

In Vitro Interferon Production

Induction in Human Leukocytes after Transfected by Human Cytomegalovirus IE

Yuan-Hua Kao Advised by: Dr. Clark E. S. Huang

INTRODUCTION

Interferons (IFN) have been discovered by Isaacs and Lindenmann since 1957. (Fig.1.) They are a group of proteins with diverse biological activities. (Table.1.) The production of IFN is observed in the cells treated with various inducers. (Fig.2.) Various viruses have been used as the inducer, but the mechanism of the induction has not been clarified yet.

It's the fact that: When cytomegalovirus (CMV) infect leukocytes can induce IFN- α . So, I am curious about if we just transfect a fragment of CMV DNA into cells, the cells can produce IFN or not. Then I design the experiment.

MATERIALS

1. Cells: We use ML-3 or HL-60, both of them are premyelocytic leukemia cell lines. (Fig.3.) When they treated by DMSO and put into bank, they will differentiate to monocyte, and when they treated by PMA (phorbol myristate acetate), they will differentiate to macrophage. They are suspension cultured cell lines, it's different from monolayer culture. The medium we used are RPMI1640 free of HEPES, which is a

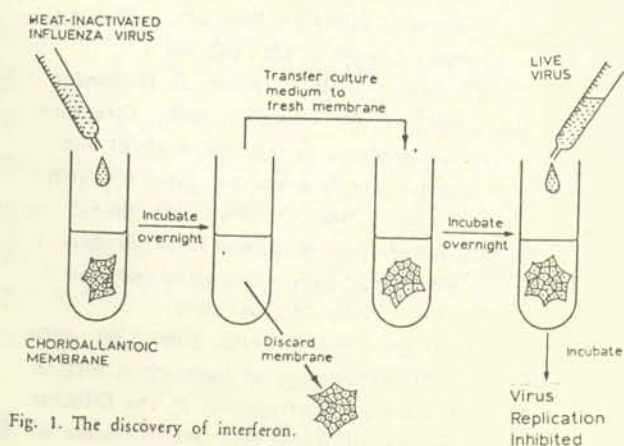


Fig. 1. The discovery of interferon.

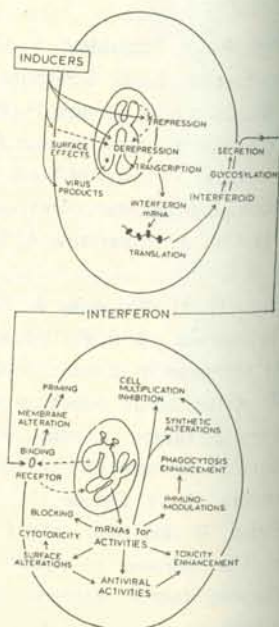


Fig. 2. The interferon system.

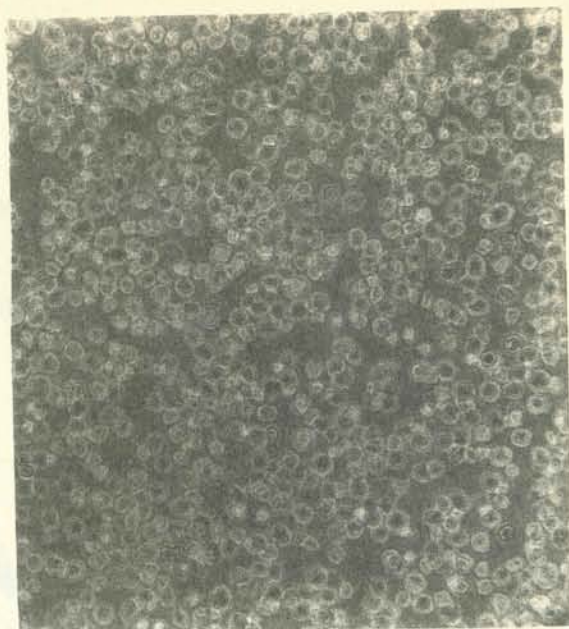
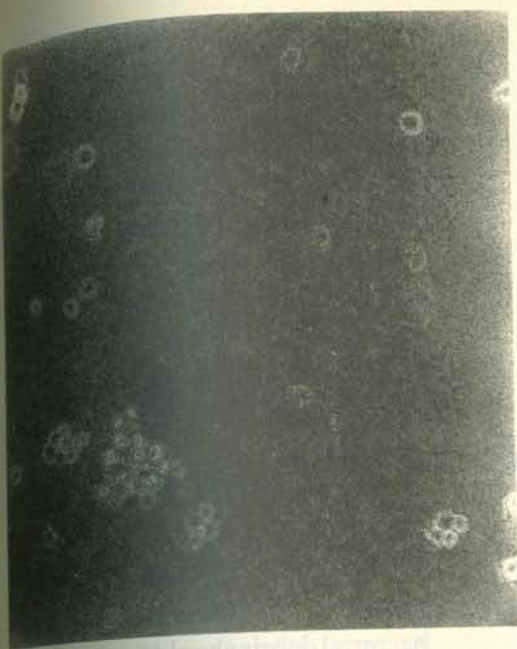


