CHAPTER III

RESULTS

Rabies neutralizing antibody

The antibody titers of all groups were shown in Fig.5 and Table 2. No subjects had detectable rabies antibody on day 0. All regimens resulted in 100 % seroconversion by day 14, except one patient in group D who had the antibody level lower than the arbitary protective value of 0.5 iu/ml.

Group A (1x6 PVRV)

By day 7, 14 of 24 patients (58%) developed detectable antibody. However, only 4 (16.7%) had antibody titer exceeding the arbitrary protective level of 0.5 iu/ml. The antibody level of this group peaked at day 28 with a geometric mean titer (GMT) of 12.67 iu/ml. All of the patients had antibody higher than 1.16 iu/ml on day 180 with a GMT of 4.62 (range : 1.16-16.9 iu/ml)

Group B (2-1-1 PVRV)

Eight of the 21 patients (38.1%) had detectable rabies antibody by day 7, but only 2 (9.5%) exceeded the 0.5 iu/ml level. The peak antibody level of this group was on day 28 with a GMT of 18,38 iu/ml. The GMTs of the 2-1-1 group were significantly higher than the 1 x 6 group (group A) on day 14 (table 2). On day 180,

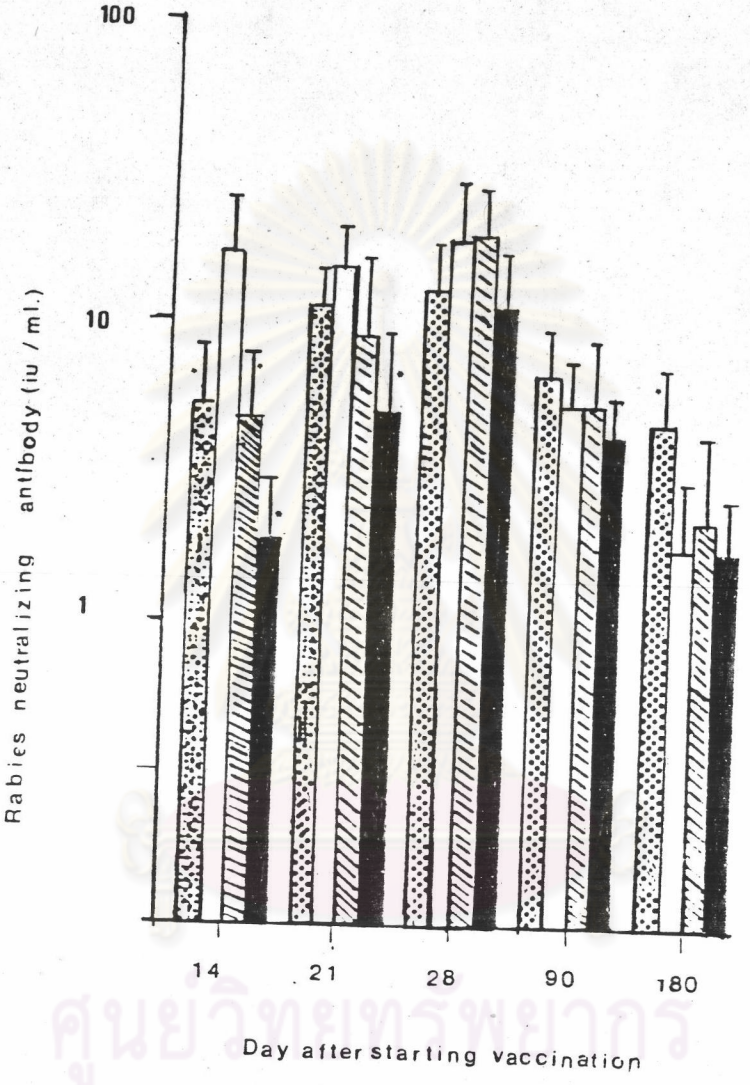

Figure 5. Neutralizing rabies antibodies in different PVRV regimens (▒) PVRV, I.M.; (□) PVRV, 2-1-1; (▓) PVRV+HRIG; (■) PVRV+ERIG.

