutralizing. Meager et al (57) used natural HuIFN-γ as the immunogen to produce MAb 5J and 4SB3 and reported that these MAbs had low neutralizing activity against HuIFN-γ whereas MAb N1B42, which was produced using rHuIFN-γ as the immunogen, had good neutralizing ability against rHuIFN-γ. The present study generally confirmed the relative

neutralizing activity among these MAbs as previously reported: 5J and 4SB3 were 5.9 NAV/EU respectively, whereas N1B42 was 14.4 NAV/EU. Although MAb B133.3 and B133.3 provided by Ziai et al (59) had high neutralizing titres (2.10×10^9 and 1.79×10^7 NAV/ml) which is generally consistent with the results from the original author, their relative neutralizing capacity were found to have more than 70 fold difference: B133.1 had the highest relative neutralizing activity (144.8 NAV/EU) among all the MAbs tested, B133.3 had low relative neutralizing activity (2.0 NAV/EU). Four MAbs provided by Pestka et al (54) subcloned in this laboratory showed differential neutralizing activity: 73A.J had high ratio (110.0 NAV/EU), 69B.J and 113B.J had intermediate ratio (17.8 and 16.1 NAV/EU respectively), 220A12.J had low ratio (3.8 NAV/EU).

Except MAb KM45, all the MAbs in this study neutralized the antiviral activity of rHuIFN-γ despite the fact that some of the MAbs were raised against the natural HuIFN-γ and that all of the MAbs raised against the rHuIFN-γ in this laboratory can neutralize the natural HuIFN-γ(NIH, Gg23-901-530), indicating that the functional structure of rHuIFN-γ is similar to that of natural IFN-γ at these functional epitopes.

In the competition sRIA, each of four MAbs from this laboratory was purified and radiolabelled with ^{125}I to a specific activity of about 20 mCi/mg and approximately 2 moles of ^{125}I per mole of immunoglobulin as previously described (63). Previous analysis indicated that the binding capacity of the ^{125}I-labelled MAb was not substantially affected by the iodination process (63). Equivalent molar amounts of labelled and unlabelled MAb were compared in a sELISA. The kinetics of binding of ^{125}I-MIF3009 compared to unlabelled MIF3009 were virtually identical. Similar results were observed for all MAbs tested. Therefore, at this moderate level of iodination, the labelling process did not significantly alter

the Ag-binding activities of the MAb. Antiviral neutralization assays also showed that the ^{125}I-labelled MAb retained their biologic activities (63) and were appropriate for this study. The optimal reaction conditions for the sRIA reaction was also determined in a previous study (63). The binding kinetics of ^{125}I-labelled MAb were monitored over a 24-h period. The binding kinetics of ^{125}I-labelled MIF3098 was typical of all the MAbs in the panel. The initial rate of binding of MIF3098 at 37^{0}C was approximately twice of that at 4^{0}C. Maximum binding of MIF3098 was achieved in 5 h at the higher temperature that was less than half the time required at the lower temperature, but the maximum level of binding was significantly lower than that attained at 4^{0}C. Based on these results and because the HuIFN-γ has a $t_{1/2}$ of more than 4 week at 4^{0}C but less than 24 h at 37^{0}C (M. J. Alfa and F. T. Jay, unpublished data), all sRIAs in this study were performed at 4^{0}C. The results of these experiments and others (63) indicated that 10 ng of MAb was sufficient to achieve saturation of the available rHuIFN-γ epitopes in the assay. The reaction time for all of the sRIA competition assays was 16 h to ensure adequate time to establish binding equilibrium.

Based on the competition sRIA results between MAbs, the competition curves derived could be grouped into three types: (i) almost straight level line type (MAb 69B.J in Fig. 5 and 5J in Fig. 6) which never produced the 50% CPM end-point, demonstrating that these MAb did not compete with the radiolabelled probe, (ii) typical sigmoidal curve (MIF3009 in Fig. 6 and B133.3 in Fig. 7) which crossed the 50% CPM end-point, (iii) curve which demonstrated downward trend but only crossed the 50% CPM end-point upon extrapolation (MAb 113B.J in Fig. 6 and B133.3 in Fig. 8). The EU required to inhibit the binding of the ^{125}I-labelled probe by 50% was determined and was compared to the homologous MAb control. Thus, the competitive binding ratios (ER) was calculated relative to the unlabelled

reference MAb (homologous to the labelled probe). This removes any minor effects which may have been caused by the labelling process or any variation between different preparations of iodinated probe. When the ER ratio (EU_{50} of test/EU_{50} of control) was > 100, the MAb was considered non-competitive with the ^{125}I-MAb probe. With the number of MAbs we obtained, we have found that 100 fold excess is an acceptable limit which is a balance between practicality in the experimentation and experimental confidence for the determination of functional relatedness of the respective epitopes.

Based on their competitive binding pattern, all of the MAbs, except KM45, could be grouped into 3 immuno-competitive groups, E1, E2 and E1/E2 (Table 6). The fact that KM45 did not compete with MAbs of any of the 3 groups is consistent with the observation that this MAb had no detectable neutralization activity.

All four MAbs, 69B.J, 220A12.J, 73A.J, 113B.J produced by Pestka et al (54) and subcloned or clone purified, in this laboratory have activities consistent with those of the E1 group which also competed with MIF3094 of the E1/E2 group, but exhibited no competition with MIF3055 of the E2 group. This is consistent with the MAbs of the E1 group described by Alfa and Jay (63). The MAbs N1B42 and B133.3, which are classified as reacting with the overlap region, competed with MAbs of both E1 and E2 groups (Table 6). This is similar to MAbs MIF3102, MIF3094 and MIF3059 previously described by Alfa and Jay (63). MAb B1, on the other hand, interacts only with MIF3094 and not with MIF3009 and MIF3055. This reflects a reactivity pattern similar to a previously described MIF3094 that competed with only a limited number of MAbs from E1 and E2 groups (63). MAb 133.1, KM48, B3, 5J and 4SB3 were assigned to the E2 group and two of them showed interaction with the overlap region (MIF3094). This observation is similar to MIF3054 or MIF3052

(63). KM61 also competed for E2 but not E1/E2 and this competition was very weak, requiring 38 fold excess to produce 50% inhibition of MIF3055 (Table 6). Together with the fact that its NAV/EU titre was very low (0.2), it is likely that KM61 is recoganizing the epitope E2 not as close to a functional domain as other E2 MAbs.

Le et al (20) demonstrated that MAb B1 and B3 recognize different epitopes from their observation that B1 does not compete with B3 binding to IFN-γ molecules. Results from the present study is consistent with that observation: B1 only competed with MIF3094 of the E1/E2 group and B3 only competed with MIF3055 of the E2 group (Table 6).

The present study confirmed that MIF3125 has no cross-reactivity with MIF3009 of the E1 group and only weak cross-reactivity with MIF3094 of the E1/E2 group (Table 6). In a previous study (63), because the MAbs of the E2 group competed with MIF3125 only very weakly (up to 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 MAbs 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; the effect of any differences in affinity, therefore, is minimized. The competition pattern of different MAbs with MIF3125 (E2') was not significantly different from that of MIF3055 (E2). On the basis of this result, it must be concluded that epitope E2' is not significantly distinct from E2 and that it should be included in the E2 group.

The fact that MAb N1B42, B133.3 can compete to varying extents with MAbs of both E1 and E2 groups (Table 6), suggests that these MAb may bind to the region (E1/E2) that overlap E1 and E2. Since there are no evidence to prove that E1/E2 is a separate

epitope, we must conclude that E1/E2 may not be a separate epitope.

Thus, there are only three epitope specificity groups: E1, E1/E2 and E2, among the anti-HuIFN-γ neutralizing MAbs surveyed. No additional immuno-competitive groups were identified amongst the neutralizing MAbs developed by these five other laboratories.

Functional neutralization by specific antibody binding can, on rare occasion, result from conformational changes at a functional domain caused by the antibody binding to a distant epitope domain, rather than direct steric hinderance of an active site at or adjacent to the MAb binding site (epitope). However, it should be pointed out that it is highly unlikely that the neutralization activity of any of the three groups is due to such "rare" incidence of MAb induced conformational changes, because there are large numbers of different MAbs isolated in this as well as others laboratories which fall into distinct reactivity groups. Steric hinderance at the functional site is more likely to be the mechanism for the neutralization. The epitopes E1 and E2, therefore, must either be the functional site(s), or very close to the functional domain(s), that is necessary for the induction of the antiviral activity.

Using somatic cell hybrids Rashidbaigi et al (39) has mapped the HuIFN-γ receptor to chromosome 6. However, it was found that in hamster-human hybrids that the expression of chromosome 6 alone was not sufficient to allow a heterologous cellular response to the HuIFN-γ. Complete functional response to HuIFN-γ required additional coding sequences from chromosome 21 (43). Similar results were found with mouse-human somatic cell hybrids (43). This suggested that the antiviral effector function may require more than the binding of HuIFN-γ to the receptor for signal transduction. It was also demonstrated that heterologous mouse cells expressing HuIFN-γ from transfected HuIFN-γ gene can develop an anti-viral state without exogenous IFN (96). The fact that endogenous HuIFN-γ can

bypass the receptor to induce the antiviral function suggested that chromosome 6 and 21 are not required for the signal transduction pathway beyond the cell surface membrane. It is likely that the function of receptor is for affinity binding and together with gene product(s) from chromosome 21 effects the internalization IFN-γ. Once it is internalized, no species specific gene product(s) is required in the remaining pathway for signal transduction or IFN-specific gene activations.

Previous data in this laboratory indicated that none of the 21 neutralizing MAbs by themselves can inhibit HuIFN-γ binding to its receptor (63). MAb B133.1 and B133.3 which are classified as E2 and E1/E2 in the present study were reported by Ziai et al (59) to be also unable to prevent binding of the HuIFN-γ to the eukaryotic receptor. None of the neutralizing MAbs thus far identified were able to inhibit attachment of the HuIFN-γ molecule to the cell surface. This suggested that the neutralizing epitopes defined in Table 6 were not essential for receptor binding. To date, there has been only one high affinity receptor for the HuIFN-γ identified (33). We hypothesize that the receptor binding function and the signal transduction functions are separate on the HuIFN-γ molecule and that the epitopes E1 and E2, separately or jointly, form the effector structure on the HuIFN-γ molecule that is essential for signal transduction either across the membrane or by other intracellular signalling mechanisms that ultimately effects the appropriate switch in gene expression that establishes the antiviral state.

In conclusion, the classification of the anti-HuIFN-γ neutralizing MAb according to the epitope specificity provides a means to correlate results from different investigators working with different neutralizing MAbs. The classification scheme proposed here serves this purpose.

Chapter III

DEVELOPMENT OF A RECOMBINANT EPITOPE LIBRARY FOR THE LOCALIZATION OF FUNCTIONAL DOMAIN ON THE HuIFN-γ

INTRODUCTION

Antigenic determinants, or epitopes, are the specific segments of antigens that are recognized by antibodies or T cells. The fact that antibodies elicited by a native protein often do not react with the denatured form (97-98) and that specific antibodies can be raised against peptides of undefined conformation (98-103) led to the definition of two classes of antigenic determinants. A segmental site (continuous or linear epitope) occurs within a continuous segment of the polypeptide. An assembled topographic site (conformational or discontinuous epitope) consists of amino acid residues located far apart in the primary sequence but brought together in the surface topography of the native protein through folding. However, it has become clear that even segmental sites bind with highest affinity in a preferred conformation (104).

The interaction of antibodies with their respective antigens has been the subject of extensive studies. However, even where the primary and tertiary structure of a protein antigen is known, we rarely know the full identity and conformation of its antigenic sites. Various approaches have been used to determine the precise location and/or amino acid sequence of protein epitopes. Two major approaches that have been widely employed for epitope characterization are competitive binding analysis using synthetic peptides and fine specificity studies with panels of evolutionary variants. Although well established, these methods have major limitations. The concept of using synthetic peptides to probe the antigenicity of a protein was first developed over 10 years ago (105). Antibodies that have stringent requirement for native conformation on the antigen, however, may not bind to the synthetic peptide. Many conformational epitopes, therefore, may not be detected by this

method. Fine-specificity studies are done with panels of evolutionary variant proteins, this approach has enabled immunodominant residues in globular proteins to be identified whose epitopes are conformationally dependent, such as in lysozyme, myoglobin, and cytochrome c (106). However, only one or two evolutionarily variant residues involved in antibody binding can be identified by this method. While numerous studies have indicated that most MAbs are commonly directed against conformational epitopes, the development of an approach that could detect continuous as well as conformational epitopes becomes challenging.