Fig. 3. Micrograph of ML-3 (left) and HL-60 (right).

useful buffer, but leukocytes are sensitive to it. With 20% Fetal Calf Serum (FCS), The serum should be heat inactivated by 56°C, 30 min. Add 100 unit/ml of streptomycin as well as 100µg/ml of penicillin. Then we add 1X of L-glutamine, the cells will grow better with it. The medium must be prepared as isotonic solution (260 mOsm/kg), and the pH value should be adjust from 7.2 to 7.4.

2. **Virus:** Human cytomegalovirus (Fig.4.), Towne strain used as positive control of IFN production.

3. **Plasmid DNA:** pBR322 (Fig.5.) used as negative control of transfection of CMV IE (immediately early) genes. And we chose IE1, IE2, and IE1+IE2 genes of CMV (Fig.6.) to tranfect leukocytes, because the IE1, IE2 genes can transactivate cellular proteins. They are inserted at the EcoR1 site of PSVOH, which is pBR322 plus SV40 HindIII fragment. (Fig.5.) Their names are: IE1-pHD101SV2, IE2-pHD1014, IE1+IE2-pHD101SV1.

We amplified the plasmid as followed:

1. Add 1µl of plasmid DNA to 20 µl of *E. coli* DH50α, which is competent cell of the plasmid.
2. Ice bath 30 min.
3. Heat shock, 42°C 90 sec. By this way, the plasmid can get into *E.coli*.
4. Ice bath 2 min.
5. Add 80µl SOC.
9. 37°C incubate over night.

B. Amplification:

1. Inoculate 10 ml of LB medium containing ampicillin with a single bacterial colony. Incubate at 37°C overnight with vigorous shaking.
2. The following days, inoculate 10

Table. 1. Physicochemical properties of human interferons

Property	α	β	γ
Previous designations	Le-IFN type I	F-IFN type I	immune IFN type II
Subtypes	> 12	1 (? more)	1 (??)
Mr			
Major subtypes	15,000–25,000	20,000	20,000–25,000
Cloned (non-glycosylated)	19,000	19,600	17,110
Glycosylation	no ^a	yes	yes
pH 2 stability	stable ^a	stable	labile
Induction	viruses	viruses	mitogens
Principal source	lymphocyte, etc.	fibroblast	lymphocyte
Activity in bovine cells	yes	no	no
Introns in gene	no	no	yes
Homology with HuIFN-α	80–95%	30–50%	< 10%

^a Most subtypes.

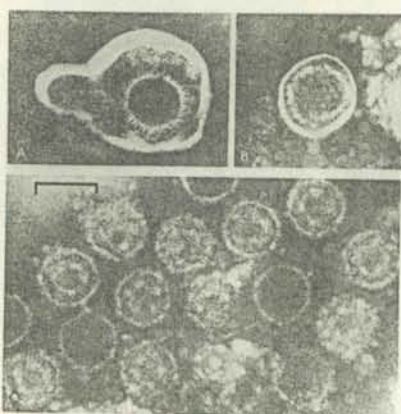


Figure 2.1. Electron micrograph of human cytomegalovirus. (A) An enveloped particle, although it is "empty." (B) A "full" enveloped particle. (C) Two "empty," many "full" but unenveloped particles. The bar is 100 nm. (Wright *et al.*, 1964. By permission of H. T. Wright, Jr., C. L. Goodheart, and editors of *Virology*.)

ml of the late log culture into 1000 ml of LB medium with ampicillin in a 2-liter flask. Incubate in 37°C for about 4 hrs with vigorous shaking. The OD₆₀₀ of the culture will be approximately 0.4.

3. Add 2.5 ml of a solution of chloramphenicol (34 mg/ml in ethanol). The final concentrate of chloramphenicol in the culture is 170 µg/ml.
4. Incubate at 37°C with vigorous shaking for a further 12-16 hrs.