Table 2. Neutralizing rabies antibodies in different PVRV regimens.

Group	Day 7	Day 14	Day 21	Day 28	Day 90	Day 180
A (1x6 PVRV)	0.17*	5.48*	11.29	12.67	6.69	4.62*
(n = 24)	(0-2.76)	(0.82-42.55)	(3.68-29.61)	(1.71-32.31)	(2.63-23.84)	(1.16-16.9)
	(n = 24)	(n = 23)	(n = 20)	(n = 23)	(n = 18)	(n = 14)
B (2-1-1 PVRV)	0.13	16.99	15.01	18.38	5.45	1.78
(n = 22)	(0-1.5)	(2.12-54.25)	(3.89-41.82)	(4.42-87.19)	(1.37-20.10)	(0.58-5.99)
	(n = 21)	(n = 21)	(n = 22)	(n = 20)	(n = 20)	(n = 16)
C (2-1-1 PVRV + HRIG)	0.2	4.72*	8.97	19.33	5.38	2.33
(n = 14)	(0-0.93)	(1.64-16.90)	(1.71-36.77)	(7.77-47.69)	(1.71-18.43)	(0.97-8.47)
	(n = 14)	(n = 14)	(n = 13)	(n = 12)	(n = 13)	(n = 8)
D (2-1-1 PVRV + ERIG)	0.1	1.86*	4.98*	11.21	4.25	1.81
(n = 17)	(0-0.48)	(0.44-8.46)	(0.29-30.93)	(2.42-41.84)	(1.58-12.44)	(0.69-6.53)
	(n = 17)	(n = 17)	(n = 16)	(n = 16)	(n = 16)	(n = 14)

(a) Geometric mean titre (in iu/ml)

* Significantly different from group B

the antibody titers of the 2-1-1 group ranged from 0.58 to 5.99 (GMT = 1.78 iu/ml). This was significantly lower than that of group A which also received a booster dose on day 90.

Group C (2-1-1 PVRV + HRIG)

Ten of the 14 individuals (71.4%) had detectable antibody on day 7 but only one (7.1%) had antibody titer higer than 0.5 iu/ml. The peak antibody level was reached on day 28 with a GMT of 19.33 iu/ml. The GMT on day 14 was significantly lowerthan group B ($p<0.05$) (table 2). All patients had antibody titers higher than 0.98 iu/ml on day 180 with a GMT of 2.33 iu/ml (range = 0.97-8.47 iu/ml).

Group D (2-1-1 PVRV + ERIG)

Five of the 17 patients (29.4%) had detectable antibody on day 7 but none exceeded the 0.5 iu/ml level. The peak antibody titer was also reached on day 28 with a GMT of 11.21 iu/ml. The GMTs of this group on day 14 and 21 were significantly lower than those of all other groups except group A,B on day 28 and group C on day 21 (table 2). On day 180, the antibody levels ranged from 0.69-6.53 with a GMT of 1.81 iu/ml.

Antigen-stimulated lymphocyte proliferative response

Antigen-stimulated lymphocyte transformation test (LTT) was carried out only in selected patients of group A (1x6 PVRV)and B (2-1-1 PVRV). There was no antigen-

stimulated LTT prior to PVRV immunization (day 0). It first became evident on day 14 in both groups (Table 3). The peak LTT was reached between day 14 and 21 (Fig. 6,7). No significant difference was noted between the 2 groups on any days.

Side-effects

No serious complication was observed in any of the patients except some complaints of pain at the infection site and local lymphadenopathy in a few patients (Table 4).

Determination of rabies antibody by indirect enzyme linked immunosorbent assay (ELISA).

Attempts were made to develop an indirect enzyme-linked immunosorbent assay (ELISA) for rabies antibody titration. ELISA has possible advantages over RIFFIT in terms of simplicity cost and convenience, especially with large-scale assay. In this study purified Vero rabies vaccine (PVRV) was used as the coating antigen.

1. Determination of the optimal conditions for rabies antibody detection by ELISA.

1.1 The optimal time for PVRV adsorption to the solid phase.

Table 3. Comparison of lymphocyte proliferative responses to the full course intramuscular PVRV regimen and the 2-1-1 PVRV regimen.