A novel approach of epitope mapping has been recently introduced which seems promising in this respect:

Limited proteolytic cleavage of immune complexes has been used for epitope characterization, based on the finding that (i) MAbs exhibit remarkable resistance towards proteolytic enzymes, (ii) in immune complexes antigenic determinants can be protected from proteolytic degradation, and (iii) proteolysis does not lead to dissociation of immune complexes (107-110). Therefore, by comparing peptides released from antigen-antibody complexes after proteolysis to peptides released from unbound antigen and antibody, the parts of the antigen in contact with the antibody could be deduced.

By means of this approach, using reverse-phase high-performance liquid chromatography (rHPLC) followed by amino acid analysis, two distinct epitopes, both conformationally well-ordered, were characterized on horse cytochrome c (110). Later the study by Sheshberadaran and Payne (111) described the use of limited proteolysis of MAb bound antigens in the analysis of the two measles virus surface glycoproteins by means of PAGE (protein "footprinting"). Most recently, a further modified approach was reported by

Suckau et al (112) in which after proteolysis, epitope peptides were dissociated from the antibody, the antigenic peptides were purified by HPLC and identified by ^{252}Cf plasma desorption mass spectrometry. Although this approach presented the advantage in terms of sensitivity and accuracy on defining epitope structures, the method is complex and requires expensive and specialized equipment that is not easily applied by other investigators.

From previous study (63), it is interesting to note that in our panel of 21 neutralizing MAbs, all five western blot positive MAbs belonged to E1 epitope group, none of MAb from E2 group are blot positive, suggesting that E2 epitope may be a comformational epitope.

The objective of this project was to attempt to develop a general method to map the segmental epitopes as well as the conformational epitopes of the HuIFN-γ.

In this approach, a recombinant DNA strategy has been used to survey the HuIFN-γ sequence for domains that encode specific protein epitopes recognized by neutralizing MAbs. The strategy involves construction of a library containing random deletions from N-terminus of HuIFN-γ gene, the expression of epitope coding sequences by recombinant bacteriophage is detected with neutralizing MAb, the specific DNA clones are isolated and characterized with restriction enzymes.

MATERIALS AND METHODS

1. Isolation of plasmid DNA

The construction and cloning of the HuIFN-γ coding sequence (91) and its efficient expression with plasmid, pJP_1R_3-IFNγ-171, in E. coli (92) have previously been described. Direct amino acid sequencing has shown that the mature natural HuIFN-γ (43) lacks the tripeptide Cys-Tyr-Cys, on the NH_2 terminus of the previously postulated amino acid sequence of the HuIFN-γ as derived from DNA sequencing (21,23). Therefore, the nona-nucleotide sequence coding for these results was deleted from the previously constructed/cloned coding sequence of pJP_1R_3-IFNγ-171 plasmid (92), and the coding sequence was expressed in a related plasmid vector as $pJP_{14}R_3$-IFN. The $pJP_{14}R_3$-IF_3 plasmid DNA used in this study was a gift from Ernest Jay, which is a modified form of $pJP_{14}R_3$-IFN.

1.1 Large scale preparation

This protocol is a modification of the procedure described by Maniatis et al (113).

E. coli strain LE392 carrying the plasmid $P_{14}R_3IF_3$ was grown at 37^0C overnight in 40 ml of TB broth containing 20 μg/ml tetracycline. The cells were harvested by centrifugation at 5,000 x g for 10 min. The cell pellet was resuspended in 2.7 ml of ice cold solution containing 25 mM Tris-HCl pH 8.0, 10 mM EDTA, 50 mM glucose, 4 mg/ml lysozyme and incubated at room temperature for 10 min. A 5.4 ml of 1% SDS in 0.2 N NaOH was added to lyse the cells and the tube was inverted rapidly three times and then placed on ice for 5 min. Four ml of ice cold 5 M potassium acetate (pH 4.8) was then added

to precipitate the protein, chromosomal DNA and RNA, the tube was inverted gently 10 times and then placed on ice for 5 min. The precipitated molecules were sedimented by centrifugation at 10,000 x g for 45 min. The supernatant was successively extracted with an equal volume mixture of buffer-equilibrated phenol and chloroform (1:1; v/v), mixture of chloroform and isoamyl alcohol (24:1; v/v) 2 to 3 times. The DNA was precipitated with 0.3 M NaCl and 2.5 volume of cold ethanol, incubated at -70^{0}C for 1 hr. The DNA was collected by centrifugation at 10,000 x g for 15 min and the pellet was washed with 70% cold ethanol. The dried pellet was resuspended in 1.3 ml of 20 μg/ml RNase A in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), incubated at 37^{0}C for 45 min. The DNA was successively extracted with an equal volume of 1:1 (v/v) mixture of buffer-equilibrated phenol and chloroform, 24:1 (v/v) mixture of chloroform and isoamyl alcohol once. The DNA was precipitated with 0.3 M NaCl and 2.5 volume of cold ethanol, incubated at -70^{0}C for 1 hr. The DNA was collected by centrifugation at 10,000 x g for 15 min and the pellet was washed with 70% cold ethanol. The dried pellet was resuspended in 3 ml of TE buffer.

Three gram of CsCl and 0.315 ml of 10 mg/ml of ethidium bromide were added and mixed. The mixture was transferred to a 13 x 51 mm polyallomer Quick-Seal (Beckman) ultracentrifuge tube and carefully balanced. The tube was sealed with Beckman tube sealer and centrifuged in Beckman L8-70M ultracentrifuge using a Beckman VTi65 rotor at 45,000 rpm at 20^{0}C for 10 hrs. The tubes were removed and the bands were visualized by a short wave ultraviolet (UV) light. The lower plasmid band was collected with a 20 gauge needle. The ethidium bromide was removed by repeated extraction with equal volume of CsCl saturated butanol. The DNA solution was dialyzed overnight against 500 ml TNE buffer (20 mM Tris HCl, 20 mM NaCl, 1 mM EDTA pH 8.0) with 2-3 changes. The DNA was

successively extracted with an equal volume of 1:1 (v/v) mixture of buffer-equilibrated phenol and chloroform, 24:1 (v/v) mixture of chloroform and isoamyl alcohol once. The DNA was precipitated with 0.3 M NaCl and 2.5 volume of cold ethanol, incubated at -70^{0}C for 1 hr. The DNA was collected by centrifugation at 10,000 x g for 15 min and the pellet was washed with cold 70% ethanol. The dried pellet was resuspended in 100 ul of TE buffer and the absorbance at 260 nm was measured.

1.2 Rapid DNA mini-preparation

This procedure was used for the preparation of small quantities of plasmid DNA. This procedure is similar to the large scale preparation except for the omission of the CsCl gradient ultracentrifugation step.

1.3 Determination of DNA concentrations

When the amount of the available DNA allowed, the concentration of nucleic acid in aqueous solution was determined spectrophometrically, as described by Maniatis et al (113). Nucleic acid solution were diluted in either double distilled water (ddH_2O) or TE buffer. The absorption at 260 nm was measured against the appropriate solvent blank and the nucleic acid concentration was calculated from a conversion equation. Nucleic acid in the form of double stranded DNA in solution at a concentration of 50 μg/ml will yield an optical density (OD) of 1. Measuring the OD at 280 nm (A_{280}) provides an estimate of the solution's purity. A ratio of A_{260}/A_{280} of 1.8 represents a relative pure preparation of DNA.

When the amount of available DNA was limiting, the quantitation of DNA was done by comparing the intensity of the band of desired DNA to the band of similar length of

molecular weight marker DNA on ethidium bromide agarose gel whose concentration was known.

2. Restriction Endonuclease Digestion

Restriction enzymes were obtained from Boehringer Mannheim (Laval, Quebec, Canada) or BRL. Restriction enzyme buffers used were as supplied by the respective companies.

Restriction enzyme digestion of double stranded DNA with a variety of enzymes was done following the manufacturer's specifications. Reactions included appropriate buffer at 1 X concentration, 0.1 - 10 μg of DNA in a final volume of 10 - 20 ul. One unit of enzyme is described as the amount required to completely digest 1 μg of a specified DNA in one hour. The reaction was incubated at 37^0C for 1-4 hours. Restriction digests were run on an agarose gel containing ethidium bromide to visualize the fragments under UV light.

3. Agarose gel electrophoresis

Fragments produced by restriction enzyme digestion were separated according to molecular size and conformation by electrophoresis in agarose gels (114). Electrophoresis was performed as described (113). A Tris-borate electrophoresis running buffer was made as a 10 X stock solution (10 X TBE). The 10 X TBE buffer consisted of 0.9 M Tris-HCl, 0.9 M boric acid, and 25 mM EDTA. The gel consisted of 0.5 - 1.5% agarose containing 1 μg/ml ethidium bromide in a 1 X TBE buffer. The restriction digested DNA samples were mixed with 0.1 volume of a 10 X gel loading buffer. The 10 X loading buffer consisted of 30% ficoll, 0.25% bromophenol blue, 0.25% xylene cyanole FF, and 0.2 M EDTA (pH 8.0)

in 10 X TBE. The samples were loaded onto the gel, and electrophoresis was carried out in 1 X TBE containing 1 μg/ml ethidium bromide at room temperature under constant voltage of 100 for 2 hrs.

After electrophoresis, the bands of DNA were visualized with an UV light source. Photographs of the gels were taken using Polaroid type 57 film and a polaroid 545 land camera (Polaroid Corporation, Cambridge, Massachusetts).

4. Recombinant DNA library construction

4.1 Deletion with Exonuclease III and Mung bean nuclease

The method for creating deletion in IFN-γ sequence utilizes the enzyme Exonuclease III (ExoIII) and Mung bean nuclease. Exonuclease III catalyzes the stepwise removal of 5' mononucleotides from the 3'-hydroxyl termini of double-stranded DNA (115). It will not degrade single-stranded DNA or double -stranded DNA with a protruding 3' terminus, but will digest 3' ends from blunt ends or 5' overhangs. Mung-bean nuclease degrades single-stranded DNA to mono- or oligonucleotides with phosphate groups at their 5' termini (116). It is, therefore, often used to convert protruding termini of DNA to blunt ends. By using ExoIII in conjunction with Mung bean nuclease, one can generate unidirectional sets of deletions of the terminal sequences of double stranded linear DNA.

ExoIII/Mung bean digestion was performed using an ExoIII/Mung Kit purchased from Strategene (La Jolla, CA). Supplied with the Kit are 1 X Mung bean dilution buffer (10 mM NaAC, pH 5.0, 0.1 mM ZnAC, 1 mM cysteine, 0.1% Triton X-100, 50% glycerol), 2 X ExoIII buffer (100 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, 20 μg/ml tRNA), 10 X Mung bean buffer (300 mM NaAC, pH 5.0, 500 mM NaCL, 10 mM $ZnCl_2$, 50% glycerol). The

digestion reaction was carried out followed Manufacturer' procedure with modifications.

The plasmid DNA was first double digested with EcoRI and Pst I restriction enzymes. The ExoIII digestion was started by adding 4 unit of Exo III for each 1 μg of double digested DNA. The reaction contained 23 ul of 0.8 μg/ul double digested DNA, 38 ul of 2 X ExoIII buffer, 7.6 ul of 100 mM β-mercaptoethanol, 7.8 ul of Exo III (73.6 Units). The Exo III reactions for all time points were started in a single tube and incubated at 23^{0}C, 3.3 ul was removed at every 2 min interval from 0 min to 44 min and put into a tube containing 16.7 ul aliquot of diluted 10X Mung Bean Nuclease buffer and placed on dry ice. When all aliquot have been removed and placed on dry ice, tubes were heated at 68^{0}C for 15 min to inactivate the ExoIII and then placed on ice. All time points were then pooled together, 1 ul (52.8 unit) of Mung bean nuclease (diluted with 1 X Mung bean dilution buffer) was added and incubated for 30 min at 30^{0}C. A solution containing 8.8 ul of 20% SDS, 22 ul of 1 M Tris-HCl pH 9.5, 4.4 ul of 8 M LiCl and 515 ul of buffer-equilibrated phenol:chloroform was added and the tube was vortexed, spined for 1 min in microfuge. The upper aqueous layer was removed and extracted with chloroform. The DNA was precipitated with 0.3 M NaCl and 2.5 volume cold ethanol, incubated at -70^{0}C for 1 hr. The DNA was collected by centrifugation at 10,000 rpm in microfuge for 15 min and the pellet was washed with cold 70% ethanol. The dried pellet was resuspended in 5 ul ddH_2O.

4.2 Ligation

4.2.1 EcoRI linkers

The 5'-phosphorylated EcoRI linker was synthesized by Regional DNA Synthesis Laboratory (University of Calgary, Calgary, AB, Canada). The sequence is: p-

d(CCGAATTCGG). The T4 DNA ligase was purchased from Strategene.

The DNA fragments after treatment with ExoIII/Mung bean was ligated with EcoRI linker. The ligation was performed using a 18 to 1 molar ratio of DNA fragment to linker and incubating at 16^{0}C overnight. A typical ligation included 5.75 ul of DNA (7 μg), 1 ul of EcoRI linker (0.5 μg), 1 ul of 10 X T4 ligase buffer, 1 ul of 10 mM ATP, 1.25 ul of T4 DNA ligase (5 unit). The tube was heated at 70^{0}C for 10 min, then chilled on ice. The ligated DNA was digested with EcoRI and electrophoresed on agarose gel.