C. Harvest:

1. Harvest the bacterial cells by centrifugation at 5000 rpm for 10 min. at 4°C. Discard the supernatant.
2. Wash in 200 ml of ice-cold STE (0.1 M NaCl, 10 ml Tris.Cl (pH7.8), and 1 mM EDTA).

D. Lysis by Alkali:

1. Resuspend the bacterial pellet from a 1-liter culture in 20 ml of solution I containing 5 mg/ml lysozyme.

Solution I

50 mM glucose
25 mM Tris.Cl (pH 8.0)
10 mM EDTA

2. Stand at room temperature for 30

min.

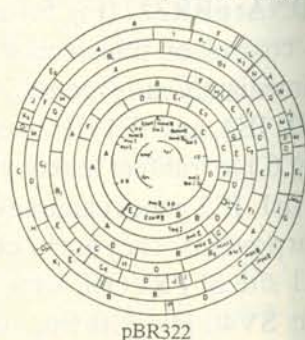
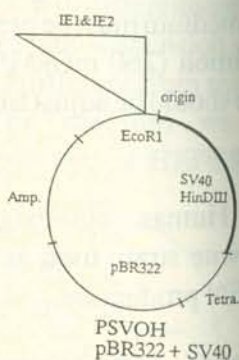
3. Add 40 ml of freshly made solution II. Cover the cap and mix the contents by gentle inverting the tube several times. Let stand on ice for 10 min.

Solution II

0.2 N NaOH

1% SDS

4. Add 30 ml of an ice-cold solution of a 5 M potassium acetate (pH 4.8).
5. Centrifuge at 9500 rpm for 25 min. at 4°C. The cell DNA and bacterial debris should form a tight pellet on the bottom of the tube.
6. Collect the supernatant, add 0.6 volumes of isopropanol to each tube. Mix well and let stand at room temperature for 15 min.
7. Recover the DNA by centrifugation at 9500 rpm for 30 min. at room temperature.



Size: 4340
Region: Col E1, relaxed
Restriction markers: Amp^r, Tet^r
Single sites: EcoRI, PstI, BamHI, XbaI, ClaI, SalI, SmaI, KpnI, HindIII
Insertion sites: Amp^r, Tet^r
References: Bolivar *et al.* (1977); Sambrook (1978, 1979)
Comments: pBR322 is the most widely used of the plasmid cloning vectors.
Its complete nucleotide sequence is known (Sussita 1979)

Fig. 5. pBR322 and PSVO11

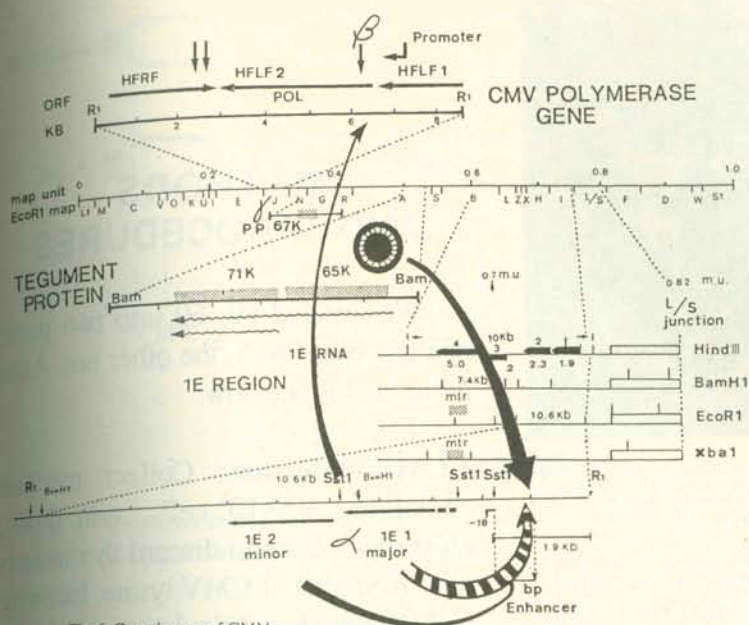


Fig. 6. Genetic map of CMV.

8. Discard the supernatant. Wash the pellet with 70% ethanol at room temperature. Discard as much ethanol as possible, then dry the nucleic acid pellet briefly in vacuum desiccator.
9. Dissolve the pellets in a total volume of 16 ml of TE (pH 8.0).