Day	Group A : I.M. PVRV (N = 11)		Group B : 2-1-1 PVRV (N = 10)	
	CPM[a]	SI[b]	CPM	SI
0	246+230[c] (N=7)	0.7+0.2	29+18 (N=8)	0.8+0.1
7	273+84 (N=10)	1.5+0.2	729+397 (N=9)	2.7+0.9
14	3,447+1,496* (N=10)	11.1+4.0*	5,548+2,087* (N=9)	12.1+3.9*
21	6,364+1,865* (N=11)	17.3+4.3*	3,619+1,501 (N=8)	11.2+4.5
28	3,556+1,627 (N=9)	12.3+5.3	4,595+1,121* (N=9)	11.1±4.3
90	1,903+1,411 (N=8)	3.7+1.9	2,120+830* (N=9)	3.8+0.9*

(a) stimulated CPM-unstimulated CPM.

(b) stimulated CPM/unstimulated CPM.

(c) mean + SEM.

* significantly higher than the corresponding values on day 0 within the group.

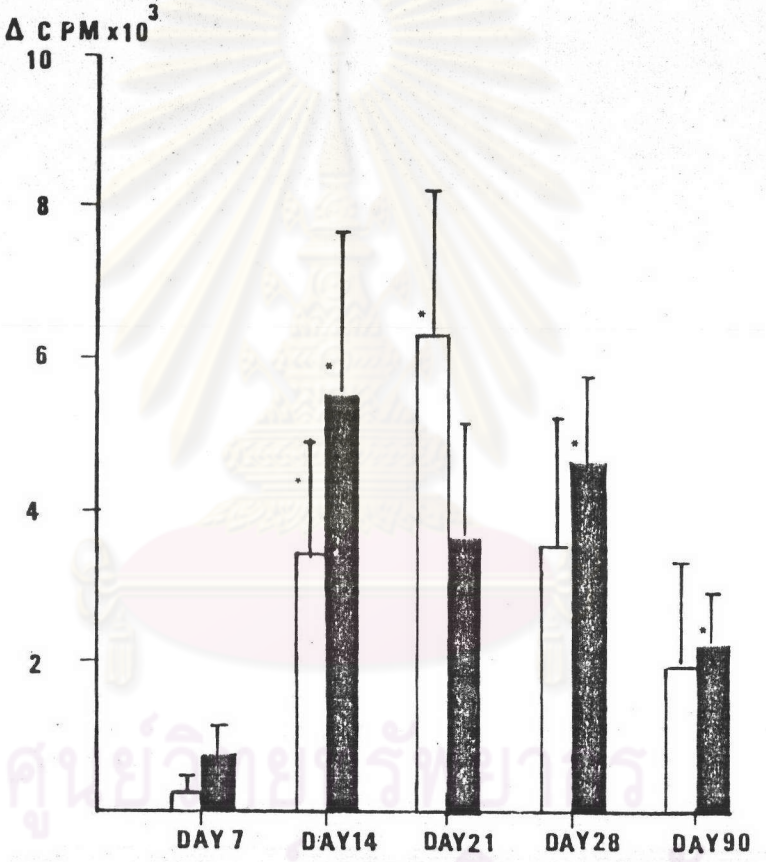

Figure 6. Kinetics of specific lymphocyte transformation in patients receiving intramuscular PVRV (□) and 2-1-1 PVRV (■). Each column represents the mean Δ CPM with bar as SEM. *P < 0.05 as compared with day 0 of the same group.