The desired fragment was purified from the agarose gel by using the Geneclean Kit from BIO/CAN Scientific INC (Mississauga, Ontario, Canada) which contains a specially formulated silica matrix that binds single and double stranded DNA without binding DNA contaminants. Briefly, the desired DNA band was excised from agarose gel, 2.5 - 3 volume of 6 M NaI was added and incubated at 50^{0}C for 5 min to dissolve the agarose gel. A volume of 5 - 8 ul of glassmilk (silica matrix) was added to absorb DNA, the tube was vortexed and incubated on ice for 5 min. The tube was then spun in a microfuge for 5 sec and the supernatant was discarded. The pellet was washed 3 times with NEW buffer (NaCl/ethanol/water). The pellet was resuspended in an equal volume of ddH_2O and incubated at 55^{0}C for 2-3 min to elute the DNA. The tube was spun in microfuge for 30 sec, the supernatant which contains the purified DNA was collected.

4.2.2 Lambda gt11 arms

Bacteriophage lambda is a double stranded DNA virus which contains a large genome of 50 Kb in size. Approximately one third of the genome is nonessential for lytic growth of the phage; therefore, it can be replaced with large quantities of foreign DNA

(113). The lambda vector, gt11 was described by Young and Davis (117). The general scheme of constructing a lambda gt11 library is shown in Fig. 9.

The ligated, EcoRI digested, phosphatased lambda gt11 arms were purchased from BRL. The E. coli host strains Y1088, Y1089, and Y1090 were also provided.

The ligation of DNA into lambda gt11 was carried out according to the manufacturers suggestions. The ligation were performed using a 1 to 1 molar ratio of DNA fragment to lambda gt11 arms and incubating at 4^0C overnight. A typical ligation included 6.5 ul of DNA (45 ng), 1 ul of Lambda gt11 arms (0.5 μg), 1 ul of 10 X T4 DNA ligase buffer, 1 ul of 10 mM ATP, 0.5 ul of T4 DNA ligase (2 unit).

Packaging of the recombinant molecules into phage heads and tails was done using the Gigapack II plus packaging extract purchased from Stratagene. The procedure followed was described by the manufacturer.

5. Screening library in lambda gt11

5.1 Plating lamda library

A single colony of E. coli strain Y1088 was used to inoculate 10 ml of sterile TYN medium (10 g tryptone, 5 g yeast exact 5 g NaCl per litre of ddH_2O containing 10 mM Tris-HCl, pH 7.2) supplemented with 10 mM $MgSO_4$ and 0.2% maltose. The Mg^{++} ions are necessary for maintenance of the phage protein coat. The maltose up-regulates bacterial maltose receptors, by which the phage particles invade the host. The culture was grown at 37^0C for 4-6 hours with good aeration (OD_{600} < 1.0/ml). Cell pellets were resuspended in cold 10 mM $MgSO_4$ by gentle hand mixing. The plating cells was prepared by diluting cells to an OD_{600} of 0.5 with sterile 10mM $MgSO_4$.

Serial dilutions of the phage library were generated and used to infect the bacterial cells. Ten microlitres of the dilutions and 200 ul of the plating cells were mixed and incubated at 37^{0}C for 15 min. During incubation, sterile soft top agar (0.7 g agar in 100 ml TYN medium) supplemented with 10 mM $MgSO_4$ was heated, then allowed to cool to approximately 50^{0}C. After incubation, 3 ml of liquid soft top agar was added to each tube, mixed, and poured onto TYN agar plates (15 g agar in 1 litre TYN medium). For the test ligation, 40 ul of 1 M isopropylthiogalactoside (IPTG) and 40 ul of 40 mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal) was added to the 3 ml soft top agar beforing pouring onto agar plates. The soft agar was allowed to solidify at room temperature, and the plates were incubated inverted at 37^{0}C for 8 - 12 hours. Recombinant plaques will be clear, non-recombinant plaques will be blue. The cloning efficiency was defined as plaque-forming units (pfu) per microgram of vector DNA. By counting the plaques, the efficiency (pfu/μg) of the cloning was calculated. The dilution which produced plaques that were almost confluent (approximately 5,000 plaques per 85 mm petri plate) was chosen to plate the library.

5.2 Library amplification

After the plates were incubated at 37^{0}C for 8 - 12 hours, the plates were overlaid with 4.5 ml of SM buffer (5.8 g NaCl, 2.0 g $MgSO_4$, 50 ml 1M Tris-HCl, pH 7.5, 5 ml 2% gelatin in 1 litre of ddH_2O) and gently rocked at 4^{0}C overnight. The bacteriophage suspension was collected from each plate, pooled into a sterile polypropylene container. Chloroform was added to 5% of total volume and incubated at room temperature for 15 min. The cell debris was removed by centrifuging for 5 min at 2000 x g. The supernatant was

transferred to a sterile polypropylene tube. Chloroform was added to 0.3% of total volume and the tube was stored in aliquots at 4^0C. The titre of a library was checked.

5.3 Immunoscreening of lambda gt11 library

The lambda gt11 library has been constructed to allow expression of high levels of insert DNA protein products. In this system, the insert DNA is cloned into the lac Z gene of the vector. Host cells are infected with the phage and the lac Z gene is induced by addition of IPTG. This results in the expression of the foreign DNA as part of a β-galactosidase fusion protein. The protein can then be detected with antibodies (118).

To remove the anti-E. coli IgG, the primary antibody used for screening was preadsorbed with an extract of Y1090 cells. The extract was prepared by growing a culture of Y1090 to saturation, harvesting the cells by centrifugation, resuspending the cells in a buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, the cells was broken by a freeze-thaw cycle followed by sonication, and the debris was removed by another centrifugation. The extract at 1 mg/ml concentration was incubated with 1:1000 dilution of antibody for overnight at 4^0C or for 60 min at room temperature.

The lambda gt11 library was titered and plated as described in "Plating lambda library" section. The E. coli strain Y1090 was used as host cell for screening. The plates were incubated at 42^0C for 3.5 hours. Nitrocellulose filter discs were saturated in 10 mM IPTG, allowed to air dry, and overlaid on the plates. The plates were then incubated at 37^0C for 3.5 hours. The 42^0C incubation allows for lytic growth of the phage and plaque formation. The 37^0C incubation allows for the expression of the fusion protein. The filters were marked with waterproof ink at 3 asymmetric locations and gently removed from the

plates. The filters were then washed briefly in TBST buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) 3 times. All subsequent incubations and washes were performed at room temperature with 10 ml solution per filter. The filter were incubated in a solution of TBST and 1% BSA for 15 min. This step blocks non-specific protein binding sites on the filters. The filters were then incubated with primary antibody for 2 hrs. The primary antibody was preadsorbed with E. coli Y1090 lysate. Rabbit polyclonal anti-HuIFN-γ immunoglobulin 3004 was used at a 1:2000 dilution or anti-rHuIFN-γ monoclonal antibody MIF3152 (63) was used at 1:1000 dilution in 1% BSA/TBST. The filters were washed in TBST three times for five minutes. The filters were then incubated with secondary antibody for 1 hr. An alkaline phosphatase conjugated goat anti-mouse IgG (Jackson Immunoresearch laboratories, Inc., PA) or alkaline phosphatase conjugated goat anti-rabbit IgG (Jackson Imunoresearch Laboratories, Inc.) was used at a 1:5000 dilution in 1% BSA/TBST. The filters were then washed in TBST three times for five minutes. The final wash was in TBS to wash off Tween 20. Filters were blotted on Whatmann 3 mm paper and placed into the BCIP (5-bromo-4-chloro-3-indolyl phosphate; BRL)/NBT (nitroblue tetrazolium; BRL) color development substrate solution. This solution consisted of 0.3 mg/ml NBT and 0.15 mg/ml BCIP in color development buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). Filters were left in the solution until positive purple plaques appeared. The filters were washed in TBS to rinse off residual precipitated dye and then were immersed in ddH_2O to stop the color development. The filters were air-dried and stored protected from light.

Positive plaques were picked from the plates with the tip of a sterile tooth pick and placed in 1 ml SM buffer containing 1 drop of chloroform. The phage was replated and screening was repeated until all plaques on a plate produced a positive signal.

6. Isolation of lambda DNA

The preparation of phage lysate was achieved by liquid culture method. Briefly, 1 ml SM buffer containing 1 positive plaque was added into 500 ml of E. coli Y1088 overnight culture and incubated at 37^{0}C for 15 min. Twenty milliliter TYN medium supplemented with 5 mM $CaCl_2$ was added and incubated at 37^{0}C for 4 - 6 hrs until complete lysis. Six hundred microliter of chloroform was added and the cells was incubated for 10 min at 37^{0}C. The debris was removed by centrifuging at 20,000 x g for 5 min at 4^{0}C. The supernatant was collected and 200 ul of chloroform was added.

Lambda gt11 DNA was purified by ammonium sulfate precipitation method described by Ziai et al (119). Ten milliliter phage lysate was mixed with an equal volume of saturated ammonium sulfate solution at 4^{0}C, placed on ice for 30 min, and centrifuged at 20,000 x g for 20 min at 4^{0}C. The pellet was resuspended with vortexing in 0.5 ml of TE-SDS buffer (10 mM Tris-HCl, 5 mM EDTA, 0.1% SDS) and transferred into a 1.5 ml microcentrifuge tube. The lysate was sequentially incubated for 60 min at 37 ^{0}C with RNase A (40 μg/ml and for 20 min at 60^{0}C with proteinase K (200 μg/ml). The lysate was placed on ice for 5 min, gently mixed with 1/10 volume of 1M NaOH at 0^{0}C, and incubated on ice for 10 min. The lysate was immediately neutralized by addition of 200 ul of 10 M ammonium acetate, pH 6.0, and centrifuged for 10 min at 4^{0}C. The supernatant was extracted twice with an equal volume of phenol, once with an equal volume of chloroform. The supernatant was recovered and mixed with an equal volume of ice-cold 2-propanol, incubated on ice for 30 min, and centrifuged at 4^{0}C for 20 min in a microcentrifuge. The pellet was rinsed twice with cold 70% ethanol, dried under vacuum for 2 min, resuspended in 20 ul of TE buffer and incubated at 65^{0}C for 10 min to dissolve the lambda gt11 DNA.

RESULTS

A lambda gt11 recombinant DNA expression library of HuIFN-γ was constructed by using the strategy depicted in Fig. 9.

ExoIII deletion

The 453-base pair (bp) synthetic human IFN-γ gene (between Hind III sites) was expressed in an expression vector which was a derivative of PBR322 (Fig. 9). The 4865-bp recombinant plasmid designated as $pJP_{14}R_3$-IF_3 had single EcoRI, Pst I, and Bam HI restriction site (Fig. 9, 10). It carried a 80 bp of functional promoter and ribosome binding site (between EcoRI and Hind III site) for IFN-γ and to which the IFN-γ gene was attached (Fig. 9).

Large scale plasmid preparation was performed to provide DNA for deletion reaction. The purified plasmid $pJP_{14}R_3$-IF_3 was double cut with EcoRI/Pst I. Two resulting DNA fragments, 4117 and 748 bp, respectively, were identified by 0.7% agarose gel (Fig. 11). Both fragments have 5' overhang at one end and 3' overhang at the other, generated by EcoRI and Pst I respective cleavages. The 4117 bp DNA fragment contained the IFN-γ gene sequence located close to the 5' protruding end (Fig. 9). The linear molecules were then subjected to digestion with ExoIII to create a series of varying extent of deletion into the IFN-γ gene sequence.