E. purification: (Fig.7.)

1. Measure the volume of the DNA solution. For every ml add exactly 1 g of solid CsCl. Mix gently until all of the salt is dissolved.
2. Add 0.8 ml of a solution of ethidium bromide (10 mg/ml in H₂O) for every 10 ml of CsCl solution. Mix well.
3. Transfer the CsCl solution to a quick sealed tube. Fill the remainder of the tube with light paraffin oil.
4. Centrifuge at 45000 rpm for 36 hrs. at 20°C.
5. AS Fig.7. show. Collect the lower band of DNA into a tube through a #21 hypodermic needle inserted into the side of the tube.
6. Remove the ethidium bromide as follows:

- a. Add an equal volume of 1-butanol.
- b. Mix the two phase by pipetting vigorously.
- c. Transfer the lower aqueous phase to a clean tube.
- d. Repeat the extraction 4-6 times until all the pink color disappears from the aqueous solution.

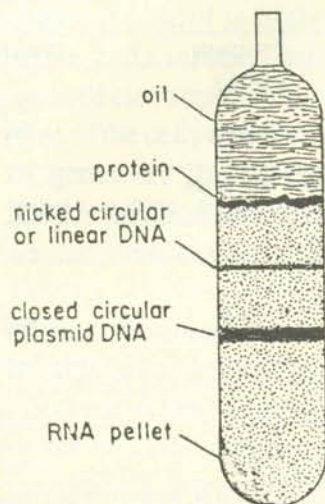


Fig. 7. Purification of plasmid DNA.

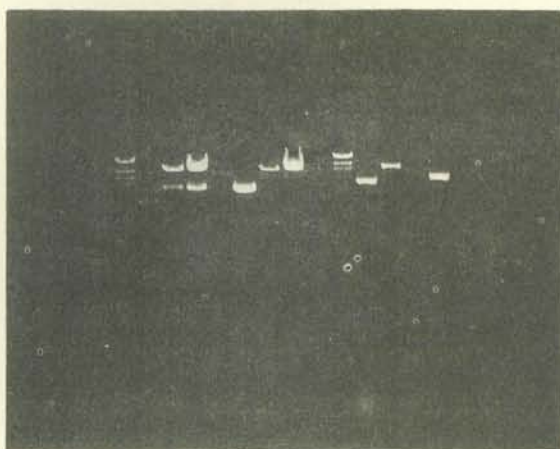


Fig. 8. Photograph of gel. From left to right: λ pBR322 digest by HindIII, SV1 (origin), SV1 (amplified), SV2, SV2, 1014, 1014, all cut by EcoRI. λ pBR322 cut by EcoRI, SV2 (origin 1), SV2 (origin 2), SV2(amplified), all cut by EcoRI; SV2, SV2, SV2, all cut by HindIII. There is something wrong with SV2 (origin 2), so we discard it.

ous phase against several changes of TE (pH 8.0).

F. Check: (Fig.8.)

1. Put dis. water, buffer, plasmid DNA, and enzyme (EcoRI, HindIII) into Eppendorf. Mix well.
2. 37°C digest 1 hr.
3. Add 2-3 μ l Dye, mix well.
4. Electrophoresis in 1% agarose gel with ethidium bromide.
5. Take picture of the gel.

4. Anti-IFN antibody with 125 I label to detect IFN.

First Incubation		
	Standards	Unknowns
Pipette IFN standard	200 μ l	
Unknown sample		200 μ l
Pipette 125 I-anti-IFN (yellow reagent)	50 μ l	50 μ l
Vortex mix tubes		
Incubate at room temperature (15-30°C) for 2 hours		
Second Incubation		
Ensure solid phase is thoroughly mixed.		
Add solid phase reagent (blue reagent).	50 μ l	50 μ l
Agitate at room temperature for 2 hours		
Separation		
Prewash	1ml	1ml
Separation Reagent	2ml	2ml
Stand tubes undisturbed for 15 minutes. Aspirate tubes (leaving approx. 0.3ml in each). Repeat separation step. Count tubes.		

Table. 2. Procedure of RIMA.

METHODS AND PROCEDURES

We divided HL-60 into two parts, one part add PMA, the other not. After 24 hr. as Fig.9. show:

1.CMV infection: Collect medium containing 3.5×10^7 cells, spin down, (1000 rpm, 5 min.) discard the supernatant. Add 3 ml of CMV lysate. Incubate in 37°C for 2 hrs. Spin down, collect the pellet. Add RPMI1640 medium to 7 ml. CO₂, 37°C incubate.

