CHAPTER IV

RESULTS

1. HCV genotyping methods

Although direct sequencing is the standard method reliably used for genotyping, there are several methods suggested for preliminary or screening the genotype as well. In this study, four approaches, RFLP with two different sets of enzymes, direct sequencing, and INNO-LiPA assay were performed as described in Materials and Methods to genotype HCV from selected thirty-five samples (as mentioned in study population).

All amplified PCR fragments covering the –21 to 383 region from 35 samples were digested with *Ava* I /*Sma* I, or *Acc* I/*Mbo* I/*Bst*N I and also sequenced directly. For INNO-LiPA assay, after hybridized labeled PCR products to immobilized oligonucleotide probes, the pattern of positive line numbers on strip were interpreted with the interpretation chart given by the manufacturer's protocol to deduce HCV genotypes. Results of all four genotyping methods were shown in Table 7. For RFLP using *Ava* I/ *Sma* I enzymes, only HCV genotype 1 (1a or 1b) from INNO-LiPA method were performed according to the purpose of the authors. The other genotypes were indicated as N.D. in the Table.

Table 7 HCV genotyping results of 35 samples* by four different methods.

Number	Sequencing	INNO-LiPA	RFLP *Ava* I/*Sma* I	RFLP *Acc* I/*Mbo* I/*Bst*N I
J1	1a	1a	1a	1a
J2	1b	1b	1b	1b
J3	3a	3a	N.D.[a]	3a
J4	1b	1b	1b	1b
J5	1a	1a	1a	1a
J6	1a	1a	1a	1a
J7	2a	2a	N.D.	Untypable
J8	1a	1a	1a	1a
J9	2	2a	N.D.	Untypable
J10	1a	1a	1a	1a
J11	3b	1	6a	1a
J12	1a	1	1a	1a
J13	1a	1a/1b	1a	1a
J14	1b	1b	1b	Untypable
J15	6a	1b	6a	6a
J16	1b	1b	1b	1b
J17	3b	3	N.D.	3a
J18	3a	3a	N.D.	3a
J19	3a	3a	N.D.	3a
J20	3a	3a	N.D.	3a
J21	6a	1b	6a	6a
J22	6a	1b	6a	3a
J23	6a	1b	6a	2c
J24	6a	1b	6a	3a
J25	6a	1b	6a	Untypable
J26	1a	1	1a	1a
J27	1a	1	1a	1a

Table 7 (continued)

Number	Sequencing	INNO-LiPA	RFLP *Ava* I/*Sma* I	RFLP *Acc* I/*Mbo* I/*Bst*N I
J28	3a	3a	N.D.	3a
J29	3a	1b/3a	Untypable	Untypable
J30	3a	3a	N.D.	1a
J31	1b	1b	1b	1b
J32	1a	1	1a	1a
J33	3a	1b	6a	Untypable
J34	3a	3a	N.D.	3a
J35	3a	3a	N.D.	3a

* Plasma samples were obtained from ten chronic HCV patients from the Erasmus MC, Rotterdam, The Netherlands (J1-J10), 18 HCV RNA positive blood donors from the National Blood Center of the Thai Red Cross, Bangkok (J11-J28), and seven chronic HCV patients from Phra Mongkutklao Hospital, Bangkok (J29-J35)

[a] N.D.= not determined

When the results of each method were compared with direct sequencing, RFLP with *Acc* I/*Mbo* I/*Bst*N I gave discordant results in several samples (J11, J17, J22, J23, J24, J30), and RFLP with *Ava* I/*Sma* I gave discordant results in two samples (J11, J33). In addition, six out of 35 samples were untypable by RFLP (J7, J9, J14, J25, J29, J33). For the INNO-LiPA assay, eight samples (J11, J15, J21, J22, J23, J24, J25, J33) gave discordant results when sequencing data were compared. All samples genotyped as 6a by sequencing were interpreted as 1b by INNO-LiPA assay as shown in Figure 7. As mentioned earlier, it has been shown by Mellor et al [12] that samples that appear to be type 1 in the INNO-LiPA may have been type 6 variant (6a) so the INNO-LiPA assay cannot distinguish some novel genotypes discovered in Thailand and Vietnam because of almost identical in 5'NCR sequences to type 1 viruses. According to these data, among all methods tested in this study, direct sequencing is still the best method for

HCV genotyping, in particular, for genotype 6 variants. For that reason, direct sequencing was used for HCV genotyping in the following experiments.

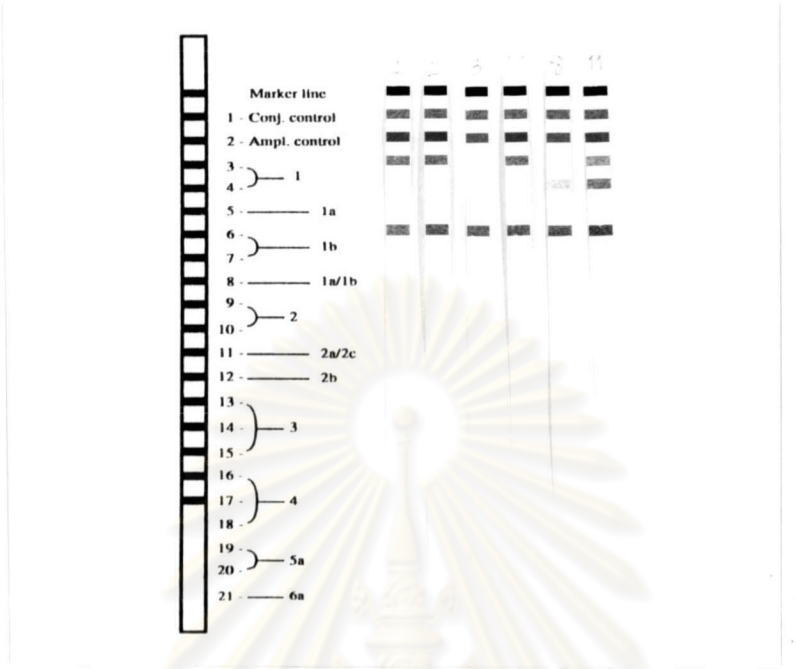


Figure 7 INNO-LiPA assay of six genotype 6a genotyped by INNO-LiPA assay as genotype 1b

In addition, the modified RFLP method by using 3 enzymes (*Ava* I/*Sma* I/*Mbo* I) was performed in order to genotype HCV commonly found in Thailand. *Ava* I and *Sma* I could distinguish genotype 1 from others according to the electropherotype patterns. The other enzyme, *Mbo* I, used for discriminating genotype 3 from 6a in non-genotype 1 group.

Examples of RFLP patterns are shown in Figure 8 and 9. I, II, III, and IV are sample numbers. In Figure 6, after being digested with *Ava* I (left) and *Sma* I (right), sample I showed pattern A1S1 (1a); sample II was A3S1 (1b); samples III and IV gave the same pattern, A12S5, which is identical to genotype 6a (pattern interpretation was described in Materials and Methods). After digestion with *Mbo* I (Figure 9), sample III and IV that gave the same pattern when being digested with *Ava* I and *Sma* I, gave different electropherotype patterns; sample III and IV were characterized as genotype 3 and 6a, respectively. However, digestion with other enzymes is needed for confirmation.

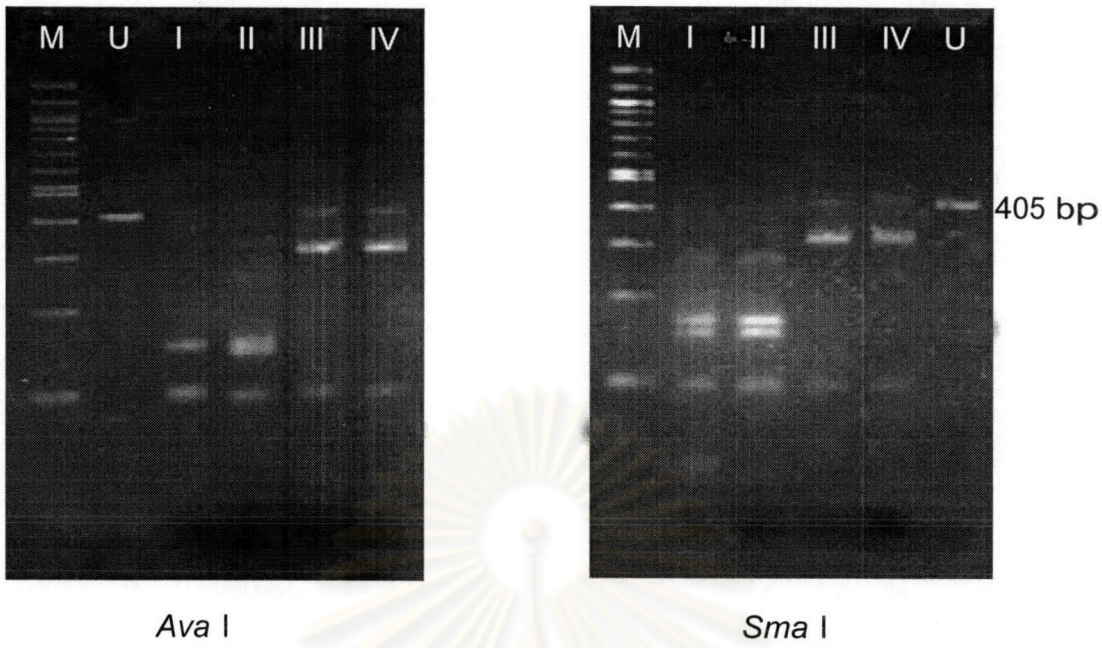


Figure 8 The examples of PCR-RFLP patterns. Four samples (I- IV) after *Ava* I (left) and *Sma* I (right) digestion. M=molecular markers, U=Uncut (405 bp) fragment

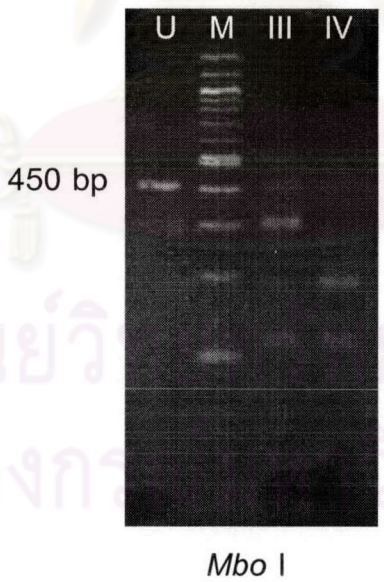


Figure 9 The examples of PCR-RFLP patterns. Samples III and IV (non genotype 1) were digested with *Mbo* I. M=molecular markers, U=Uncut (405 bp) fragment.