The ExoIII/Mung bean nuclease was employed for making the IFN-γ deletion library. ExoIII would not digest the 3' protruding end generated by Pst I, but only digest the 5' protruding end of the DNA fragments generated by EcoRI so that the N-terminus of the

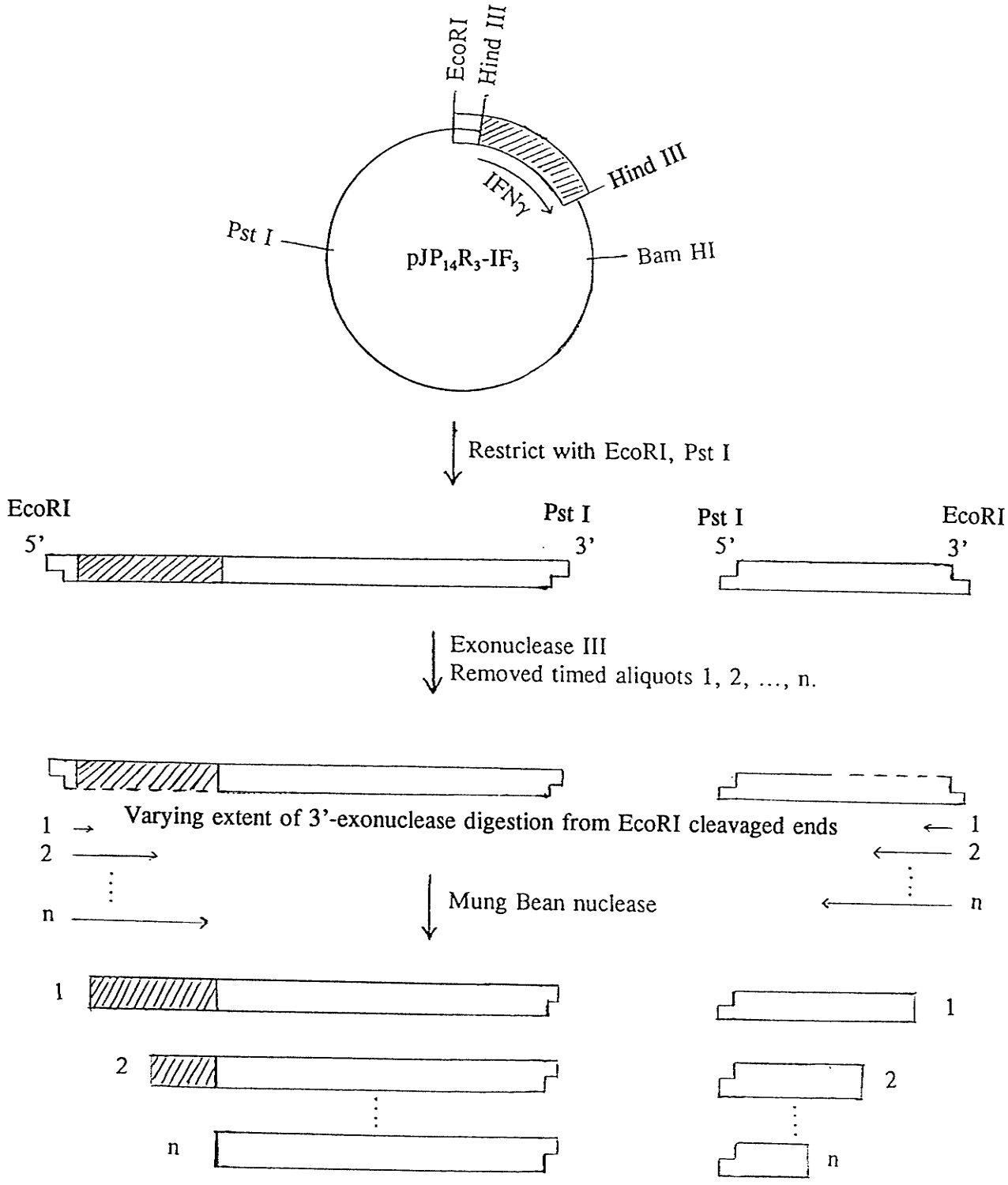


Figure 9. General scheme of the library construction

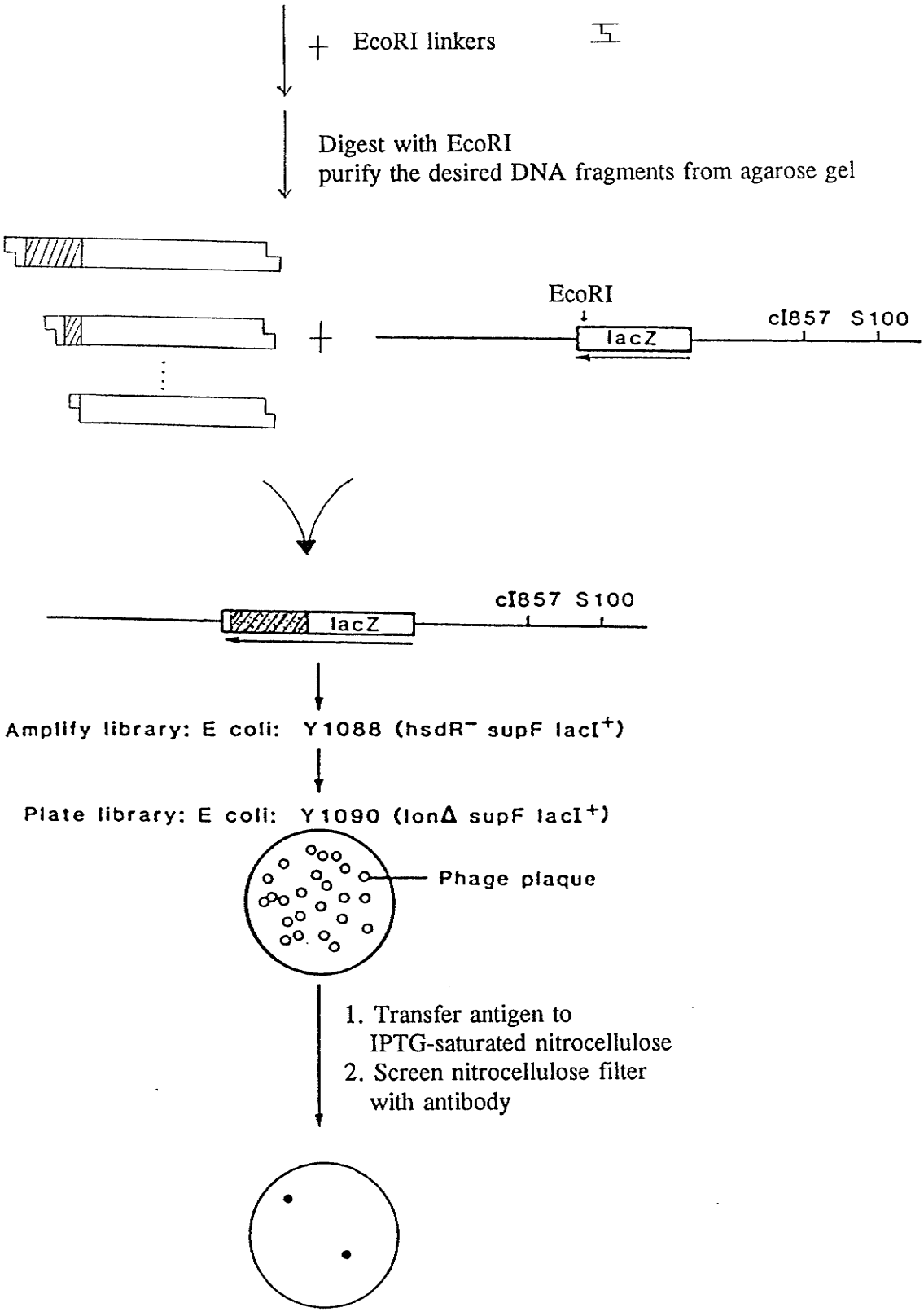

+ EcoRI linkers
Digest with EcoRI
purify the desired DNA fragments from agarose gel
EcoRI
lacZ
cI857 S100
lacZ
cI857 S100
Amplify library: E coli: Y1088 (hsdR⁻ supF lacI⁺)
Plate library: E coli: Y1090 (lonΔ supF lacI⁺)
Phage plaque
1. Transfer antigen to
IPTG-saturated nitrocellulose
2. Screen nitrocellulose filter
with antibody

Figure 10.

Agarose gel analysis of $pJP_{14}R_3$-IF_3.

Lane 1 Standard: Hind III digest of lambda DNA (bp: 21226, 5148, 4973, 4277, 3530, 2027, 1904, 1584, 1330, 983, 831, 564).

Lane 2 Native $pJP_{14}R_3$-IF_3.

Lane 3 EcoRI digest of $pJP_{14}R_3$-IF_3.

Lane 4 Hind III digest of $pJP_{14}R_3$-IF_3.

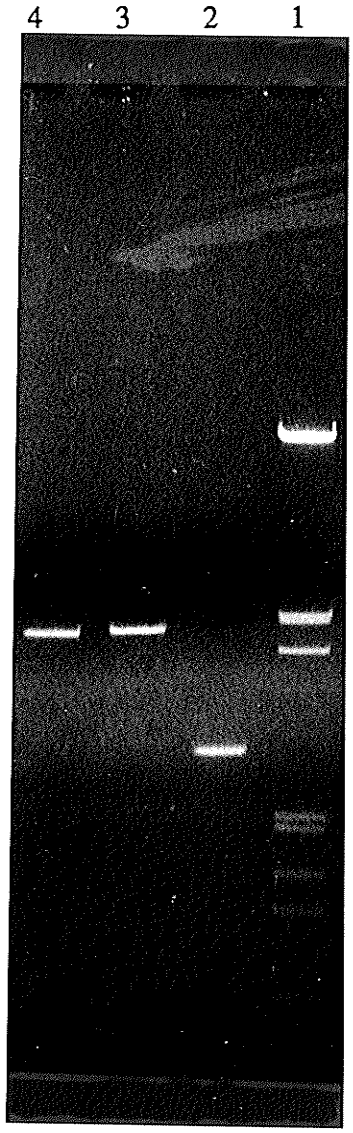
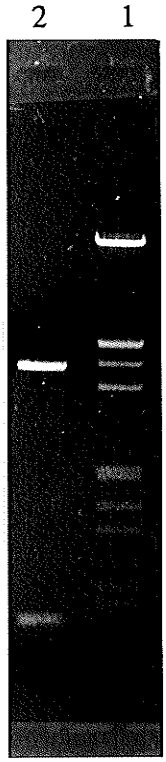

4
3
2
1

Figure 11.

Double digestion of plasmid $pJP_{14}R_3$-IF_3 analysed on 7% agarose gel.

Lane 1 Standard: Hind III digest of lambda DNA (bp: 21226, 5148, 4973, 4277, 3530, 2027, 1904, 1584, 1330, 983, 831, 564).

Lane 2 EcoRI/Pst I digest of $pJP_{14}R_3$-IF_3.


2
1

HuIFN-γ gene sequence would be deleted to various extents. The length of DNA converted from double strand to single strand by ExoIII can be controlled by the reaction temperature and time of incubation. The ideal deletion library for the purpose of this study should contain as many as possible of various degree of N-terminus deleted IFN-γ sequence. Optimal digestion conditions for generating this library was determined by a series of preliminary experiments with varying reaction temperature and times of incubation. Constant concentration of ExoIII (4 units of ExoIII for 1 ug of DNA) was used as recommended by the manufacturer. The optimal condition of the ExoIII digestion for this study's purpose was considered to be that which generated deletion at about every 30 bp throughout the IFN-γ sequence (approximately 600 bp deleted). The preliminary experiments were carried out by setting up a single reaction for ExoIII and removing aliquots at varying time points. Each time point was then treated with Mung bean nuclease and analyzed on agarose gel. From the gel, the length of DNA from each time point was calculated. The deletion rate was determined. The result of one of the preliminary ExoIII digestions are shown in the agarose gel in Fig. 12. It demonstrated that at 4 unit of ExoIII per 1 ug DNA at 23^0C resulted in a deletion rate of 14 bp/min which was considered the optimal reaction condition for generating DNA fragments of the size range desired.

The deletion library was created by setting up the large scale digestion using 4 units of ExoIII for 1 ug of DNA incubating at 23^0C. The ExoIII reactions for all time points were started in a single tube. At 0 time and at every 2 min interval, an aliquot was withdrawn and put into a tube containing Mung bean nuclease buffer which stops the ExoIII digestion and then treated with Mung bean nuclease to remove single strand DNA. The last time point was 44 min in which 616 bp were deleted from both fragments. By pooling the fractions from

Figure 12.

Calibration of optimal reaction condition of ExoIII deletion.

A 4.8 μg of double digested DNA was incubated with 19.2 units of ExoIII at 23^{0}C. At each time point, 2.5 ul was taken out and treated with Mung bean nuclease, and analysed on 0.7% agarose gel.

Lane 1 Standard: Hind III digest of lambda DNA (bp: 21226, 5148, 4973, 4277, 3530, 2027, 1904, 1584, 1330, 983, 831, 564).

Lane 2 0 min fraction.

Lane 3 1 min fraction.

Lane 4 4 min fraction.

Lane 5 8 min fraction.