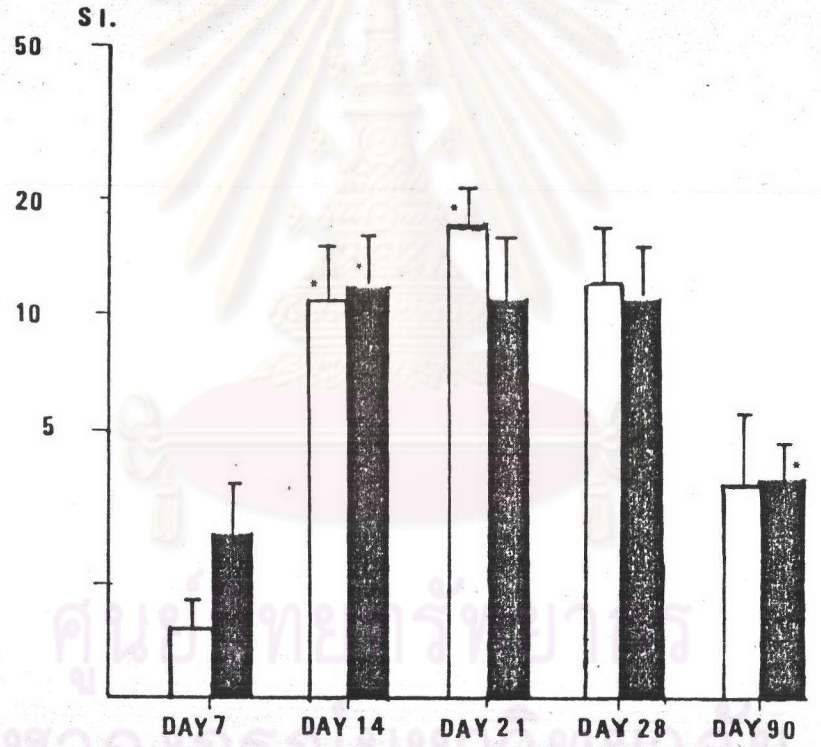

Figure 7. Kinetics specific lymphocyte transformation in patients receiving intramuscular PVRV (□) and 2-1-1 PVRV (■). Each column represents the mean S.I. with bar as SEM. *P < 0.05 as compared with day 0 of the same group.

Table 4. Side-effects from various PVRV vaccination regimens.

	Group A (n = 24)	Group B (n = 22)	Group C (n = 14)	Group D (n = 17)
Local Lymphadenopathy	0	1	0	1
Pain	3	1	0	1
Local itching	0	0	0	0
Headache	0	0	0	0
Malaise	0	0	0	0
Dizziness	0	0	0	0
Nausea, Vomiting	0	0	0	0

In view of the optimal antigen and conjugate concentrations employed in the rabies antibody detection by Bhatia et al. (128), 1:150 dilution fo PVRV and 1:4,000 dilution of rabbit anti-human immunoglobulin-peroxidase conjugate were used in this study. To find the proper time of antigen adsorption to the solid phase, two (neutralizing antibody) positive sera and one negative serum at a dilution of 1:100 were incubated with PVRV which had been pre-coated at 37°C for 3 hours compared with overnight coating. The overnight coated PVRV gave higher OD with positive sera than the 3 hour coating as shown in Table 5.

1.2 Checkerboard determination of working dilutions of reagents

To determine the optimal dilutions of PVRV and rabbit antihuman immunoglobulin-peroxidase conjugate in overnight PVRV coating the antigen and conjugate dilutions of PVRV were determined to be 1:100 and 1:200 where those of the conjugate were varied as illustrated in Fig 8. The optimal of PVRV were determined to be 1:100 and 1:200 where those of the conjugate were 1:2000 and 1:4000. Thus, PVRV at 1:150 dilution and conjugate at 1:4000 were selected as the working dilutions in the test system.

2. Antirabies antibody titration by ELISA.

Standard curves were constructed by using various concentrations of commercial human rabies immune globulin (Imogam) at 150 iu/ml as determined by RIFFIT.

Table 5 The adsorption time of rabies antigen to the solid phase.