2. Distribution of HCV genotypes

In order to identify HCV genotypes from samples collected for this study and also provide the information on HCV genotype distribution in Thai blood donors, genotyping by direct sequencing was performed.

One hundred anti-HCV positive blood donors from at the National Blood Center, Thai Red Cross, Bangkok, Thailand were included in this study. All samples were kept anonymous, indicated only by the code number as shown in APPENDIX B. Ninety were found HCV-RNA positive, however, there were only 77 samples available for further study. The data obtained from 77 samples by direct sequencing were aligned with the 11 known genotype sequences and analyzed. HCV sequences on the same node were interpreted to belong to the identical genotypes. The distribution of HCV genotype observed in our cohort are shown in Figure 10. New sequences obtained in the study have been submitted to GenBank and have been assigned accession numbers as shown in APPENDIX B. All details with respect to the sequencing were previous described by Theamboonlers et al [148].

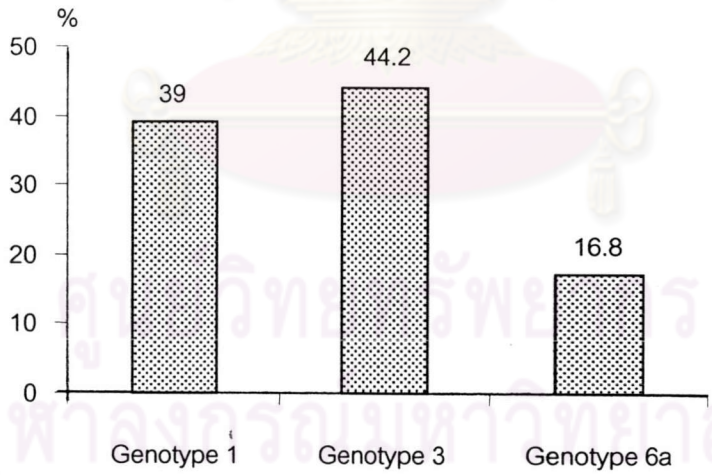


Figure 10 HCV genotypic distribution generated by direct sequencing from 77 HCV RNA positive blood donors used in genotyping study.

3. HCV specific immune response studies

3.1 by using soluble proteins

Since the study of immune response to HCV proteins required PBMCs which could not be recovered following frozen and transportation processes from some samples collected. According to the availability of samples, only 41 samples were selected for further studies. HCV genotypes from 41 samples were 15 genotype 1, 18 genotype 3, and 8 genotype 6a, respectively. In genotype 1 group, 6 samples were 1a and 9 were 1b. For genotype 3 group, all except 2 samples were 3a as shown in Table 8. The genotypic distribution of the samples included in HCV specific response study is shown in Figure 11 which the distribution was similar to the distribution obtained from 77 samples used in genotyping study. This indicated that the samples used in both genotyping and HCV specific response study had the similar distribution of HCV genotypes. The phylogenetic tree of 41 core based sequences is shown in Figure 12.

Table 8 The HCV genotype of 41 blood donors included in HCV specific response study.

genotype	1		3		6
	1a	1b	3a	3b	
number	6	9	16	2	8

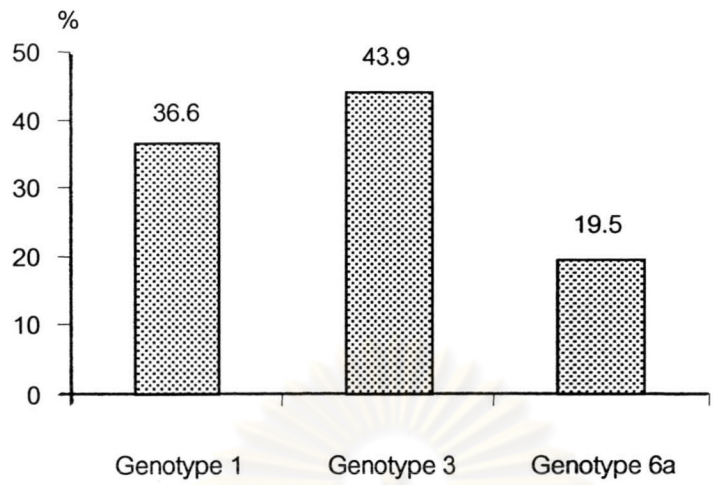


Figure 11 HCV genotypic distribution in 41 selected blood donors used for HCV specific response study.

In order to determine immune response of isolated PBMC to HCV proteins, 2 methods, a lymphocyte proliferation and an IFN-γ ELISPOT assay, were performed. The result of all samples from both assays are summarized in Table 9.

3.1.1 HCV specific proliferation assay

The ability of HCV core (c22-3), NS3/4 (c-200) and NS5 proteins to induce proliferation of PBMCs from 41 selected samples and 10 control subjects was determined by ^{3}H-thymidine proliferation assay. SOD protein (SOD-SDS), influenza antigen (FLU) and PHA were used as controls.

The SI and Δcpm of all positive samples for antigens used are shown in Table 10 and Figure 13. There was no significant difference in proliferation among samples with different HCV genotypes. The results (SI and Δcpm) from 10 anti-HCV negative blood donors were lower than 3 and 2000, respectively when tested with HCV antigens, but they still had positive result with PHA. (data not shown)

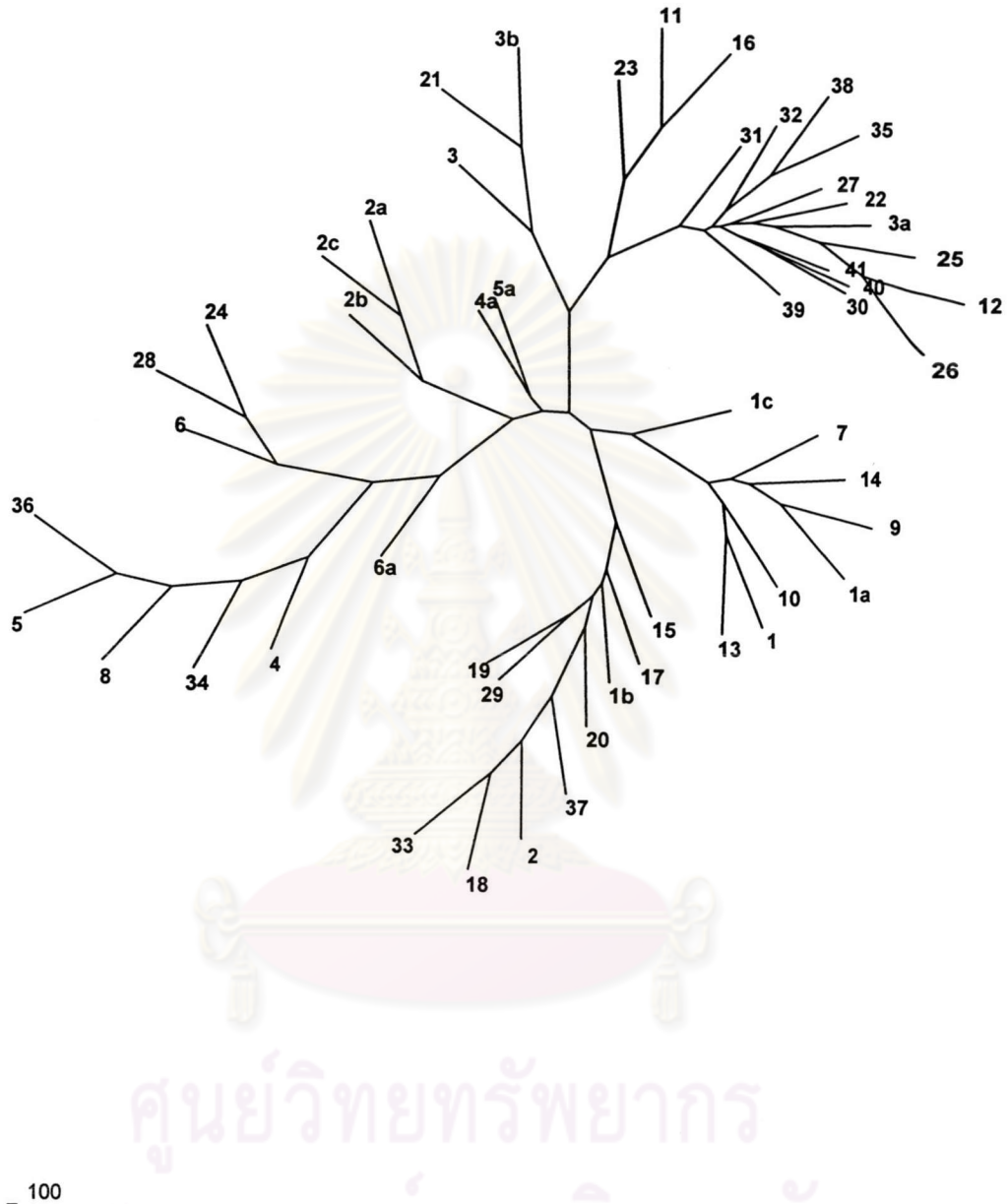


Figure 12 Phylogenetic tree of 41 core sequences included in HCV specific response study