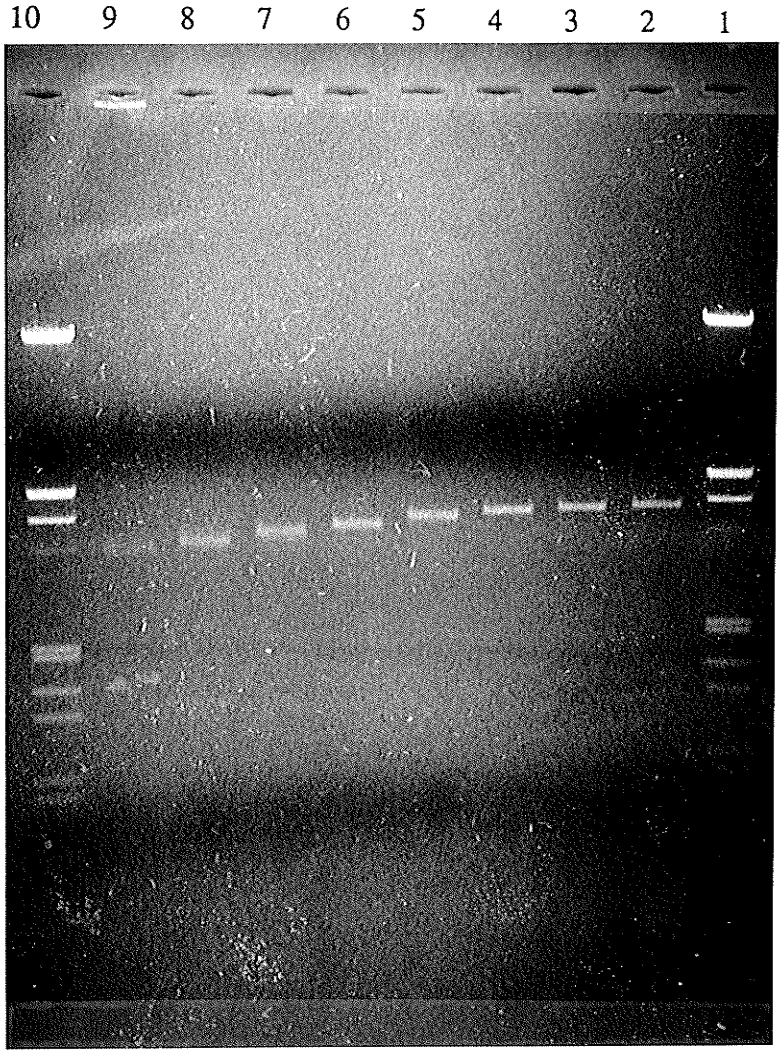
Lane 6 16 min fraction.

Lane 7 24 min fraction.

Lane 8 32 min fraction.

Lane 9 40 min fraction.

Lane 10 Standard: Hind III digest of lambda DNA.


10
9
8
7
6
5
4
3
2
1

each time point, a DNA library containing various lengths of DNA fragments (3500 - 4117 bp and 132 - 748 bp) were generated.

Recombinant DNA library construction

The deleted DNA were then ligated with phosphorylated EcoRI linker (10 bp) and digested with EcoRI which produced the DNA fragments with EcoRI end to allow subsequent insertion at the unique EcoRI site of the lambda gt11 phage.

To purify the desired DNA library from the unligated linkers and unrelated DNA fragments, the digested DNA was electrophoresed on agarose gel, the DNA fragments in the size range of 3500 to 4117 bp were cut and purified from the gel using the Geneclean Kit as described in Materials and Methods (Fig. 13).

The purified DNA was inserted into the vector lambda gt11. The recombinant molecules were packaged into phage heads and tails and adsorbed onto E. coli host cells. Recombinants were detected as clear plaques, in the presence of X-gal, indicating that the expression of β-galactosidase has been interrupted by insertion of foreign DNA within the gene. The cloning efficiency of the library was 2.25×10^5 (pfu/ug) (clear plaque), the ligation positive control DNA included had the cloning efficiency of 6.25×10^7 (pfu/ug). The library generated in this manner contained 4.35×10^4 individual recombinant phage, consisted of 62% recombinants.

Screening library in lambda gt11

The library was initially immunoscreened with rabbit polyclonal anti-HuIFN-γ imunoglobulin 3004 which was produced by immunizing rabbits with purified native

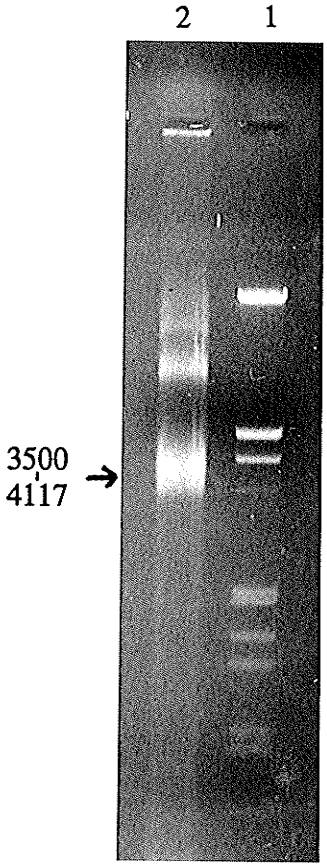
Figure 13.

Purification of DNA deletion library from agarose gel.

ExoIII treated DNA was ligated with EcoRI linker, digested with EcoRI and electrophoresed on agarose gel. The DNA fragments in the size range of 3500 to 4117 bp were cut and purified from the gel.

Lane 1 Standard: Hind III digest of lambda DNA (bp: 21226, 5148, 4973, 4277, 3530, 2027, 1904, 1584, 1330, 983, 831, 564).

Lane 2 DNA sample.


2
1
3500
4117

recombinant HuIFN-γ. The initial screening with polyclonal antibody 3004 produced positive signals on all the plaques including negative control (ligated lambda gt11 without inserts). The same results were observed with anti-rHuIFN-γ monoclonal antibody MIF3152 (63). The further study using immunodot blot analysis (result not shown) demonstrated that antibody 3004 and MIF3152 bound to the E. coli cell lysate, suggesting that antibody contained anti-E. coli IgG. The antibody used for screening, therefore, were preadsorbed with an extract of Y1090 cells to remove the anti-E. coli IgG. As shown in Fig. 14, on the nitrocellulose filter screened with untreated antibody, the positive signals were unable to be distinguished from the background of other plaques (a), wherease, on the nitrocellulose filter screened with preadsorbed antibody, although numerous background plaques are seen, the enhanced signal to noise ratio obtained is clearly evident (b).

The detection method used for immunoscreening was based on the binding of species-specific antiimmunoglobulins conjugated with alkaline phosphatase to the primary antibody, followed by a color development reaction with the phosphatase substrate BCIP in combination with NBT. A comparison study of the sensitivity of detection obtained with alkaline phosphatase- and peroxidase-conjugated second antibodies was done (results not shown). Results revealed several advantages of this approach over the use of antiimmunoglobulins conjugated with horseradish peroxidase: the alkaline phosphatase conjugate was more sensitive on detection, the precise signal-producing plaque could be located by comparing the faint background pattern produced by neighboring plaques on the nitrocellulose filter with the pattern of plaques on the agar plate.

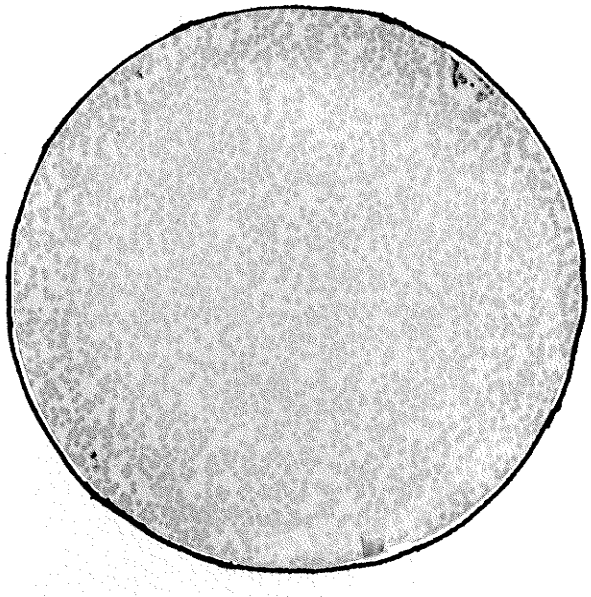
The lambda gt11 library was screened with preadsorbed polyclonal antibody 3004, approximately 150 positive signals were produced in each 85 mm petri plate (about 5000

Figure 14.

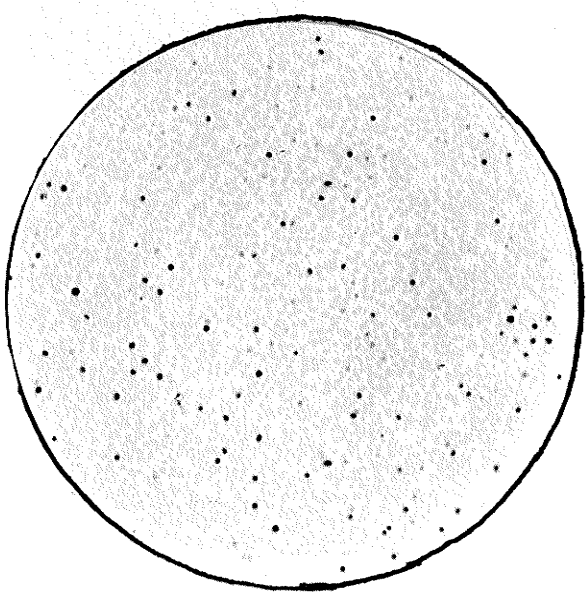
Comparison of signals produced with untreated antibody 3004 and preadsorbed antibody 3004 on nitrocellulose.

a. Immunoblot of lambda library plaques screened with untreated 3004.

b. Immunoblot of lambda library plaques screened with preadsorbed 3004.



a



b

pfu). In order to increase the ratio of positive to be able to screen the library more efficiently, a sublibrary was made by picking 500 positive plaques produced by polyclonal antibody 3004. The library was amplified in E. coli Y1088 by producing plate stock. The resulting sublibrary produced 500 positive signals in each plate when screened with polyclonal antibody 3004.

The sublibrary was screened with monoclonal antibody MIF3152 which in a previous study (63) demonstrated to recognize E1 epitope group and was immunoblot positive. In a screening of 5 x 10^4 plaques, 30 independent clones that produced strong signals were isolated and purified to homogeneity.

Restriction analysis

Lambda phage from these 30 clones were grown in quantity. Recombinant DNA were purified as described in Materials and Methods and then subjected to restriction enzyme analysis.

The DNA inserts were cloned into an EcoRI restriction site within the lambda lac Z gene of the lambda gt11 vector. Once the lambda DNA was isolated, the cloned inserts can be excised with the restriction enzyme EcoRI. Recombinant DNA from 30 positive clones were digested with EcoRI and were subjected to agarose gel electrophoresis to determine the sizes of inserted DNA fragments. By this analysis, it was determined that 7 clones contained inserts ranging from 3800 to 4117 bp, clone 12 had the shortest insert (Fig. 15). These results agreed with the insert size range expected (3500 - 4117 bp). No inserts were identified in the rest of 23 positive clones, in spite of the efforts of digesting these DNA for prolonged time.

These 7 clones were further characterized by double digestion with EcoRI and Bam HI (Fig. 16). Two fragments derived from inserts were observed in all 7 clones, one was 900 bp in all 7 clones, the other one ranged from 2600 - 3300 bp. These results did not agree with the size (3238 bp and 263 - 879 bp) predicted from the restriction map of the recombinant molecule.

Figure 15.

Restriction analysis of positive clones with EcoRI on 0.5% agarose gel.

Lane 1 Standard: Hind III digest of lambda DNA (bp: 21226, 5148, 4973, 4277, 3530, 2027, 1904, 1584, 1330, 983, 831, 564).

Lane 2 Native vector lambda gt11.

Lane 3 EcoRI digest of vector lambda gt11.

Lane 4 Native clone 17.

Lane 5 EcoRI digest of clone 17.

Lane 6 EcoRI digest of clone 25.

Lane 7 EcoRI digest of clone 29.

Lane 8 EcoRI digest of clone 22.

Lane 9 EcoRI digest of clone 20.

Lane 10 EcoRI digest of clone 12.

Lane 11 EcoRI digest of clone 23.

Lane 12 Standard: Hind III digest of lambda DNA.