2. Transfection of plasmid DNA: Get 3.5×10^7 cells as pellet, add 0.5 ml pure RPMI1640 without serum. Add 30 μ g plasmid DNA, which has been precipitated by adding alcohol and dissolve in dis. water. Put the mixture into cuvette. Then electroporation (25 mF, 1000 volt, 0.001 sec.), by this way, the cell membrane will get some pores, the plasmid DNA will enter the cells. Then stand on ice for 10 min. Put the mixture into flask, add medium RPMI1640 with FCS to 7 ml CO₂, 37°C incubate.

When the infection and transfection have been done. We collect medium as well as cell pellets and count cells at 0, 24 hrs, 48 hrs, 72 hrs, and 96 hrs. later. We check the transfection works or not by:

1. Detect IE1, IE2 genes: Extract DNA from the cells, Then digest it with restriction enzyme. Transfer the DNA fragment to nylon paper by

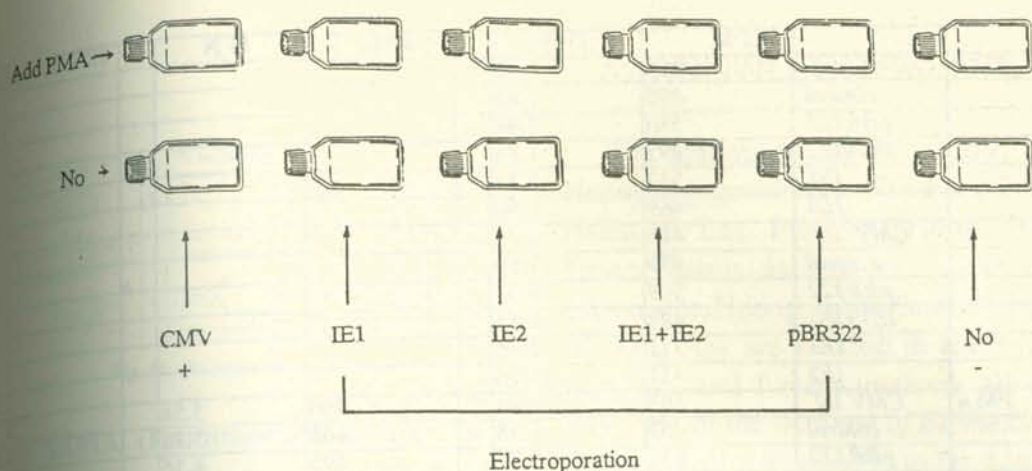


Fig. 9. Infection and transfection.
(+: positive control; -: negative control.)

southern blot. Hybridization with CMV IE gene probes.

2. Detect CMV-early nuclear Ag by indirect Immunofluorescence Assay. The procedure is:

1. Apply the cell onto slide, fix the cells by immersing the slides in 3% formaldehyde and acetone.
2. Wash with PBS. Apply the 1st Ab. Incubate at 37°C for 1 hr.
3. Wash with PBS. Apply the 2nd Ab (FITC). Incubate at 37°C for 1 hr.
4. Wash with PBS. Ready for investigation under fluorescent microscope.

And detect IFN- α , γ by Immunoradiometric Assay (IRMA), as Fig.10. and Table.2. show:

RESULTS

1. Cell count:

The results are shown into the next two pages.

2. Immunofluorescence Assay:

3. IRMA (Immunoradiometric Assay):

We did the assay for two times, then got the standard curve. (Fig.11.) We used the standard curve to interpolate IFN concentrations for unknown samples, which are list in the table 3.

DISCUSSION

1. Cell counts:

The survival rate of the cells for negative control is higher than that of the cells infected by CMV. because of electroporation, the survival rates of the cells transfected by CMV IE genes are lower, especially for IE2 transfection. But without repeat, we can't make any conclusions.

2. Immunofluorescence Assay:

By this way, we know that: CMV infect HL-60 and the same cells transfected by CMV IE genes are able to translate CMV-early nuclear protein. While as the negative control and pBR322 transfection cannot.