Incubation temperature and time	Serum	OD at 492 nm. (triplicates)		
37°c x 3 hour	positive 1	0.662	0.687	0.599
	positive 2	0.897	0.876	0.867
	negative	0.165	0.180	0.175
	positive 1	0.829	0.818	0.900
37°c overnight	positive 2	1.250	0.181	1.329
	negative	0.191	0.184	0.220

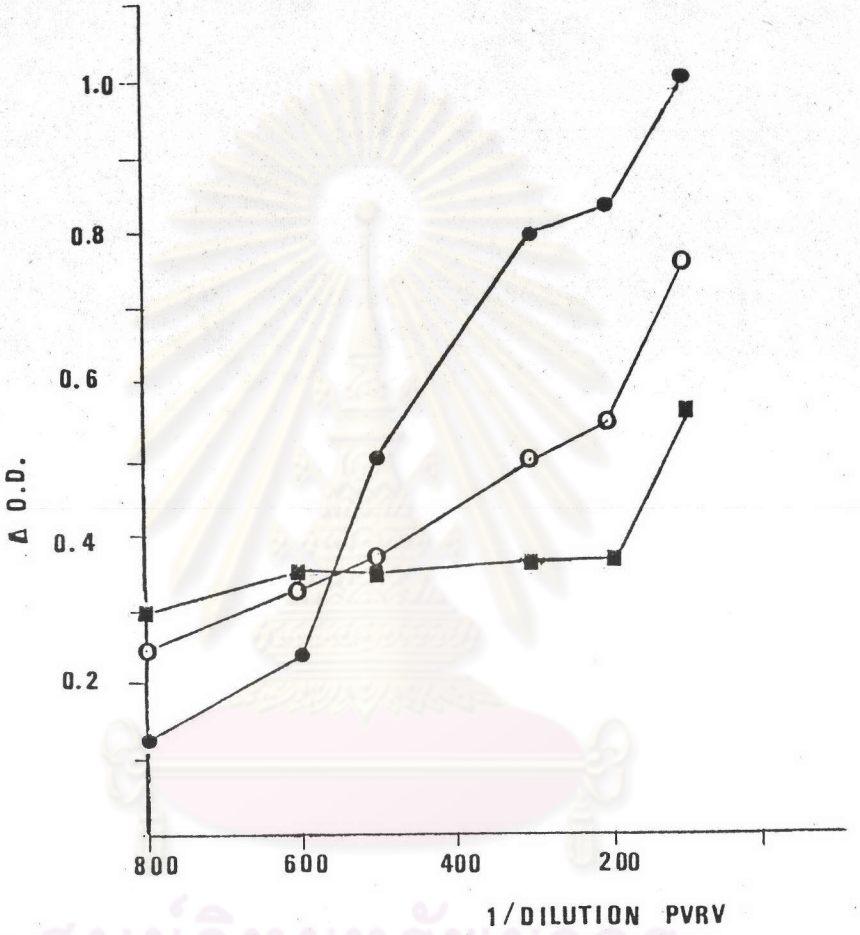

Figure 8. The checkerboad titration of PVRV and rabbit anti-human immunoglobulin peroxidase conjugate by fixed dilution (1:100) of antirabies positive and negative sera. Conjugate dilutions : 1:2,000 (●),1:4,000 (○) and 1:5,000 (■). The OD represents the difference in OD between positive and negative sera.

As shown in Figure 9, from 3 standard curves constructed in 3 assays, a test serum had 3 different antibody values; 0.34, 20 and 17 iu/ml whereas this serum was found to have an antibody titer of 30.0 iu/ml by RIFFIT. Table 5 illustrates the same finding of lack of reproducibility when other serum samples were repeatedly assayed by ELISA.