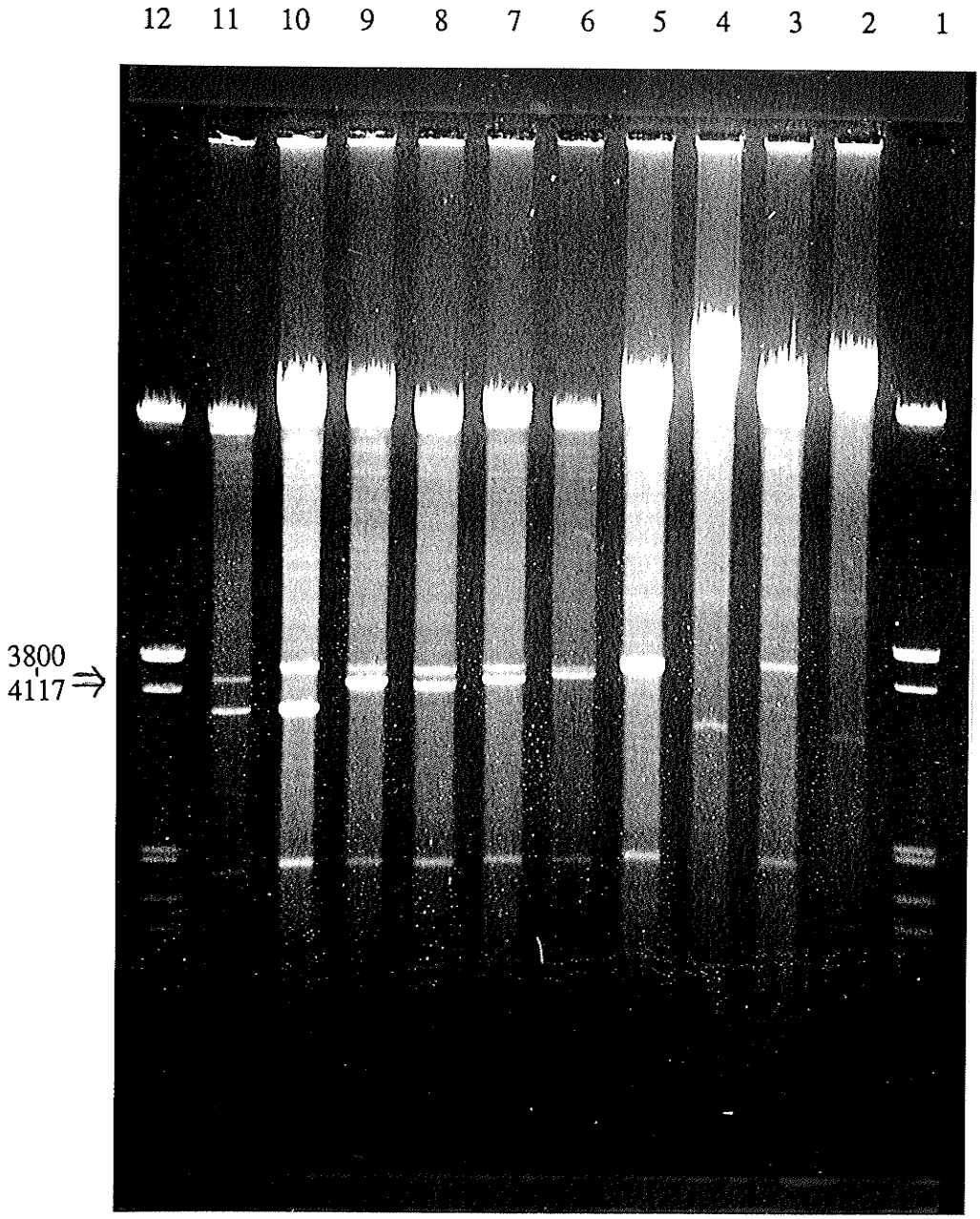

12 11 10 9 8 7 6 5 4 3 2 1
3800
4117

Figure 16.

Restriction analysis of positive clones with EcoRI/Bam HI.

Lane 1 Standard: Hind III digest of lambda DNA (bp: 21226, 5148, 4973, 4277, 3530, 2027, 1904, 1584, 1330, 983, 831, 564).

Lane 2 EcoRI/Bam HI digest of vector lambda gt11.

Lane 3 EcoRI/Bam HI digest of clone 17.

Lane 4 EcoRI/Bam HI digest of clone 25.

Lane 5 EcoRI/Bam HI digest of clone 29.

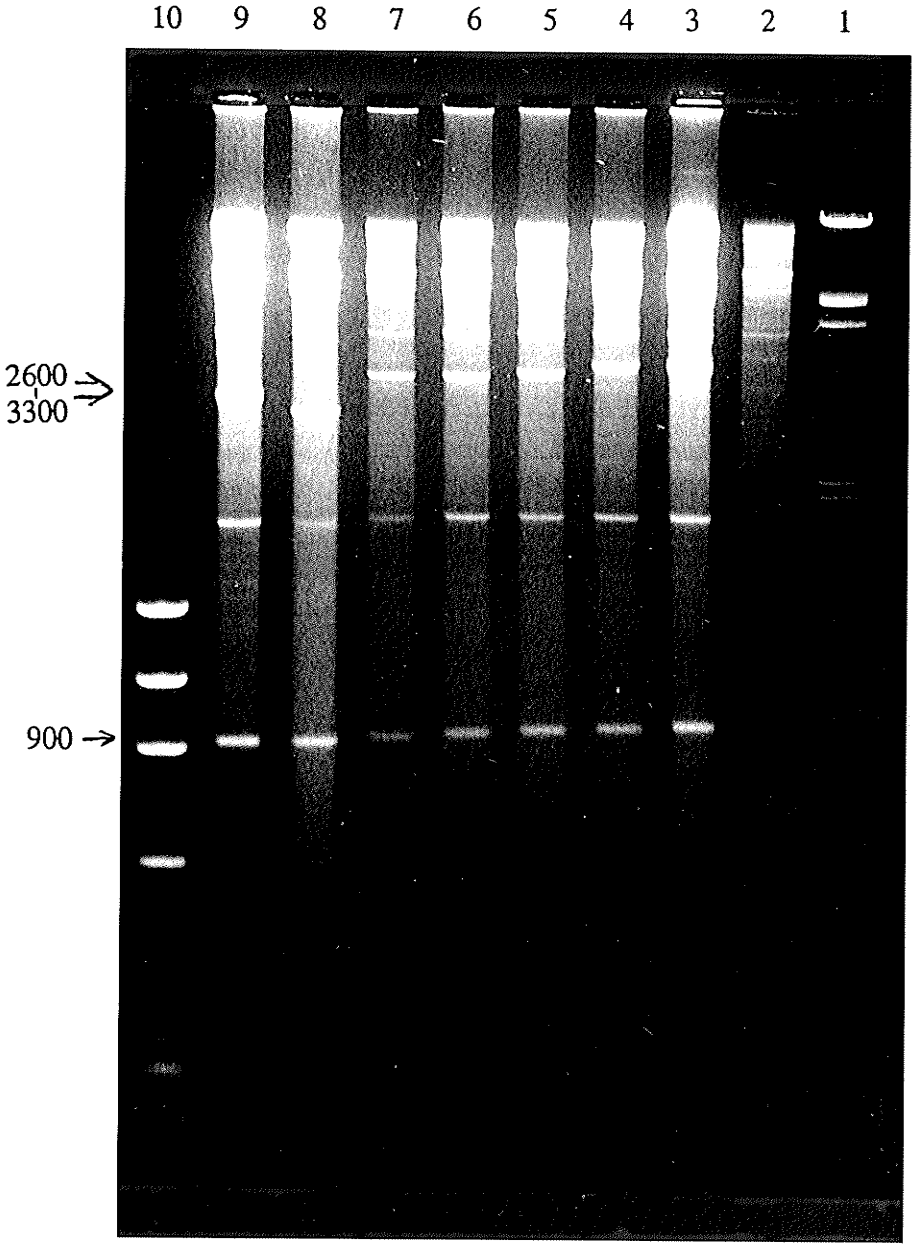
Lane 6 EcoRI/Bam HI digest of clone 22.

Lane 7 EcoRI/Bam HI digest of clone 20.

Lane 8 EcoRI/Bam HI digest of clone 12.

Lane 9 EcoRI/Bam HI digest of clone 23.

Lane 10 Standard: Hae III digest of phi X 174 DNA (bp: 1353, 1078, 872, 603, 310, 271, 234, 194).


10
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6
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4
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2
1
2600
3300
900

DISCUSSION

Human interferon-γ is an interesting protein that exhibits a number of different biological activities. From a previous study (63) of the characterization of 21 antiviral neutralizing MAbs, it was determined that at least two distinct epitopes (E1, E2) are present. However, the location of these epitopes have not been identified.

The potential limitation of most of strategies used to define epitopes is the difficulty presented by the class of antibodies that recognize conformational epitopes. A means to survey all of the functional epitopes of the HuIFN-γ would be useful for the better understanding the structure/function relationship of the HuIFN-γ and also could facilitate the epitope mapping techniques.

The present study described the attempt to construct a recombinant epitope library for the localization of functional domain(s) on the HuIFN-γ. A recombinant DNA strategy was designed as depicted in Fig. 9. Exonuclease III/Mung bean nuclease was used to create the N-terminus deletion in the HuIFN-γ sequence. A library containing fragments of N-terminus deleted IFN-γ with random endpoints was constructed in the bacteriophage expression vector lambda gt11. The expression of epitope coding sequences by individual recombinant bacteriophage was detected with MAb probes and the appropriate DNA clones were isolated and characterized with restriction enzymes to determine the general location of the epitope. Further characterization with other approaches would identify the exact sequence of the epitope. A number of features of the design of this strategy are important:

Recombinant DNA expression technology offers an effective strategy to thoroughly and systematically examine the epitopes of the protein when antibodies are available for use

as probes.

Using ExoIII for creating the unidirectional deletion of HuIFN-γ, the construction of both N-terminus and later C-terminus deletion library would complete the search for the functional epitopes of HuIFN-γ.

Because bacteria rapidly degrade most unusual polypeptides (120-122), eukaryotic proteins, and especially portions therefore, are potentially unstable in prokaryotic cells. IFN-γ deletion fragments were inserted into the C-terminus of the β-galactosidase of lambda gt11 gene, the fusion of the eukaryotic moiety with all but a small portion of prokaryotic protein β-galactosidase, encoded by lambda gt11, would enhance the stability of IFN-γ connected, since it was shown that fusion proteins are often more resistant to proteolytic degradation than is the foreign polypeptide alone (123-124).

Because IFN-γ sequence is expressed and probed in its native form by MAb, part of the power of this approach lies in the potential to detect epitopes that are conformationally organized.

The plasmid $pJP_{14}R_3$-IF_3 containing IFN-γ gene was double cut with EcoRI/Pst I, resulting two DNA fragments with 5' overhang at one end and 3' overhang at the other. These two DNA fragments were digested with ExoIII/Mung bean nuclease. The ExoIII reaction was set up by using 1 unit ExoIII for 4 ug DNA, incubating at 23^0C, the fractions were taken out at every 2 min interval until 44 min. Determined from preliminary experiments, under this condition, the deletion rate was 14 bp/min. Thus, the DNA would contain fragments representing deletion at every base. Resulting DNA fragments with the size range from 3500 bp to 4117 bp and 132 bp to 748 bp. The fraction taken at 0 min contained full length IFN-γ sequence, whereas the fraction taken at 44 min had IFN-γ

completely deleted. By this means, the N-terminus deleted IFN-γ library with random endpoint was generated. This was confirmed by analysing the DNA library on agarose gel (Fig. 13). The big band ranging from 3500 - 4117 bp represented this DNA library. The other range size of DNA fragments (132 bp to 748 bp) was too small to be visualized on this gel.

EcoRI linker was added to the ends of the DNA fragments to allow insertion at the unique EcoRI site of the lambda gt11 phage. After EcoRI digestion, the DNA library (3500 - 4117 bp) was cut and purified from the agarose gel.

The EcoRI digested lambda gt11 arms were dephosphorylated to reduce the chance of self ligation. Lambda gt11 contains a unique EcoRI cloning site near the end of β-galactosidase coding sequence which can accept up to 7.2 Kb DNA insert. With the deletion IFN-γ library at 3500 - 4117 bp in size, the chance of multiple insertion was greatly reduced. The lambda gt11 recombinant DNA expression library of IFN DNA constructed in this manner contained 4.35×10^4 individual recombinant phage. The cloning efficiency was 2.25×10^5 (pfu/ug) which was 100 fold lower than that of the ligation control DNA (6.25×10^7) provided by the manufacturer. The reason could be that certain percentage of the insert DNA did not have EcoRI end resulting from either the failure of EcoRI linker ligation or incomplete digestion afterwards with EcoRI.

Immunoscreening of the library employed preadsorbed primary antibody and the alkaline phosphatase conjugated secondary antibody. The method produced good signal to noise ratios to precisely detect positive plaques (Fig. 14). The proper expression of IFN epitope library in lambda gt11 recombinant will depend on the orientation and reading frame of the insert DNA with respect to those of Lac Z. Thus, one-sixth of the lambda gt11

recombinants containing a IFN sequence will produce the fusion protein of β-galactosidase fused to the IFN protein. This was reflected by the low percentage (3%) of positive signals produced by polyclonal antibody 3004.