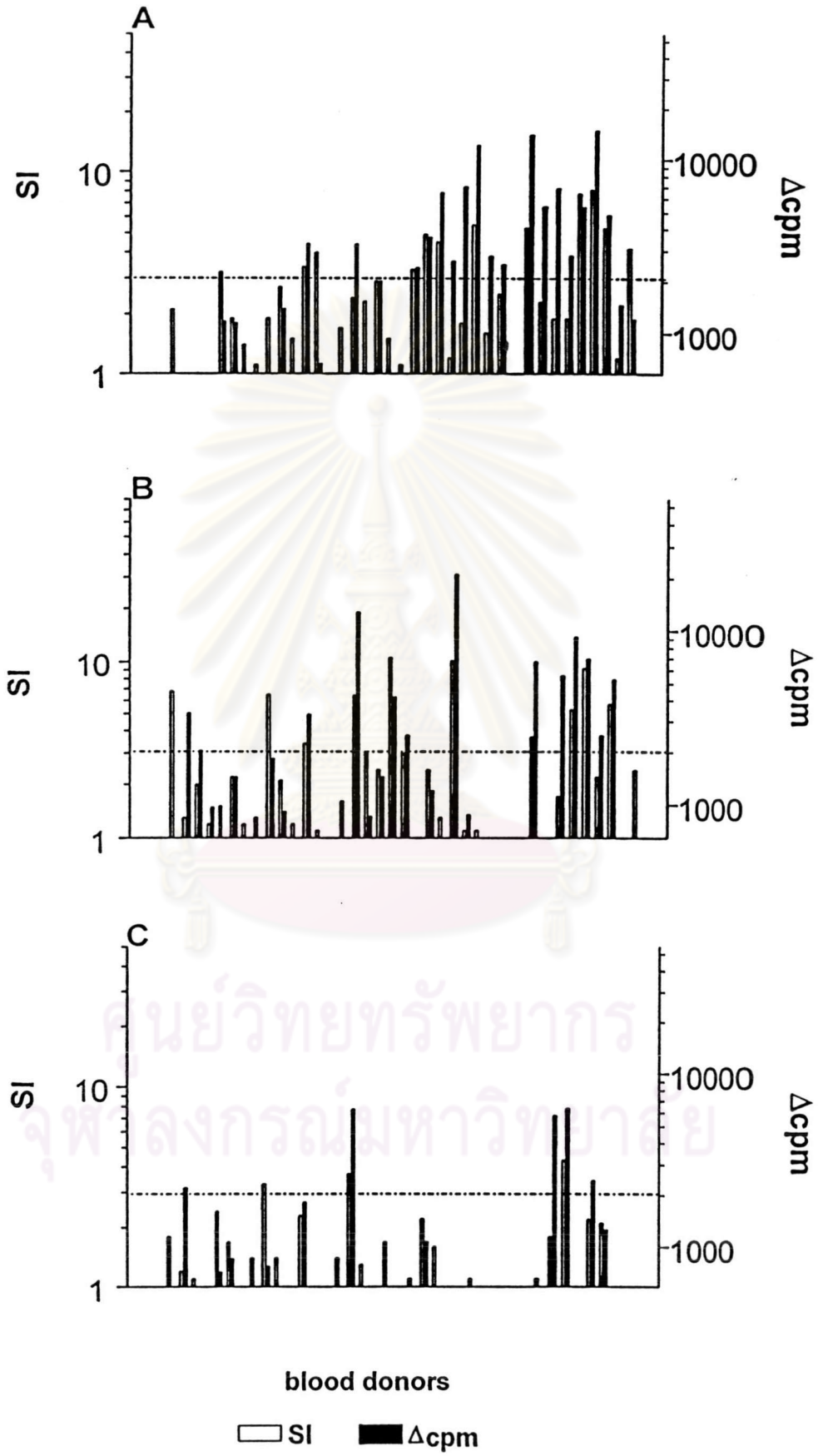
Table 9 Characterization and HCV specific responses against different HCV proteins obtained from 41 selected blood donors.

number	code	genotype	Proliferation			IFN-γ production		
			core	NS3/4	NS5	core	NS3/4	NS5
1	07265	1a	-	-	-	-	-	-
2	06804	1b	-	-	-	-	-	-
3	05927	3b	-	-	-	-	-	-
4	70646	6a	-	-	-	-	-	-
5	74822	6a	-	-	-	-	-	-
6	11885	6a	-	-	-	-	-	-
7	11352	1a	-	-	-	-	-	-
8	10442	6a	-	-	-	-	-	-
9	08166	1a	-	-	-	-	-	-
10	09723	1a	-	-	-	-	-	-
11	09715	3a	-	-	-	-	-	-
12	07893	3a	-	-	-	-	-	-
13	09955	1a	-	-	-	+	-	-
14	11882	1a	-	-	-	-	-	-
15	09612	1b	+	+	-	+	+	-
16	09555	3a	-	-	-	-	+	+
17	87190	1b	-	-	-	-	-	-
18	08035	1b	-	-	-	-	-	-
19	88283	1b	-	+	+	-	+	-
20	09896	1b	-	-	-	+	-	-
21	11025	3b	-	-	-	-	+	-
22	11671	3a	-	+	-	-	-	-
23	08782	3a	-	+	-	-	-	-
24	08795	6a	+	-	-	+	-	-
25	08720	3a	+	-	-	+	+	-
26	92597	3a	+	-	-	-	+	-
27	08629	3a	-	+	-	-	+	-
28	92855	6a	-	-	-	-	-	-
29	95263	1b	+	-	-	+	+	+

Table 9 (continued)

number	code	genotype	Proliferation			IFN-γ production		
			core	NS3/4	NS5	core	NS3/4	NS5
30	13590	3a	-	-	-	+	-	-
31	11224	3a	-	-	-	-	+	-
32	97197	3a	-	-	-	-	-	-
33	09152	1b	+	+	-	-	+	+
34	02234	6a	-	-	-	-	-	-
35	16800	3a	-	-	-	-	-	-
36	05458	6a	-	+	+	-	+	-
37	10335	1b	+	+	-	-	-	-
38	14304	3a	+	-	-	-	-	-
39	11614	3a	+	+	-	-	-	+
40	12001	3a	-	-	-	-	-	-
41	12208	3a	-	-	-	-	-	-

Note : - = negative result (SI<3 and Δcpm <2000 in proliferation test, no spot in ELISPOT assay)