			CPM	IFN	CPM	IFN	IFN(Average)
0	PMA	CMV inf.	459	1.50			1.50
		- control	495	1.86			1.86
		pBR322	546	2.44			2.44
		IE1+IE2	493	1.84			1.84
		IE1	532	2.27			2.27
		IE2	469	1.60			1.60
0		CMV inf.	485	1.75			1.75
		- control	479	1.69			1.69
		pBR322	509	2.01			2.01
		IE1+IE2	493	1.84			1.84
		IE1	571	2.76			2.76
		IE2	431	1.26			1.26
1	PMA	CMV inf.	493	1.84	460	3.58	3.58
		- control	467	1.58	468	3.71	3.71
		pBR322	532	2.27	495	4.16	4.16
		IE1+IE2	506	1.97	447	3.38	3.38
		IE1	576	2.83	478	3.87	3.87
		IE2	532	2.27	442	3.31	3.31
1		CMV inf.	596	3.11	458	3.55	3.55
		- control	510	2.02	438	3.25	3.25
		pBR322	556	2.56	482	3.94	3.94
		IE1+IE2	479	1.69	588	5.88	5.88
		IE1	512	2.04	571	5.54	5.54
		IE2	531	2.26	510	4.41	4.41
2	PMA	CMV inf.	558	2.59	496	4.17	4.17
		- control	468	1.59	446	3.37	3.37
		pBR322	568	2.72	538	4.92	4.92
		IE1+IE2	500	1.91	442	3.31	3.31
		IE1	567	2.71	455	3.51	3.51
		IE2	473	1.64	433	3.17	3.17
2		CMV inf.	552	2.51	445	3.35	3.35
		- control	486	1.76	438	3.25	3.25
		pBR322	522	2.15	495	4.16	4.16
		IE1+IE2	613	3.36	453	3.48	3.48
		IE1	544	2.41	457	3.54	3.54
		IE2	536	2.32	489	4.05	4.05
3	PMA	CMV inf.	579	2.87	465	3.66	3.66
		- control	535	2.30	469	3.73	3.73
		pBR322	546	2.44	558	5.29	5.29
		IE1+IE2	537	2.33	469	3.73	3.73
		IE1	520	2.13	583	5.78	5.78
		IE2	535	2.30	530	4.77	4.77
3		CMV inf.	472	1.63	485	3.99	3.99
		- control	504	1.95	399	2.69	2.69
		pBR322	486	1.76	682	7.93	7.93
		IE1+IE2	544	2.41	496	4.17	4.17
		IE1	479	1.69	639	6.95	6.95
		IE2	531	2.26	436	3.22	3.22
4	PMA	CMV inf.	512	2.04			
		- control	633	3.68			
		pBR322	506	1.97			
		IE1+IE2	545	2.42			
		IE1	541	2.38			
		IE2	523	2.16			
4		CMV inf.	485	1.75			
		- control	546	2.44			
		pBR322	550	2.49			
		IE1+IE2	659	4.11			
		IE1	476	1.66			
		IE2	490	1.80			

Table. 3. Titer of IFN production (U/ml).

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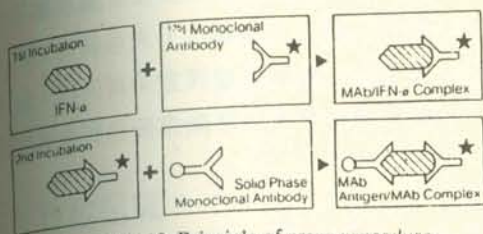


Fig. 10. Principle of assay procedure.

3. IRMA (Immunoradiometric Assay):

Because the titers of interferon production (U/ml) are very low, we can't make any conclusions. The reasons may be: 1). The cell numbers of the medium for assay are too low. 2). CMV is a dsDNA virus, which can't induce much IFN. (By others' research, CMV can induce about 40 U/ml IFN.) So, if we want to get more reliable data, we have to repeat the whole experiment.

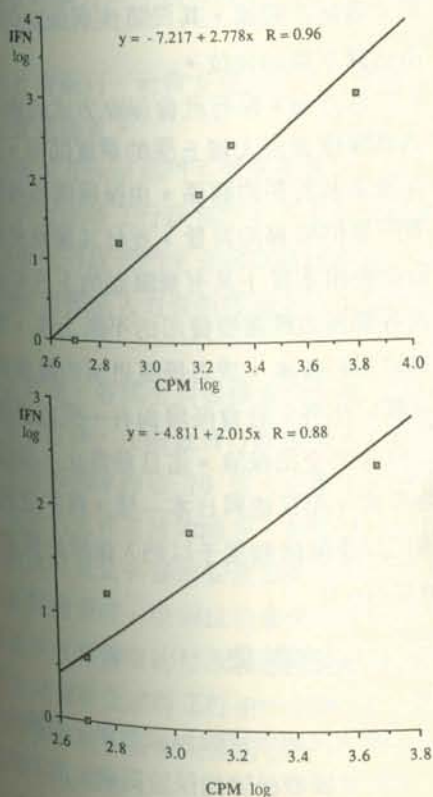


Fig. 11. Standard curves of IFN production.