The library was screened with MAb MIF3152 which was demonstrated (63) to bind to the epitope of E1 group and was immunoblot positive. Thirty positive plaques were isolated and purified to homogeneity. The recombinant DNA was purified and analyzed with restriction enzymes. When digested with EcoRI, only 7 clones produced inserts (Fig. 15). The rest of 23 clones failed to produce inserts in spite of the efforts of prolonged digestion time. This could be the indication that these are false positive clones, one possibility is that the fusion proteins resulting from these clones expressed an unrelated polypeptide containing a similar or identical epitope with IFN-γ. The size of the EcoRI inserts from these 7 clones ranged from 3800 to 4117 bp determined from the agarose gel which agree with the insert size expected. However, further analyses of these DNA with double digestion of EcoRI/Bam HI revealed two DNA fragments resulted from the insert: 900 bp and 2600 to 3300 bp. This result did not agree with the size predicted from the restriction map of the insert. From the restriction map, digestion of insert with EcoRI/Bam HI should result a DNA fragment of constant size of 3238 bp (between Bam HI and Pst I in Fig. 9) and DNA fragments ranging 263 to 879 bp since ExoIII does not digest 3' protruding end generated by Pst I.

It is not clear in the present study if these positive clones contain IFN-γ gene.

Further studies are needed to identify these inserts: one approach is to determine if HuIFN-γ sequence is present by hybridization with cloned HuIFN-γ sequence and locate the insertion site and orientation to confirm if the construction is as expected; alternatively, the recombinant fusion protein could be produced through lysogeny of the individual phage clone

for SDS-Page analysis, and analyzed in western blot by reacting with MAb; last, DNA sequencing technique would directly reveal the exact sequence of these DNA inserts.

REFERENCES

1. Isaacs A and Lindenmann J. (1957) Virus interference. Proc R Soc Lond B Biol Sci 147:258

2. Kirchner H. (1984) Interferons, a group of multiple lymphokines. Springer Semin Immunopathol 7:347

3. De Maeyer-Guignard J and De Maeyer E. (1985) Immunomodulation by interferons. In: Interferon 6:69 Gresser (Eds) New York, Academic Press

4. Paucker K, Cantell K, and Henle W. (1962) Quantitative studies on viral interference in suspended L-cells III. Effect of interfering viruses and interferon on the growth rate of cells Virology 17:324

5. Gaffney EV, Picciano PT, and Grant CA. (1973) Inhibition of growth and transformation of human cells by interferon. J Natl Cancer Inst 50:871

6. Gresser I, Coppey Y, Falcoff E, and Fontaine D. (1967) Interferon and murine leukemia I. Inhibitory effect of interferon preparations on development of Friend leukemia in mice. Proc Soc Exp Biol Med 124:84

7. Berman LD. (1970) Inhibition of oncogenicity of murine sarcoma virus (Harvey) in mice by interferon. Nature 227:1349

8. Pestka S and Baron S. (1981) Definition and classification of the interferons. In Methods in Enzymology Vol 78, ed. S. Pestka pp 3-14 New York: Academic

9. Gray PW and Goeddel DV. (1982) Structure of the human immune IFN gene. Nature 298:859

10. Pestka S, Langer JA, Zoon KC, and Samuel CE. (1987) Interferons and their actions. Annu Rev Biochem 56:727

11. Mannering GJ and Deloria LB. (1986) The pharmacology and toxicology of the interferons: an overview. Ann Rev Pharmacol Toxicol 26:455

12. Yip YK, Pang RHL, Urban C, and Vilcek J. (1982) Partial purification and characterization of human γ (immune) interferon. Proc Natl Acad Sci USA 78:1601

13. Havell EA, Yip YK, and Vilcek J. (1977) Correlation of physicochemical and antigenic properties of human leukocyte interferon subspecies. Archs Virol 55:121

14. Nathan I, Groopman JE, Quan SG, Bersch N, and Golde DW. (1981) Immune interferon produced by a human T lymphoblast line. Nature 292:842

15. Trinchieri G, Matsumoto-kobayashi M, Clark SV, Sheera J, London L, and Perussia B. (1984) Response of resting peripheral blood natural killer cells to interleukin 2. J Exp Med 160:1147

16. Steward WE. II (1981) The Interferon System, Springer-Verlag New York Inc., New York

17. Le J and Vilcek J. (1987) Accessory function of human fibroblasts in mitogen-stimulated interferon-γ production by T lymphocytes: Inhibition by interleukin 1 and tumor necrosis factor. J Immunol 139:3330

18. Reem GH and Yeh N-H. (1984) Interleukin 2 regulates expression of its receptor and synthesis of gamma interferon by human T lymphocytes. Science 225:429

19. Hochkeppel HK and De Ley M. (1982) Monoclonal antibody against human IFN-γ. Nature 296:258

20. Le J, Barrowclough BS, and Vilcek J. (1984) Monoclonal antibodies to human immune interferon and their application for affinity chromatography. J Immunol Methods 69:61

21. Gray PW, Leung DW, Pennica D, Yelverton E, Najaran R, Simonsen CC, Derynck R, Sherwood PJ, Wallace DM, Berger SL, Levinsen AD and Goeddel DV. (1982) Expression of human immune interferon cDNA in E. coli and monkey cells. Nature 295:503

22. Rinderknecht E, O'Conner BH, and Rodriguez H. (1984) Complete amino acid sequence of human interferon and determination of sites of glycosylation. J Biol Chem 259:6790

23. Devos RH, Cheronte Y, Taya W, Degrave H, Van Heuverswyn, and Fiers W. (1982) Molecular cloning of human immune interferon cDNA and its expression in eukaryotic cells. Nucleic Acids Res 10:2487

24. Kelker HC, Le J, Rubin BY, Yip YK, Nagler C, and Vilcek J. (1984) Three molecular weight forms of natural human interferon-γ revealed by immunoprecipitation with monoclonal antibody. J. Biol. Chem. 259:4301

25. Kelker HC, Yip YK, Anderson P, and Vilcek J. (1983) Effects of glycosidase treatment on the physicochemical properties and biological activity of human interferon-γ. J Biol Chem 258:8010

26. Hogrefe HH, McPhie P, Bekisz JB, Enterline JC, Doreen D, Webb DSA, Gerrard TL, and Zoon KC. (1989) Amino terminus is essential to the structural integrity of recombinant human interferon-γ. J Biol Chem 264:12179

27. Arakawa T, Hsu YR, and Yphantis DA. (1987) Acid unfolding and self-association of recombinant Escherichia coli derived human interferon gamma. Biochemistry 26:5428

28. Windsor WT and Trotta PP. unpublished observations

29. Ealick SE, Cook WJ, Vijay-Kumar S, Carson M, Nagabhushan TL, Trotta PP, and Bugg CE. (1991) Three-dimensional structure of recombinant human interferon-γ Science 252: 698

30. Arakawa T, Hsu Y-R, Chang D, Stebbing N, and Altrock B. (1986) Structure and activity of glycosylated human interferon-gamma. J Interferon Res 6:687

31. Arakawa T, Hsu Y-R, Parker CG, and Lai P-H. (1986) Role of Polycationic C-terminal portion in the structure and activity of recombinant human interferon-γ. J Biol Chem 261:853431.

32. Hsu Y-R, Ferguson B, Narachi M, Richards RM, Stabinsky Y, Alton NK, Stebbing N, and Arakawa T. (1986) Structure and activity of recombinant human interferon-γ analogs. J Interferon Res 6:663

33. Langer JA and Pestka S. (1988) Interferon receptors. Immunol Today 9:393

34. Brance AA and Baglioni C. (1981) Evidence that type I and type II interferon have different receptors. Nature 294:768

35. Rashidbaigi A, Kung H-F, and Pestka S. (1985) Characterization of receptors for immune interferon in U937 cells with ^{32}P-labeled human recombinant immune interferon. J biol Chem 260:8514

36. Finbloom DS, Hoover DL, and Wahl LM. (1985) The characteristics of binding of human recombinant interferon-γ to its receptor on human monocytes and human monocyte-like cell lines. J Immunol 135:300

37. Ucer U, Bartsch H, Scheurich P, and Pfizenmaier K. (1985) Biological effects of γ-interferon on human tumor cells: Quantity and affinity of cell membrane receptors for γ-IFN in relation to growth inhibition and induction of HLA DR expression. Int J Cancer 36:103

38. Langer JA, Rachidbaigi A, and Pestka S. (1986) Preparation of ^{32}P-labeled murine immune interferon and its binding to the mouse immune interferon receptor. J Biol Chem 261:9801

39. Rashidbaigi A, Langer JA, Jung V, Jones C, Morse HG, Tischfield JA, Trill JJ, Kung H-F, and Pestka S. (1986) The gene for the human immune interferon receptor is located on chromosome 6. Proc Natl Acad Sci USA 83:384

40. Branca AA, Faltynek CR, D'Allessandro SB, and Baglione C. (1982) Interaction of interferon with cellular receptors, internalisation and degradation of cell-bound interferon. J Biol Chem 257:13291

41. Celada A and Schreiber RD (1987) Internalization and degradation of receptor-bound interferon-γ by murine macrophages. Demonstration of receptor recycling. J Immunol 139:147

42. Aguet M, Dembic Z, and Merlin G. (1988) Molecular cloning and expression of the human interferon-γ receptor. Cell 55:273

43. Jung V, Rashidbaigi A, Jones C, Tischfield JA, Shows TB, and Pestka S. (1987) Human chromosome 6 and 21 are required for sensitivity to human interferon gamma. Proc Natl Acad Sci USA 84:4151

44. Weil J, Epstein CJ, Epstein LB, Sedmak JJ, and Grossberg SE. (1983) A unique set of polypeptides is induced by gamma interferon in addition to those in common with alpha and beta interferons. Nature 301:437

45. Kerr IM, Brown RE. (1978) pppA2'p5'A2'p5'A: An inhibitor of protein synthesis synthesized with an enzyme fraction from interferon-treated cells. Proc Natl Acad Sci USA 75:256

46. Lebleu B, Sen GC, Shaila S, Cabrer B, and Lengyel P. (1976) Interferon, double-stranded RNA and protein phosphorylation. Proc Natl Acad Sci USA 73:3107

47. Moore G, Gewert DR, and Clemens MJ. (1984) Inhibition of cell proliferation by interferons. Eur J Biochem 139:627

48. Einert M, Resnitzky A, and Kimchi A. (1985) Close link between reduction of c-myc expression by interferon and G_0/G_1 arrest. Nature 313:597

49. Kawade Y and Watanabe (1986) On the nature of neutralization reaction between interferon and antibody. In The Biology of the Interferon System W.E. Stewart II and H. Schellekens eds. Elsevier Science Publishers, New York

50. Kohler G and Milstein C (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256:495

51. Rubin BY, Bartal AH, Anderson SL, Millet SK, Hirshaut Y, and Feit C. (1983) The anticellular and protein-inducing activities of human γ interferon preparations are mediated by the interferon. J Immunol 130:1019

52. Wang CY, Bushkin Y, Chen P-D, Plastsoucas CD and Long C. (1984) Preparation and characterization of monoclonal antibodies directed at epitopes of human IFN-γ. Hybridoma 3(4):32146.

53. Stefanos S, Wietzerbin J, Catinot L, Devos R, and Falcoff R. (1985) Characterization of antibodies against recombinant HuIFN-γ produced by hybridoma cells. J Interferon Res 5:45557.

54. Kelder B, Rashidbaigi A, and Pestka S. (1986) A sandwich Radioimmunoassay for human IFN-γ. In Methods in Enzymology edited by S. Pestka 119 pp582 New York: Academic Press

55. Liang C-M, Herren S, Sand A and Jost T. (1985) Study of antigenic epitopes recognized by monoclonal antibodies to recombinant interferon-gamma. Biochem Biophys Res Commun 128:171

56. Oda S, Akinaga S, Kumagai A, Inoue A, Nakamizo N, and Yoshida H. (1986) Generation of a new monoclonal antibody and its application for determination and purification of biologically active human gamma-interferon. Hybridoma 5:32957.

57. Meager A, Parti S, Barwick S, Spragg J, and 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 interferon. J Interferon Res 4:619

58. Van der Meide PH, Dubbeld M and Schellekens H. (1985) Monoclonal antibodies to human immune interferon and their use in a sensitive solid-phase ELISA. J Immunol Method 79:293

59. Ziai MR, Imberti L, Kobayashi M, Perussia B, Trinchieri G, and Ferrone S. (1986) Distinct functional domains on the recombinant human immune interferon molecule. Cancer Res 46:6187

60. Oleszak E, Feickert H-J, Mecs I, and Fox F. (1983) Mouse monoclonal antibody with specificity for human interferon gamma. Hybridoma 2:439

61. Farre C, Wijdenes J, Cabriliat H, Ojossou O, Banchereau J, and DE Vries JE. (1989) Epitope mapping of recombinant human gamma interferon using monoclonal antibodies. Mol Immunol 26(1):17

62. Alfa M, Dembinski J, and Jay FT. (1987) Production of monoclonal antibodies against recombinant human interferon-gamma: screening of hybridomas without purified antigen. Hybridoma 6(5):509

63. Alfa M and Jay FT. (1988) Distinct domains of recombinant human IFN-γ responsible for anti-viral effector function. J Immunol 141:2474

64. Schreiber RD, Hicks LJ, Celada A, Buchmeier NA, and Grey PW. (1985) Monoclonal antibodies to murine γ-interferon which differentially modulate macrophage activation and antiviral activity. J Immunol 134:1609

65. Ichimori Y, Suzuki N, Kitada C, and Tsukamoto K. (1987) Monoclonal antibodies to human interferon-γ II: antibodies with neutralizing activity. Hybridoma 6:173

66. Johnson HM, Langford MP, Lachohausa B, Chan T, and Stanton GJ. (1982) Neutralization of native human gamma interferon (HuIFN-γ) by antibodies to a synthetic peptide encoded by the 5' end of HuIFN-γ cDNA. J Immunol 129:2357

67. Fish EN, Banerjee K, Krakawa T, and Stebbing N. (1988) Structure/function studies on recombinant human gamma interferon. Drug Design and Deliv 2:19159.