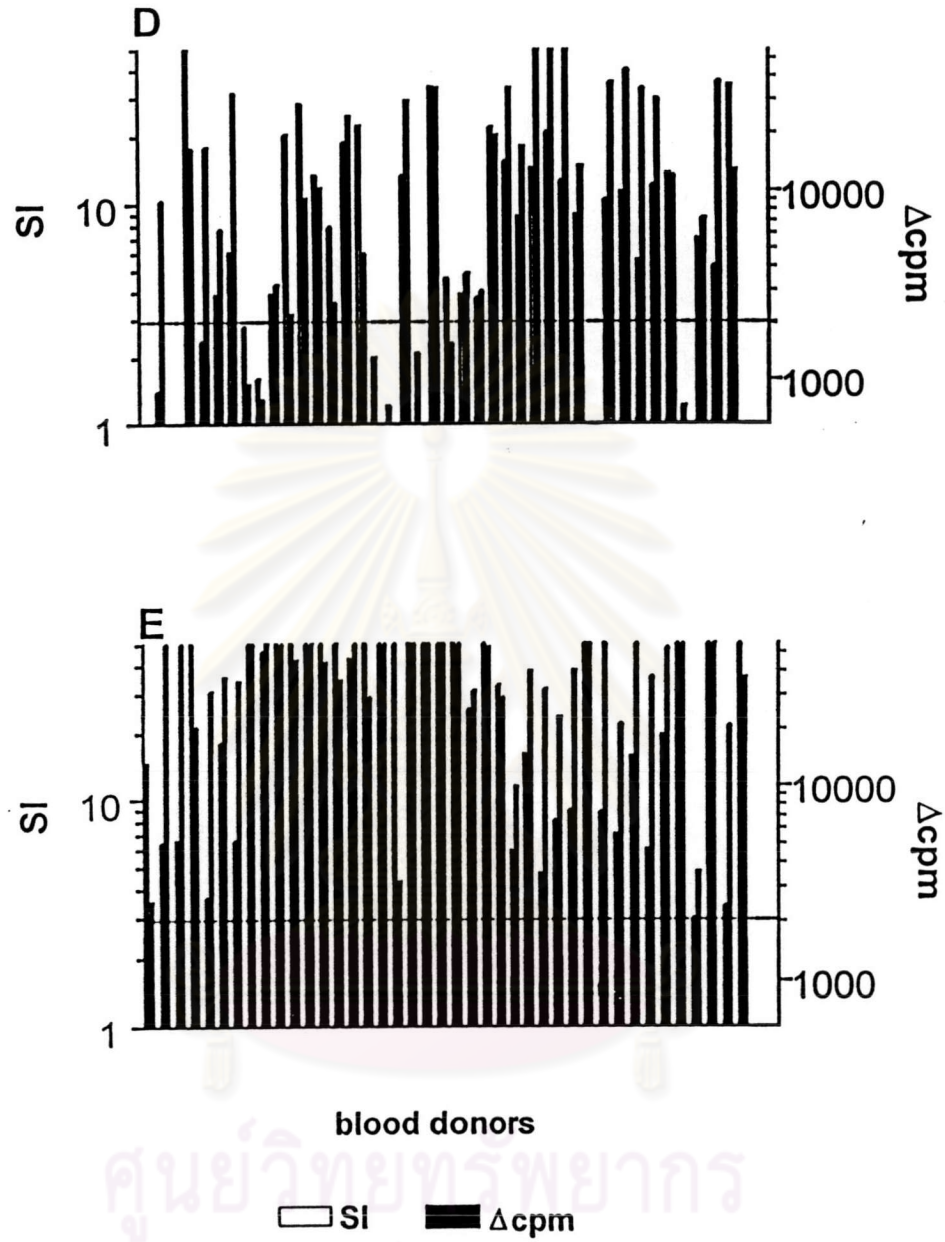


Figure 13 The SI and Δcpm obtained from HCV specific proliferation assay in 41 selected blood donors. Proliferative response to core (c22-3) (A), NS3/4 (c-200) (B), NS5 (C), FLU (D) and PHA (E) were shown individually. The cut off of the positive response was SI≥3 and Δcpm>2000

Table 10 SI and Δcpm of all samples positive by proliferation assay. The criteria for positive response were SI≥3 and Δcpm >2000

Number	Code	C22-3		C200		NS5	
		SI	Δcpm	SI	Δcpm	SI	Δcpm
15	09612	3.4	3,251.6	3.4	3,295.6	N	N
19	88283	N	N	6.4	12,753	3.7	6,280.5
22	11671	N	N	10.5	4,173.3	N	N
23	08782	N	N	3	2,485	N	N
24	08795	3.3	2,383.5	N	N	N	N
25	08720	4.9	3,557	N	N	N	N
26	92597	4.5	6,391.2	N	N	N	N
27	08629	N	N	10.1	20,938	N	N
29	95263	3.7	11,952	N	N	N	N
33	09152	5.3	13,815.5	3.7	6,672.5	N	N
36	05458	N	N	5.3	9,216.5	4.3	6,347.3
37	10335	7.8	5,320.5	9.2	6,939.5	N	N
38	14304	8.2	14,761.2	N	N	N	N
39	11614	5.3	4,801.5	5.7	5,273	N	N

Note: 1. all samples were positive against PHA as shown in Figure 13

2.The average SI and Δcpm of negative control were lower than 3 and 2000,respectively.

3. N=Negative result, the criteria as in 2

3.1.2 IFN-γ ELISPOT assay

The ability of HCV proteins to induce IFN-γ production by PBMCs was also determined by ELISPOT assay. All control antigens used were the same as in the proliferation assay. The number of positive spots obtained from positive were shown in Table 11. There was no significant difference in IFN-γ production observed in subjects infected with different genotypes.

Table 11 The number of IFN-γ spots/2x10^5 PBMCs of all samples positive by ELISPOT assay

Number	Code	Number of IFN-γ spots/2x10^5 PBMCs				
		c-22-3	c200	NS5	PHA	FLU
13	09955	20	-	-	44	12
15	09612	26	14	-	40	12
16	09555	-	18	20	45	-
19	88283	-	15	-	42	-
20	09896	16	-	-	40	-
21	11025	-	12	-	58	17
24	08795	18	-	-	74	-
25	08720	13	12	-	64	-
26	92597	-	17	-	60	-
27	08629	-	14	-	80	16
29	95263	25	25	20	92	22
30	13590	11	-	-	60	15
31	11224	-	16	-	80	-
33	09152	-	15	14	85	12
36	05458	-	15	-	82	-
39	11614	-	-	12	75	16

Note : 1. FLU = Influenza virus

2.There was no spot detected from negative control

3. – = no spot in well

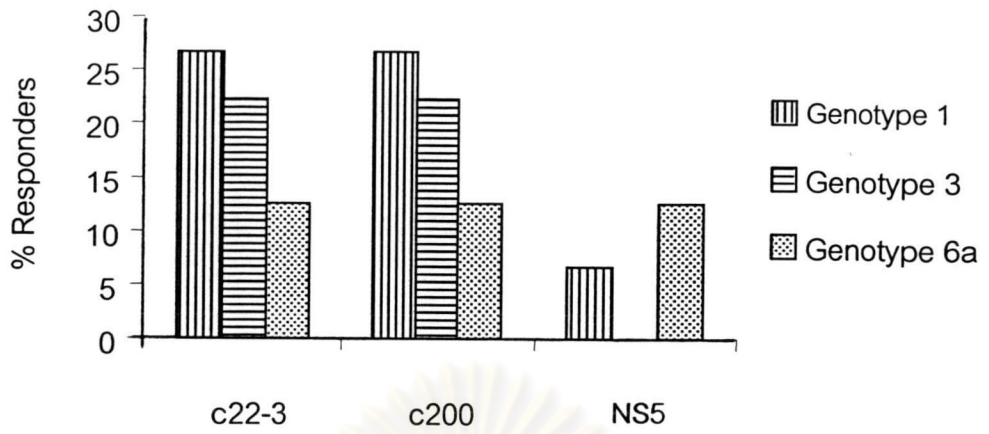


Figure 14 Proliferation assay of PBMCs from 41 blood donors. Data represent percentages of individuals infected with different genotypes showed a significant response (SI≥3, Δcpm>2000) to 3 HCV antigens ; core (c22-3), NS3/4 (c200), NS5

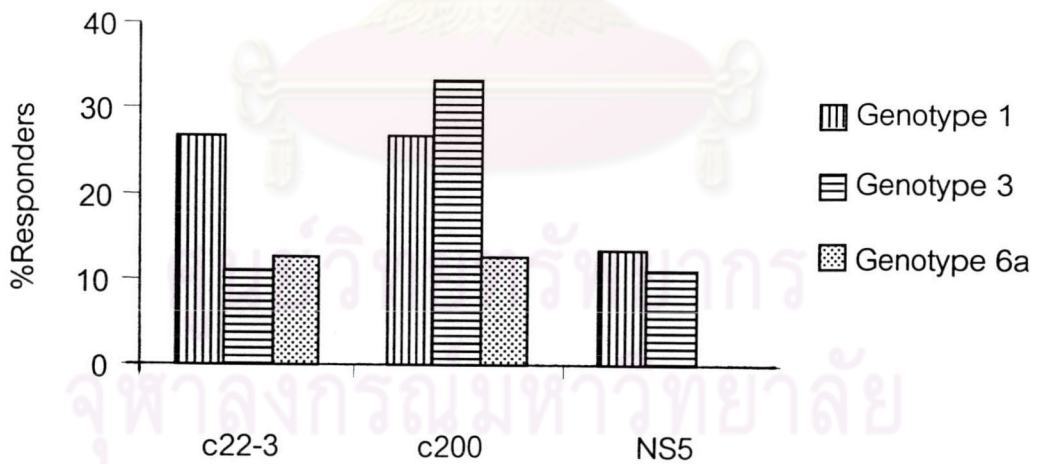


Figure 15 IFN-γ ELISPOT assay of PBMCs from 41 blood donors. Data represent percentages of individuals infected with different genotypes and showed a significant response to 3 HCV antigens ; core (c22-3), NS3/4 (c200), NS5.

The results from both proliferation and IFN-γ production are also summarized in Table 12, Figure 14 and 15. There was no significantly different (p>0.05) among different HCV genotypes in responses to HCV 1a antigens. The proliferation and the IFN-γ production were not correlated such as found in genotype 6a (13% vs 0%) and also found in genotype 3 (0% vs 10%) when tested with NS5 protein as shown in Figure 14 and 15. In proliferation assay, % responders of each genotype when tested with core (c-22-3) and NS3/4 (c-200) were the same. There was the lowest of % responders observed in all genotypes when tested with NS5 protein when compared with core and NS3/4 in both assays.

If a positive result from one assay is considered there is a response, from 41 selected samples, number of samples showed responses to c22-3 and c-200 were 26.8% and 36.6%, respectively. However, only 12.2% of samples showed a significant response to NS5 as shown in Figure 16. Six out of 14 samples (42.8%) gave positive results in proliferation test induced by more than one antigen (Table 10) and 5 out of 16 (31.2%) gave positive results by IFN-γ ELISPOT assay induced by more than one antigen (Table 11). The NS3/4 (c-200) may be the most immunogenic proteins observed in this study.