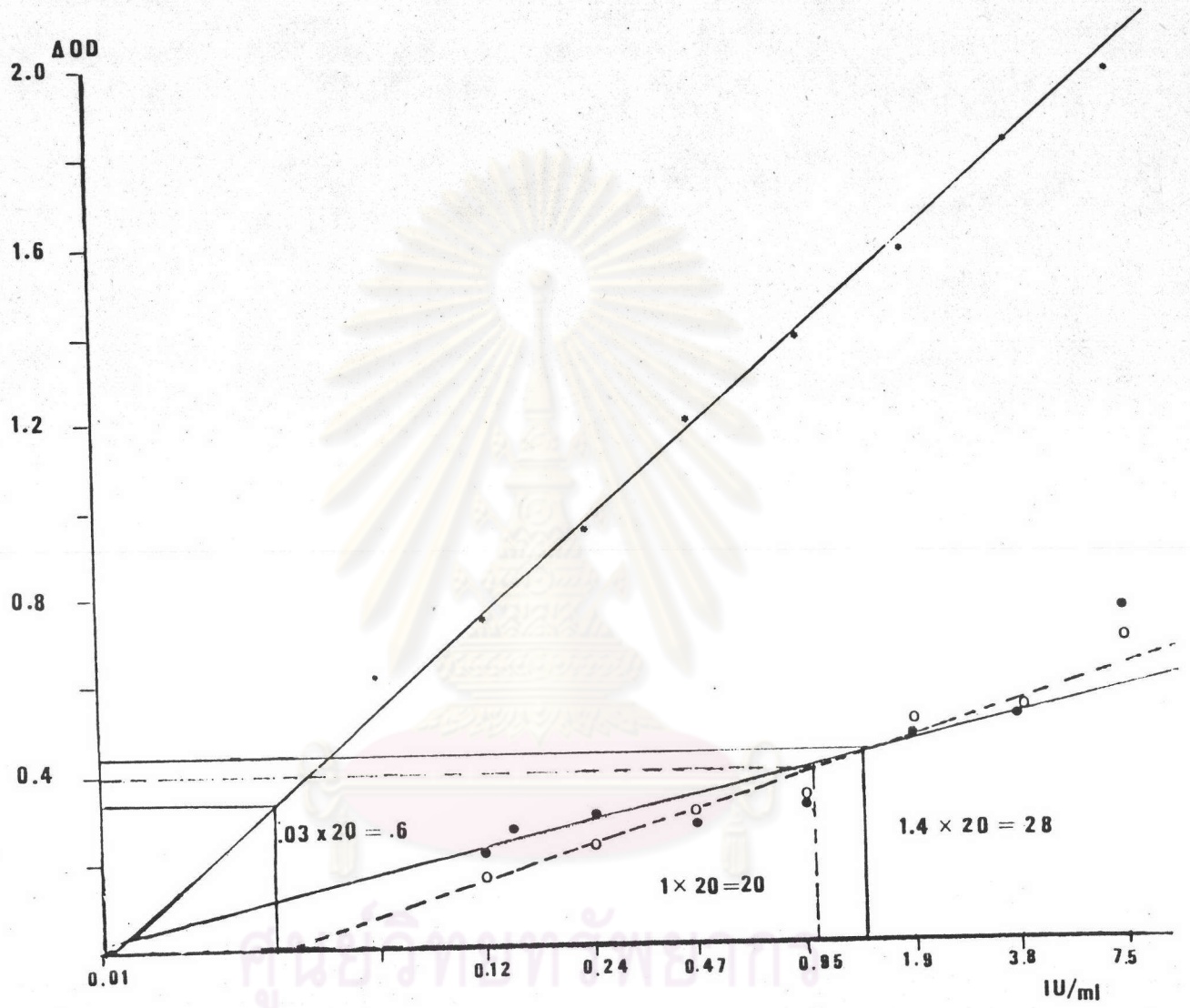

Figure 9 Lack of reproducibility when a serum sample was tested on 3 occasions. The values read from 3 standard curves were 0.6 , 20.0 and 28.0 iu/ml whereas this sample was found to have an antibody level of 30.0 iu/ml by RIFFIT.

Table 6. Estimation of antirabies antibody titer by ELISA : lack of reproducibility

Serum No	RIFFIT iu/ml	ELISA Run 1	2	3	4	5
		iu/ml				
1	16.0	4.9	6.4	7.4	36.6	1.49
2	20.0	5.2	5.4	2.6	12.7	1.43
3	11.0	6.2	1.8	0.8	-	-
4	2.5	0.0	2.5	1.2	4.1	-
5	4.2	0.3	1.8	6.8	-	-
6	30.0	17.0	28.0	0.6	-	-
7	3.0	2.1	2.0	3.0	1.6	-
8	13.0	4.9	11.6	25.7	0.22	-
9	16.8	1.3	3.36	2.9	-	-
10	13.0	8.9	0.13	-	-	-
11	15.5	6.3	0.07	-	-	-
12	14.8	16.6	0.04	-	-	-