68. Ichimori Y, Kurokawa T, Honda S, Suzuki N, Wakimasu M, and Tsukamoto K. (1985) Monoclonal antibodies to Human Interferon-γ I: Antibodies to a synthetic carboxyl-terminal peptide. J Immunol Methods 80:55

69. Lord SC, McMullen GL, Marzuki S, and Cheetham B. (1989) Functional domains of human interferon gamma probed with antipeptide antibodies. Mol Immunol 26(7):637

70. Arakawa T, Horan TP, Mcginoley M, and Rohde MF. (1990) Effect of amino-terminal processing by Staphylococcus aureus V-8 protease on activity and structure of recombinant human interferon-γ. J Interferon Res 10:321

71. Rose K, Simona MG, Offord RE, Prior CP, Otto B, and Thatcher DR. (1983) A new mass-spectrometric C-terminal sequencing technique finds a similarity between γ-interferon and α2-interferon and identifies a proteolytically clipped γ-interferon that retains full antiviral activity. Biochem J 215:273

72. Luk S K-S, Jay E, and Jay FT. (1990) Structure-function analysis of the human interferon γ. J Biol Chem 265:13314

73. Sakaguchi M, Honda S, Ozawa M, and Nichimura O. (1988) Human interferon-γ lacking 23 COOH-terminal amino acids is biologically active. FEBS Letts 230:201

74. Leinikki PO, Calderon J, LuQuette MH, and Schreiber RD. (1987) Reduced receptor binding by a human interferon-γ fragment lacking 11 carboxyl-terminal amino acids. J Immunol 139:3360

75. Arakawa T, Narachi MA, Hsu Y-R, Everett RR, Lai P-H, and Fish EN. (1989) The effect of C-terminal processing on the activity of human interferon-γ. Drug Design Deliv 4:217

76. Seelig GF, Wijdenes J, Nagabhushan TL, and Trotta PP. (1988) Evidence for a polypeptide segment at the carboxyl terminus of recombinant human γ interferon involved in expression of biological activity. Biochem 27:1981

77. Burton LE, Gray PW, Goeddel DV, and Rinderknecht E. (1985) Modifications of recombinant human and murine interferon-γ and their biochemical characteristics. In The Biology of the IFN System 1984 (Kirchner H and Schellekens H, eds) pp.403-409 Elsevier Science Publishers B.V., Amsterdam

78. Russell JK, Hayes MP, Carter JM, Torres BA, Dunn BM, Russel SW, and Johnson HM. (1986) Epitope and functional specificity of monoclonal antibodies to mouse interferon-γ: The synthetic peptide approach. J Immunol 136:332472.

79. Langford MP, Weight DA, Chan TS, Johnson HM, and Stanton GJ. (1987) Antibodies to the carboxyl terminus of mouse interferon-γ neutralize its immunoregulatory and antiviral activities. J Interferon Res 7:95

80. Langford MP, Gray PW, Stanton GJ, Lakhcharura B, Chan T-S, and Johnson HM. (1983) Antibodies to a synthetic peptide corresponding to the N-terminal end of mouse gamma interferon(IFN). Biochem Biophy Res Commun 117(3):866

81. Zavodny PJ, Petro ME, Chiang T-R, Narula SK, and Leibowitz PJ. (1988) Alterations of the amino terminus of murine interferon-γ: Expression and biological activity. J Interferon Res 8:483

82. Magazine HI, Carter JM, Russell JK, Torres BA, Dunn BM, and Johnson HM. (1988) Use of synthetic peptides to identify an N-terminal epitope on mouse γ interferon that may be involved in function. Proc Natl Acad Sci USA 85:1237

83. Jarpe MA and Johnson HM. (1990) Structure of an epitope in an immunodominant region of the interferon-γ molecule that is involved in receptor interaction. J Interferon Res 10:243

84. Evinger M, Rubinstein M, and Pestka S. (1981) Antiproliferative and antiviral activities of human leukocyte interferons. Arch Biochem Biophys 210:319

85. Eife R, Hahn T, De TAvera M, Schertel F, and Holtman H. (1981) A comparison of the antiproliferative and antiviral activities of α-, β- and γ- interferons: description of a unified assay for comparing both effects simultaneously. J Immunol Methods 47:339

86. Ortaldo JR, Mantovani A, Hobbs D, Rubinstein M, Pestka S, and Herberman RB. (1983) Effects of several species of human leukocyte interferon on cytotoxic activity of NK cells and monocytes. Int J Cancer 31:285

87. Ortaldo JR, Mason A, Rehberg E, Moschera J, Kelder B, and Pestka S. (1983) Effects of recombinant and hybrid recombinant human leukocyte interferons on cytotoxic activity of natrual killer cells. J Biol Chem 258:15011

88. Greiner JW, Fisher PB, Pestka S, and Schlom J. (1986) Differential effects of recombinant human leukocyte interferons on cell surface antigen expression. Cancer Res 46:4984

89. Giacomini P, Aguzzi A, Pestka S, Fisher PB, and Ferrone S. (1984) Modulation by recombinant DNA leukocyte (alpha) and fibroblast (beta) interferons of the expression and shedding of HLA- and tumor- associated antigens by human melanoma cells. J Immunol 133:1649

90. Ortaldo JR, Herberman RB, Harvey C, Osheroff P, Pan Y-C, Kelder B, and Pestka S. (1984) A species of human alpha interferon that lacks the ability to boost human natural killer activity. Proc Natl Acad Sci USA 81:4926

91. Jay E, Macknight D, Lutze-Wallace C, Harrison D, Wishart P, Lui W-Y, Asundi V, Pomeroy-Cloney L, Rommens J, Eglington L, Pawlak J, and Jay F. (1984) Chemical synthesis of a biologically active gene for human immune interferon-γ. J Biol Chem 259:6311

92. Jay E, Rommens J, Pomeroy-Cloney L, MacKnight D, Lutze-Wallace C, Wishart P, Harrison D, Lui W-Y, Asundi V, Dawood M, and Jay F. (1984) High-level expression of a chemically synthesized gene for human interferon-γ using a prokaryotic expression vector. Proc Natl Acad Sci. USA 81:2290

93. Tartof KD and Hobbs AC (1987) Focus (Idaho) 9:12

94. Schaffner W and Weissmann C. (1973) A rapid, sensitive, and specific method for the determination of protein in dilute solution. Anal Biochem 56:502

95. Fraker PJ and Speck JC. (1978) Protein cell membrane iodinations with a sparingly soluble chloramide 1,3,4,6-tetrachloro 3α, 6α, diphenylglycoluri. Biochem Biophys Res Commun 80:849

96. Sanceau J, Sondermeyer P, Florence B, Falcoff R, and Vaquero C. (1987) Intracellular human γ-interferon triggers an antiviral state in transformed murine L cells. Proc Natl Aced Sci USA 84:2906

97. Landsteiner K. (1936) The specificity of serological reactions. Springfield, IL: Charles C. Thomas

98. Brown RK, Delaney R, Levine L, Van Vunakis H. (1959) Studies on the antigenic structure of ribonuclease. I. General role of hydrogen and disulfide bonds. J Biol Chem 234:2043

99. Lerner RA. (1982) Tapping the immunological repertoire to produce antibodies of predetermined specificity. Nature 299:592

100. Shinka S, Imanishi M, Miyagawa N, Amano T, Inouye M, Tsugita A.)1967) Chemical studies on antigenic determinants of hen egg white lysozyme. I. Biken J 10:89

101. Gerwing J, Thompson K. (1968) Studies on the antigenic properties of eggwhite lysozyme. I. Isolation and characterization of a trypic peptide from reduced and alkylated lysozyme exhibiting haptenic activity. Biochemistry 7:3888

102. Young JD, Leung CY. (1970) Immunochemical studies on lysozyme and carboxymethylated lysozyme. Biochemistry 9:2755

103. Sela M. (1969) Antigenicity: some molecular aspects. Science 166:1365

104. Berzofsky JA. (1985) Intrinsic and extrinsic factors in protein antigenic structure. Science 229:932

105. Arnon R, Maron E, Sela M, Anfinsen CB. (1971) Antibodies reactive with native lysozyme elicited by a completely synthetic antigen. Proc Natl Acad Sci. USA 68:1450

106. Benjamin DC, Berzofsky JA, East IJ, Gurd FRN, Hannum C, Leach SJ, Margoliach E, Michael JG, Miller A, Prager EM, Reichlin M, Sercarz EE, Smithgill SJ, Todd PE, and Wilson AC. (1984) The antigenic structure of proteins: a reappraisal. Ann Rev Immunol 2:67

107. Schwyzer M, Weil R, Frank G, and Zuber H. (1980) Amino acid sequence analysis of fragments generated by partial proteolysis from large simian virus 40 tumor antigen. J Biol Chem 255:5627

108. Moelling K, Scott A, Dittmann KEJ, and Owada M. (1980) Effect of p15-associated protease from an avian RNA tumor virus on avian virus-specific polyprotein precursors. J Virol 33:680

109. Eisenberg RJ, Long D, Pereira L, Hampar B, Zweig M, and Cohen GH. (1982) Effect of monoclonal antibodies on limited proteolysis of native glycoprotein gD of herpes simplex virus type I. J Virol 41:478

110. Jammerson D and Paterson Y. (1986) Mapping epitopes on a protein antigen by the proteolysis of antigen-antibody complexes. Science 232:1001

111. Sheshberadaran H and Payne LG. (1988) Protein antigen-monoclonal antibody contact sites investigated by limited proteolysis of monoclonal antibody-bound antigen: Protein "footprinting" Proc Natl Acad Sci. USA 85:1

112. Suckau D, Kohl J, Karwath G, Schneider K, Casaretto M, Bitter-Suermann D, and Przybylski M. (1990) Molecular epitope identification by limited rpoteolysis of an immobiliqed antigen-antibody complex and mass spectrometric peptide mapping. Proc Natl Acad Sci. USA 87:9848

113. Maniatis T, Fritsch EF, Sambrook J (eds) (1982) Molecular cloning: A laboratory manual New York. Cold Spring Habor Laboratory

114. Aaij C and Borst P. (1972) The gel electrophresis of DNA. Biochem Biophys Acta 269:192

115. Weiss B. (1976) Endonuclease II of Escherichia coli is exonuclease III. J Biol Chem 251:1896

116. Laskowski M, Sr. (1980) Purification and properties of the mung bean nuclease.

Methods Enzymol 65:263

117. Young RA and Davis RW. (1983) Efficient isolation of genes using antibody probes. Proc Natl Acad Sci. USA 80:1194

118. Young RA and Davis RW. (1985) Immunoscreening lambda gt11 recombinant DNA expression libraries. In : Genetic Engineering 7, Selow JK, and Hollaender A; eds. Plenum Press, New York: 29-41

119. Ziai MR, Giordano A, Armandola E, and Ferrone S. (1988) Purification by ammonium sulfate precipitation of bacteriophage lambda gt11 DNA for restriction analysis of cloned cDNA inserts. Analytical Biochemistry 171:192

120. Goldberg AL and St. John AC. (1976) Intracellular protein degradation in mammalian and bacterial cells: part 2. Annu Rev Biochem 45:747

121. Charnay P, Gervais M, Louise A, Galibert F, and Tiollais P. (1980) Biosynthesis of hepatitis B virus surface antigen in E. coli. Nature 286:893

122. Edman JC, Hallewell RA, Valenzuela P, Goodman HM, and Rutter WJ. (1981) Synthesis of hepatitis B surface and core antigens in E. coli. Nature 291:503

123. Goeddel DV, Kleid DG, Boliver F, Heynecker HL, Yansura DG, Crea R, Hirose T, Krsszewski A, Itakura K, and Riggs A. (1979) Expression in Escherichia coli of chemically synthesized genes for human insulin. Proc Natl Acad Sci. USA 76:106

124. Davis AR, Nayak DP, Veda M, Hiti AL, Dowbenko D, and Kleid DG. (1981) Expression of antigenic determinants of the hemagglutinin gene of a human influenza virus in E. coli. Proc Natl Acad Sci. USA 78:5376