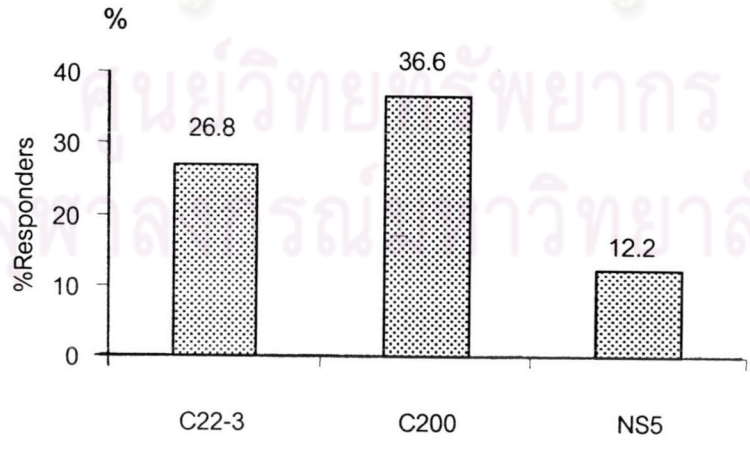


Figure 16 The percentages of samples positive by either proliferation or IFN-γ ELISPOT assay against HCV antigens.

Table 12 Percentages of samples in each genotype showed positive responses for either proliferation assay or IFN-γ production, for both assays and showed negative response for both assays.

Genotype (number)	positive response by (%)			no response at all (%)
	proliferation assay	IFN-γ production	both assays	
1a (6)	0	16.6	0	83.4
1b (9)	11.1	11.1	44.5	33.3
3a (16)	18.75	18.75	25	37.5
3b (2)	0	50	0	50
6a (8)	0	0	25	75

Despite the fact that the antigens were derived from HCV genotype 1a, the PBMCs from donors infected with HCV 1a had the highest percentages of no response from both assays.

3.2 by using constructed plasmid containing NS3 gene

For study of T cell response to certain antigens, soluble antigens can be added in the presence of antigen presenting cells and T cell response can be measured. However, antigens can be made so it can be expressed and introduced into certain cells; then these cells are used to stimulate T cell response. In general, stimulator cells used are autologous BLCLs infected with recombinant vaccinia virus carrying a gene encoding antigen of interest have been used as stimulators for studying of class I-restricted effector function. However, with vaccinia virus, manipulation such as making a new recombinant is difficult and the virus may cause cytopathic effect on the stimulators. Plasmids constructs expressing proteins of interest has become promissing tools for induction of class I antigen presentation. This study had attempted to construct a plasmid expressing HCV protein, transfect it into cells and use these transfected cells

as stimulators for T cell stimulation. The stimulation efficiency was compared with that of recombinant vaccinia virus.

The pNS plasmid carrying part of NNRd region was constructed as described in Materials and Methods. Liver-infiltrating lymphocytes (LILs) of patient number 816 was used since the preliminary data demonstrated that only LILs from this patient showed positive response only to NNRd measured by IFN-γ production (as shown in Table 13) when they were stimulated with rVV-HCV infected BLCLs. (Figure 17)

Table 13 Proliferative response of liver-infiltrating lymphocytes (LILs) from 7 chronic HCV patients used for immunological study against HCV infection.

Chronic HCV patients (LILs)	HCV genotype	HCV specificity* (region)	T cell profiles (%)		
			$CD4^+$	$CD8^+$	Others
809	1a	-	35	23	43
811	3b	-	28	46	26
812	3a	-	30	66	23
814	4a	-	24	54	22
816	1b	+ (NNRd)	34	54	12
822	3a	-	3	71	27
823	2b	-	23	71	5

* detected by IFN-γ production ELISPOT assay

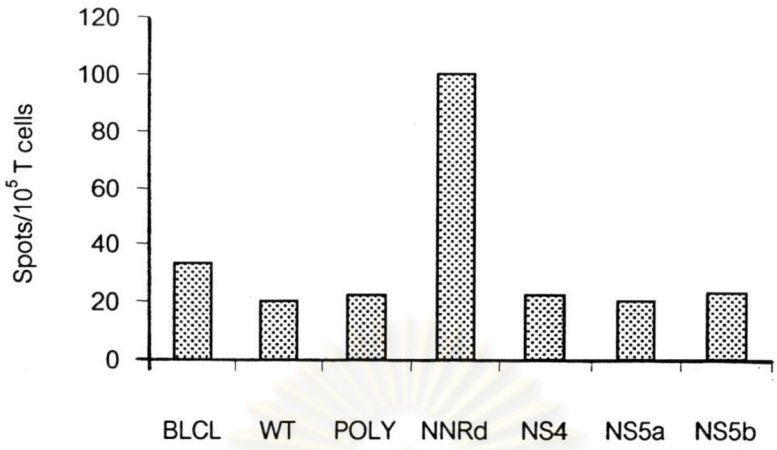


Figure 17 IFN-γ ELISPOT assay of 816 LILs stimulated with rVV-HCV infected autologous BLCLs. All rVV-HCV used had been previously shown in Figure 6. LILs only have specific response against NNRd region. WT=vaccinia virus wild type

VR1012Neo+ was selected to compare the efficiency of HCV expression with pNS plasmid because of its strong CMV promoter and highly express in mammalian cells. The positive clones from both plasmids were tested for correct direction of NS3 gene by using enzyme digestion. For pNS plasmid, 3 enzymes were used, 2 for each time of digestion. For VR1012Neo+ plasmid, only 1 enzyme used. From 33 clones of the pNS transfectant, only 4 clones had the correct direction of the gene. For VR1012Neo+ transfectant, there were 31 positive clones and at least 4 clones had correct direction of the gene. Only 1 clone for 1 plasmid was selected which named pNS/N16 and VR/N8 to use in the further experiments.

- Expression of pNS/N16 and VR/N8

Expression of NS3 protein was determined by transfection of pNS/N16 and VR/N8 into NKNT-3 cells using Lipofectamine 2000 (LF2000). NS3 protein expression was detected by immunostaining method using mAb to NS3. The results are shown in Figure 18. NKNT-3 cells infected with rVV-NNRd were used as positive control and NKNT-3 cells mixed with only LF2000 were also included as negative control. NKNT-3 cells transfected with pNS and VR1012Neo+ (mocked) were negative for NS3

expression (data not shown). As shown in Figure 18, NS3 expression could be detected in both pNS/N16 and VR/N8 transfected cells. This indicated that the constructed plasmids, pNS/N16 and VR/N8, could be transfected and expressed protein in transfected cells and the protein expressed was detectable by using antibody to NS3

In order to demonstrate the ability of pNS/N16 and VR/N8 to induce cellular response, BLCLs was used instead of NKNT-3 cells. The plasmids were transfected into BLCLs and the expression was also tested as described for NKNT-3 cells. However, by using LF2000 method, all transfected BLCLs died. Another transfection method, electroporation, was selected and used as previously described.[136]

After 3 days of transfection, plasmid containing BLCLs were selected by using 1 mg/ml Geneticin (G418). pNS/N16 and pNS transfected BLCLs were able to survive throughout the drug selection process. In contrast, the VR1012Neo+ transfected BLCLs died after the first time of drug selection. For that reason, only pNS/N16 and pNS transfected cells were used in the following experiments.

The expression of NS3 protein in BLCLs transfected with pNS/N16 was detected by flow cytometry using mAb to NS3. The results are shown in Figure 19. BLCL infected with rVV-NNRd were used as positive control. Non-trasfected BLCLs, pNS transfected BLCL, and BLCL infected with VV wild type (VV-WT) were used as negative controls. The results shown that 12.5% of rVV-NNRd infected BLCLs and 6.32% of pNS/N16 transfected BLCLs expressed NS3 protein.

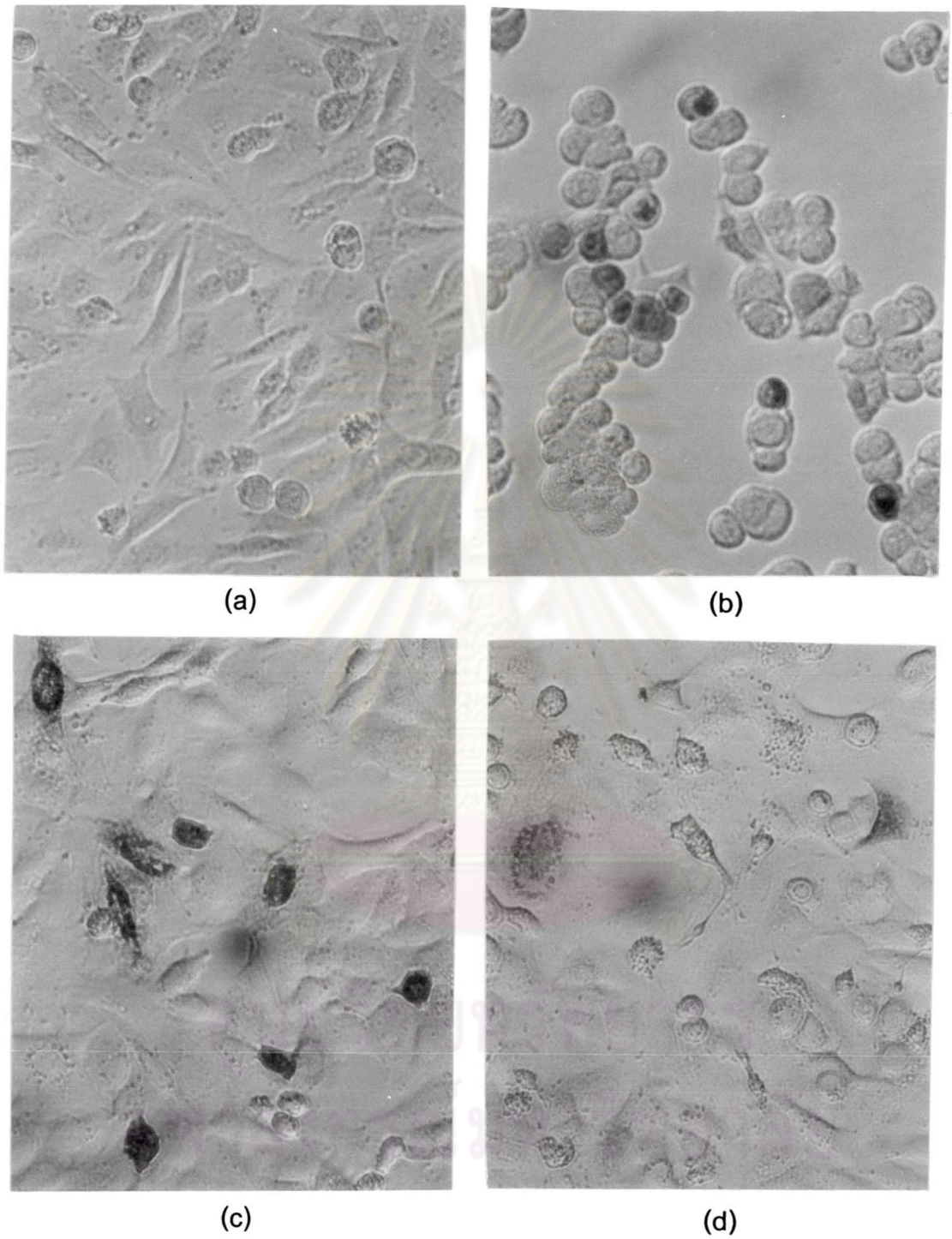
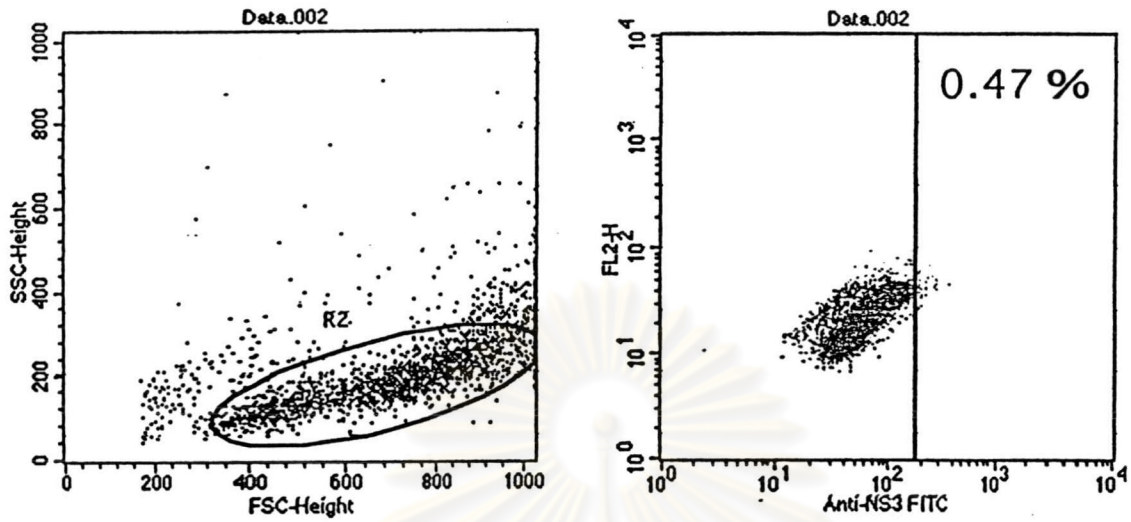
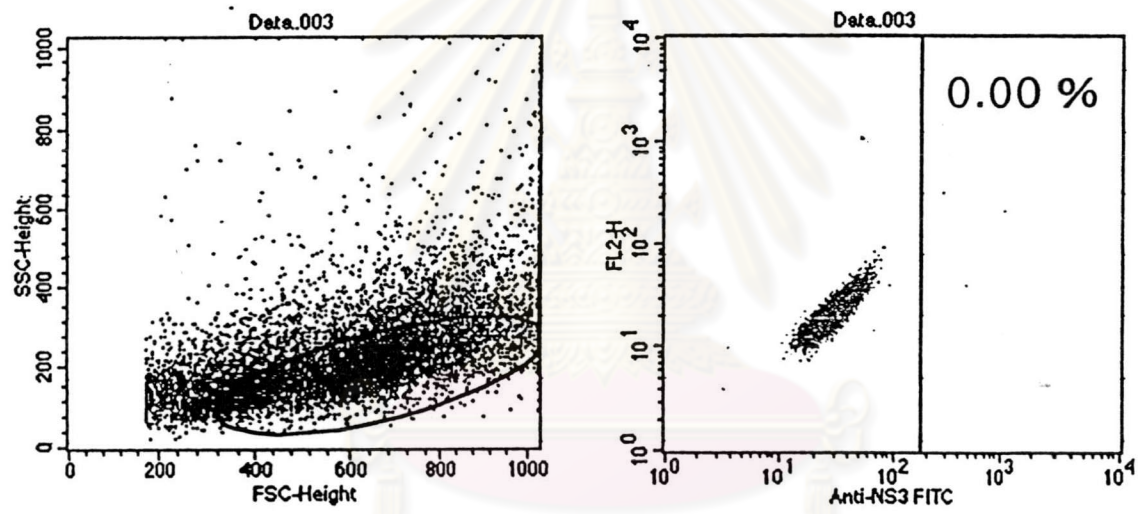


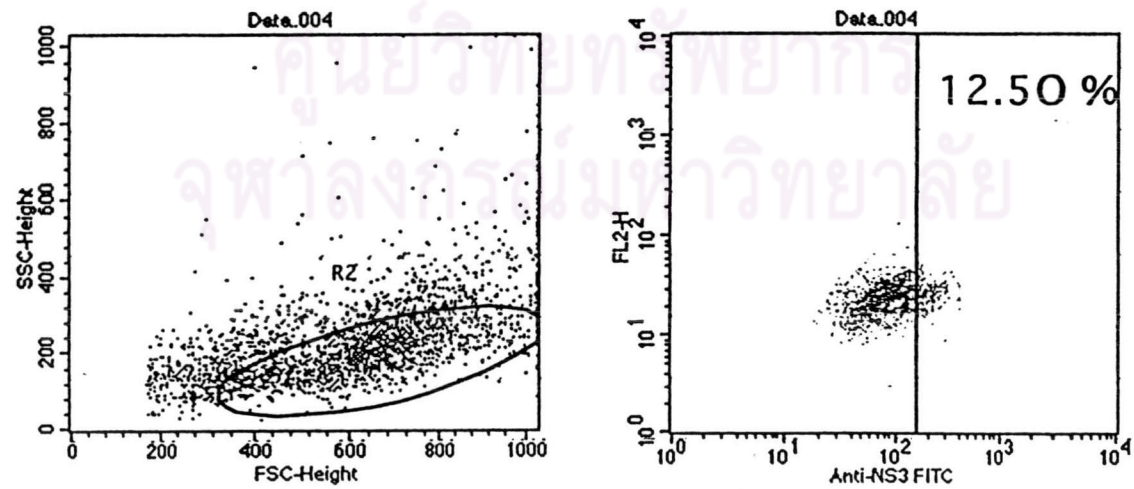
Figure 18 NS3 expression in NKNT-3 cells detected by immunostaining. (a) NKNT-3 cells, (b) rVV-NNRd infected NKNT-3 cells used as positive control, (c) VR/N8 transfected NKNT-3 cells, (d) pNS/N16 transfected NKNT-3 cells. Magnification x200.



(a)



(b)



(c)

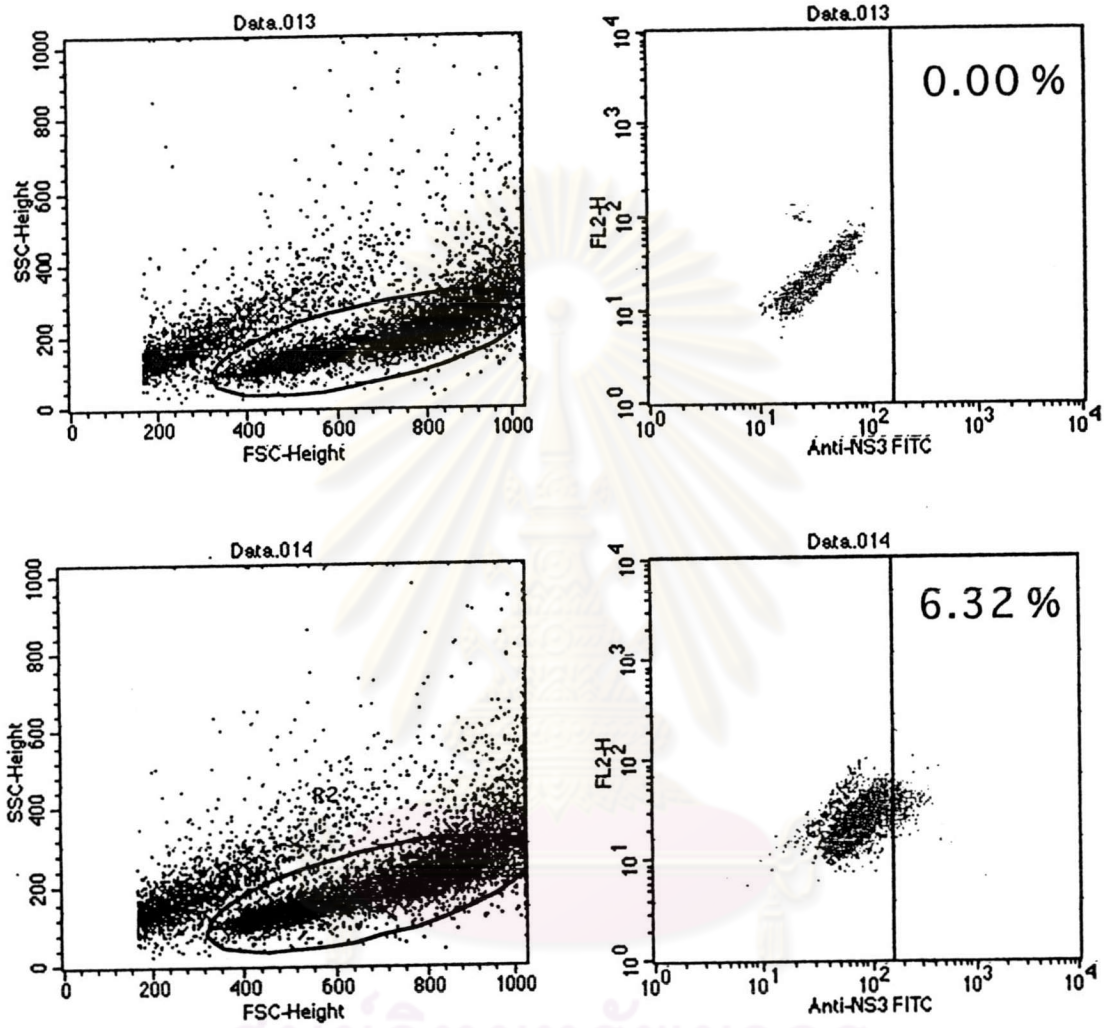


Figure 19 NS3 expression in 816 BLCLs detected by flow cytometry. (a) non-transfected BLCLs, (b) VV-WT infected BLCLs, (c) rVV-NNRd infected BLCLs, (d) pNS transfected BLCLs, (e) pNS/N16 transfected BLCLs.

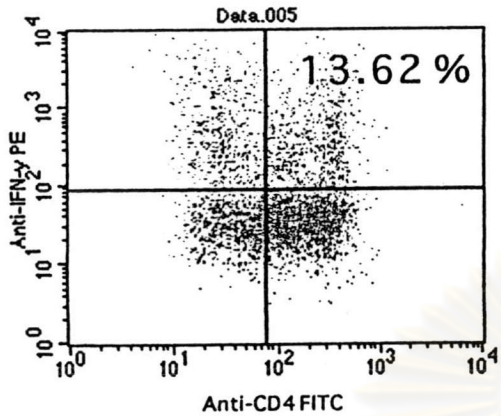
- Induction of IFN-γ production by pNS/N16

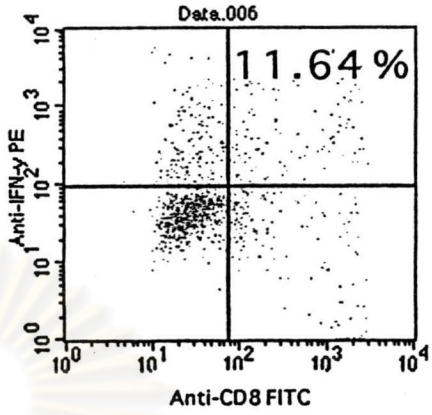
The ability of pNS/N16 in induction of cellular response was measured by IFN-γ production. Besides detection of IFN-γ by ELISPOT assay, intracellular IFN-γ in $CD4^+$ and $CD8^+$ T cells was also detected by flow cytometry.

BLCLs containing pNS/N16 plasmid was used to stimulate liver-infiltrating lymphocytes (LILs) of the patient 816 as described earlier and IFN-γ production was measured. By flow cytometry, when rVV-NNRd infected BLCLs were used as stimulator cells, IFN-γ was detected in 1.77% of $CD8^+$ T cells. When pNS/N16 transfected BLCLs were used, 2.18% of $CD8^+$ T cells expressed intracellular IFN-γ. Even though there were more IFN-γ producing $CD4^+$ T cells than IFN-γ producing $CD8^+$ T cells when stimulated with PHA (13.62% vs 11.64%), when pNS/N16 transfected BLCLs were used as stimulator cells, it was vice versa. No HCV specific $CD4^+$ T cells was detected. This may be due to the pathway of antigen presentation. The results of intracellular IFN-γ staining are shown in Figure 20.

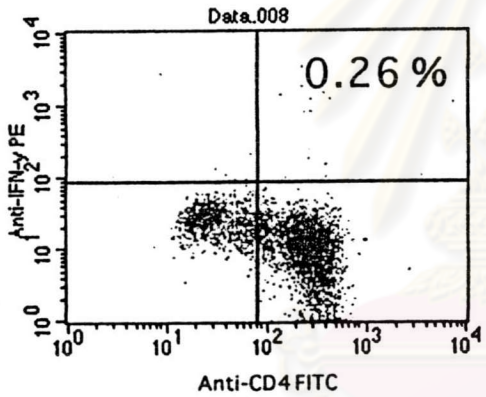
The positive controls of the test were LILs of 816 stimulated with rVV-NNRd infected BLCLs and the negative controls were LILs of 816 stimulated with 3 types of BLCLs: non-transfected BLCLs, VV-WT infected BLCLs and pNS transfected BLCLs. All negative controls gave negative results which not more than 0.5 % of $CD8^+$ T cells expressing IFN-γ were detected.

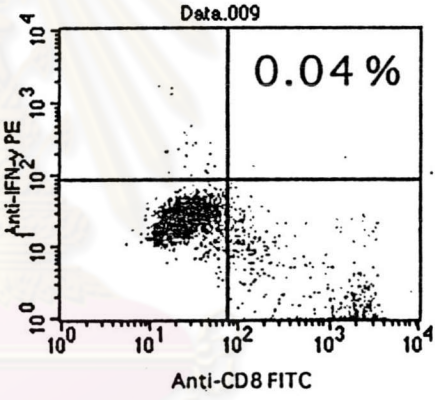
The results of ELISPOT assay for detection of IFN-γ production are shown in Figure 21. There was no significant difference in numbers of positive spots observed in LILs of 816 stimulated with rVV-NNRd infected BLCLs or pNS/N16 transfected BLCLs. It can be concluded from these finding that pNS/N16 transfected BLCLs could be used to induce IFN-γ production of 816 LILs and gave the similar results as when rVV-NNRd infected BLCLs were used.



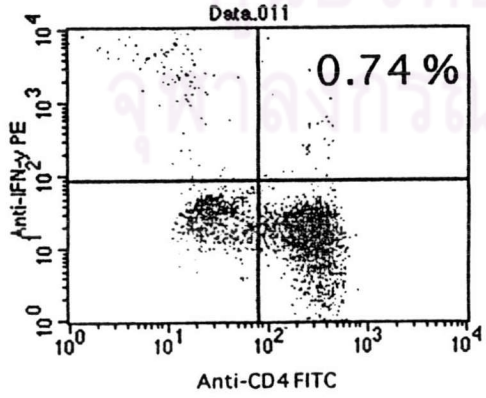


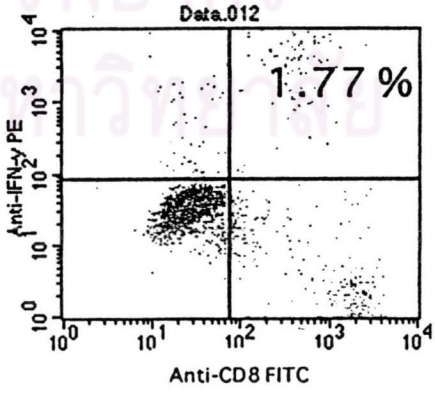
(a)





(b)





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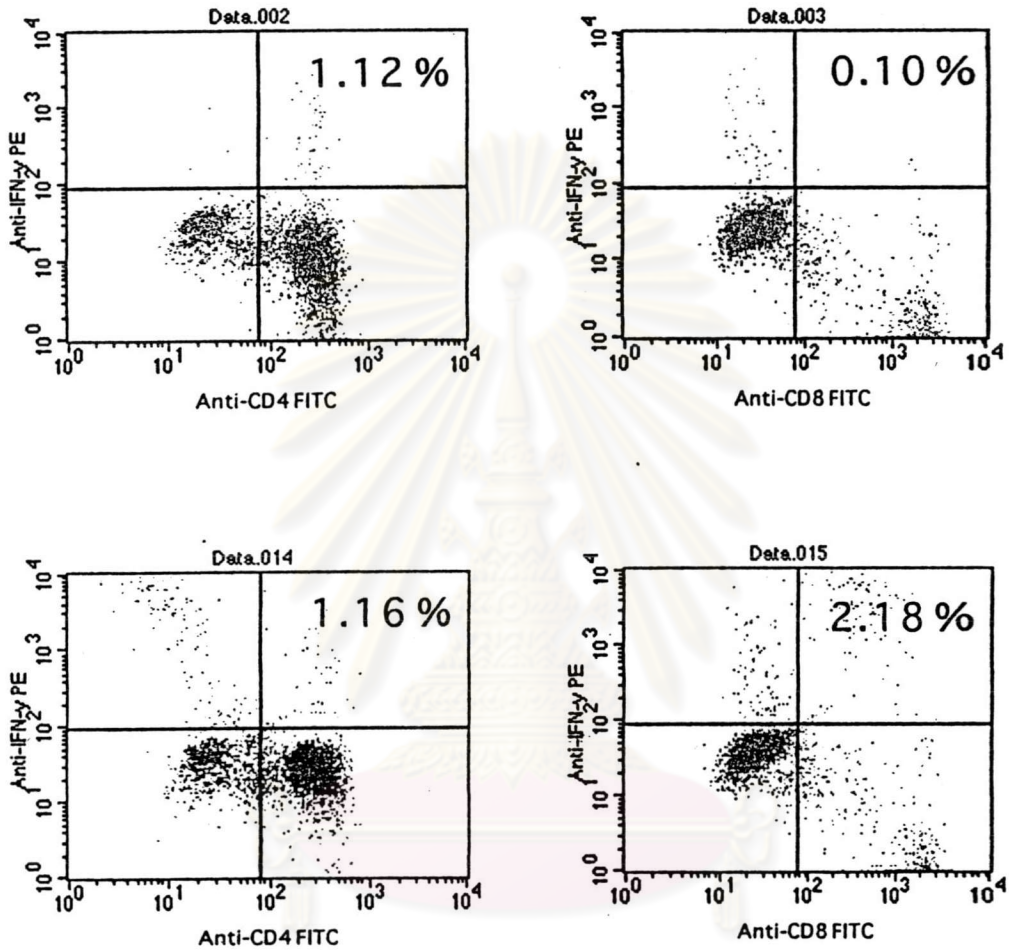

Figure 20 Intracellular IFN-γ detection by flow cytometry. The left column showed $CD4^+$ T cells that were positive for intracellular IFN-γ and the right column showed $CD8^+$ T cells with intracellular IFN-γ detected. Stimuli used in each panel were (a) PHA activated LILs, (b) VV-WT infected BLCLs, (c) rVV-NNRd infected BLCLs, (d) pNS transfected BLCLs, (e) pNS/N16 transfected BLCLs.

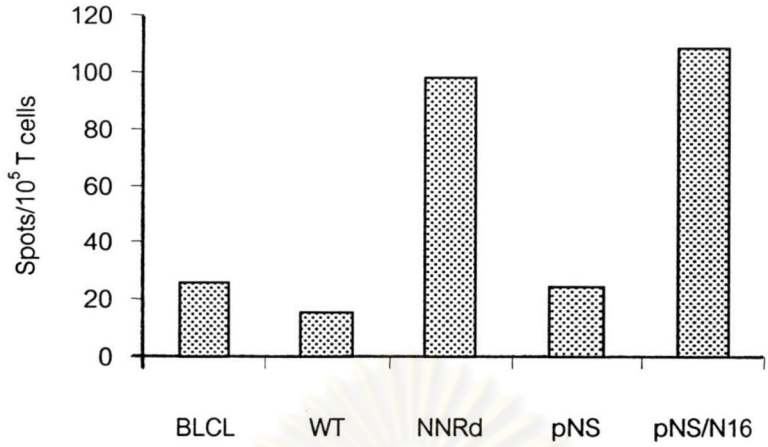

Figure 21 IFN-γ ELISPOT assay from 816 LILs stimulated with rVV-NNRd infected or pNS/N16 transfected autologous BLCLs. WT=vaccinia virus wild type

In addition to liver infiltrating lymphocytes, PBMCs were also used to determine whether the pNS/N16 plasmid was capable to induce detectable IFN-γ production. Three PBMCs from blood samples (05458, 09152, 09612) which gave positive responses to SOD c-200 tested by proliferation and ELISPOT assays performed earlier, were selected.

BLCLs were prepared from PBMCs and transfected with pNS/N16 or pNS by the electroporation method and drug resistance selection was performed as mentioned above. The expression of NS3 protein was demonstrated by flow cytometry. The results are shown in Figure 22. BLCLs from all blood samples transfected with pNS/N16 plasmid could express NS3 protein. For blood sample number 09152, there were not enough pNS transfected BLCLs.

Transfected BLCLs were used to stimulate autologous PBMCs and IFN-γ production was measured by ELISPOT assay. Since the survival of thawed PBMCs was very low, PHA was used to stimulate PBMCs and cells were rested for 7 days prior to stimulation with BLCLs transfected with pNS/N16 or pNS plasmid.

The number of positive spots are shown in Table 14. There was no difference in the number of IFN-γ spots obtained from PBMCs stimulated with non-transfected, pNS-transfected and pNS/N16-transfected BLCLs.

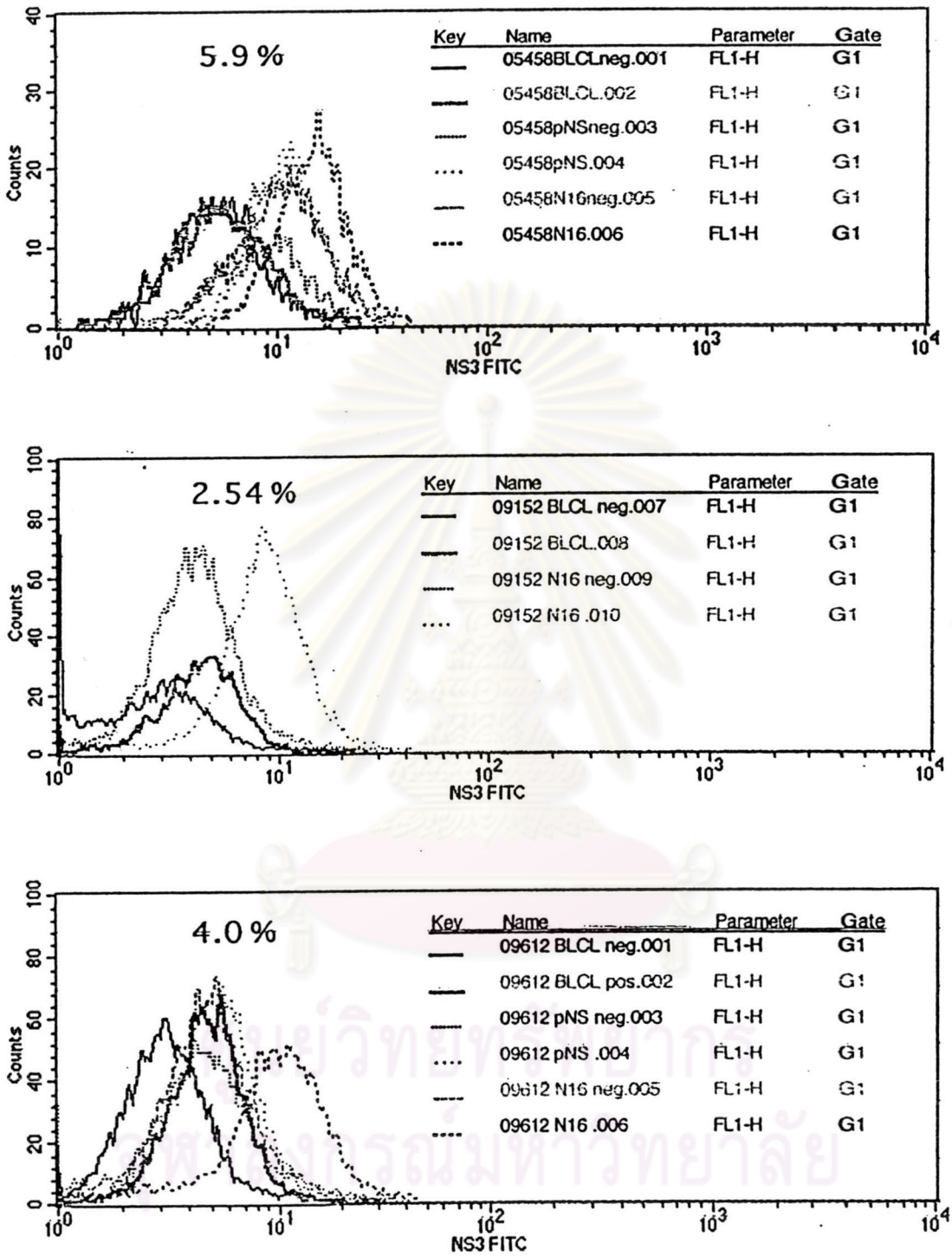

Figure 22 NS3 expression in BLCLs from three blood donors. The percentage of NS3 expression were indicated in the Figure. (a) 05458 BLCLs, (b) 09152 BLCLs, (c) 09612 BLCLs

Table 14 The number of IFN-γ spots/1x10^5 PHA-stimulated PBMCs found in 3 blood donors ; 05458, 09152 and 09612

Blood donors	Number of IFN-γ spots/1x10^5 PHA-stimulated PBMCs			
	non-transfected BLCLs	pNS-BLCLs	pNS/N16-BLCLs	PHA
05458	158	179	162	222
09152	87	77	63	150
09612	20	33	30	50

The numbers of positive spots in the test wells (pNS/N16-transfected BLCLs) and in control wells (non-transfected BLCLs, pNS-transfected BLCLs) were also compared. There was no significant difference (spots in test wells were not more than 2 times of spots in the control wells) was observed. The reason that pNS/N16 could not induce the response that was significantly different from negative control, may be that there is lower frequency of T cells specific to antigen expressed by pNS/N16 in PBMCs than in LILs.