IV. RESULTS

In vitro lymphocyte transformation test

1. Determination of the suboptimal and the optimal concentration of PHA-P

In order to evaluate the suboptimal and optimal concentration of PHA-P used to activate PBMC, lymphocytes were stimulated with various concentrations of PHA-P (0.0156, 0.03125, 0.0625, 0.125, 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 μg/ml). The results demonstrated that all concentrations of PHA-P could stimulate lymphocyte to synthesize the new deoxyribonucleic acids, and the PHA-P concentration 1.0 μg/ml provided the maximal stimulation (Table 1a and 1b, Fig. 1a and 1b). The concentration of 0.125 and 1.0 μg/ml of PHA-P were used as the suboptimal and optimal concentration, respectively in the further assay.

2. Determination of the optimal concentration of PBMC

In order to titrate for the appropriate concentration of PBMC, various concentrations of PBMC (0.25, 0.5, 1.0, 1.5, 2.0 and 4.0 x 10^6 cells/ml) were individually cultured in the presence or absence of 1 μg of PHA-P. It was found that the PBMC concentration at 1.0 x 10^6 cells/ml to 2.0 x 10^6 cells/ml gave the maximal results in incorporation of the radioactivity (Table 2, Fig. 2), therefore the average concentration of 1.5 x 10^6 cells/ml was used in further assays.

Table 1a. ^{3}H-thymidine incorporation of PBMC stimulated with various concentrations of PHA-P (0 to 8 μg/ml).

No. of donor	^{3}H-TdR incorporation of PBMC (cpm)						
	concentration of PHA-P (μg/ml)						
	0	0.25	0.5	1	2	4	8
1	443	26,576	29,924	30,580	29,956	24,632	26,908
2	297	28,233	29,128	29,650	26,517	30,785	29,586
3	597	25,862	31,764	33,050	24,449	27,208	23,257

Table 1b. ^{3}H-thymidine incorporation of PBMC stimulated with various concentrations of PHA-P (0 to 1 μg/ml).

No. of donor	^{3}H-TdR incorporation of PBMC (cpm)							
	concentration of PHA-P (μg/ml)							
	0	0.0156	0.0313	0.0625	0.125	0.25	0.5	1
1	666	546	533	1,173	9,661	25,718	31,596	33,839
2	323	388	1,921	5,630	21,418	35,475	41,509	41,764
3	579	523	677	1,542	2,846	16,793	26,891	36,725

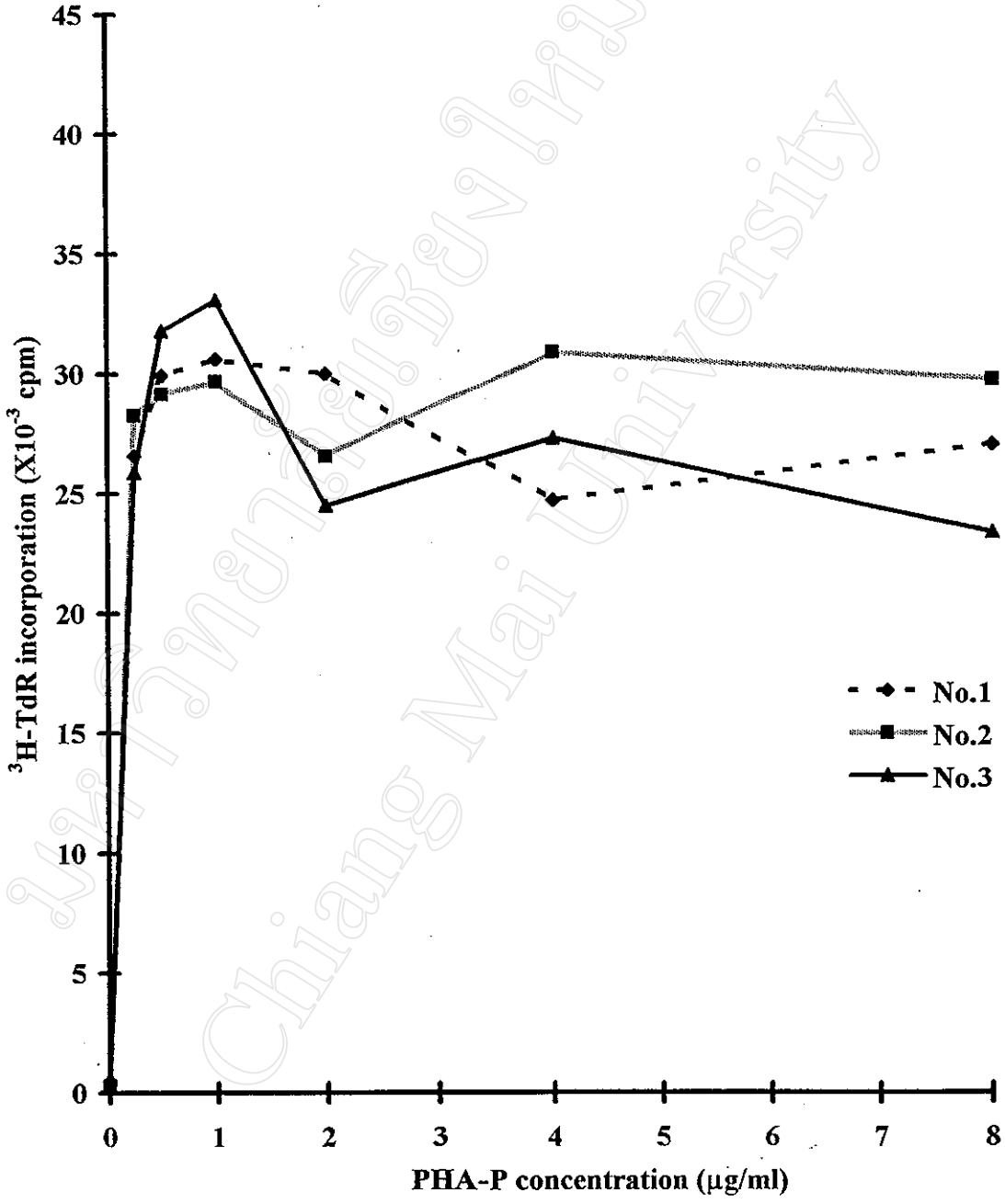

Figure 1a. ^{3}H-thymidine incorporation of PBMC stimulation with various concentrations of PHA-P (0 to 8 µg/ml). The radioactive was expressed in term of Δcpm (cultures without PHA-P were subtracted).

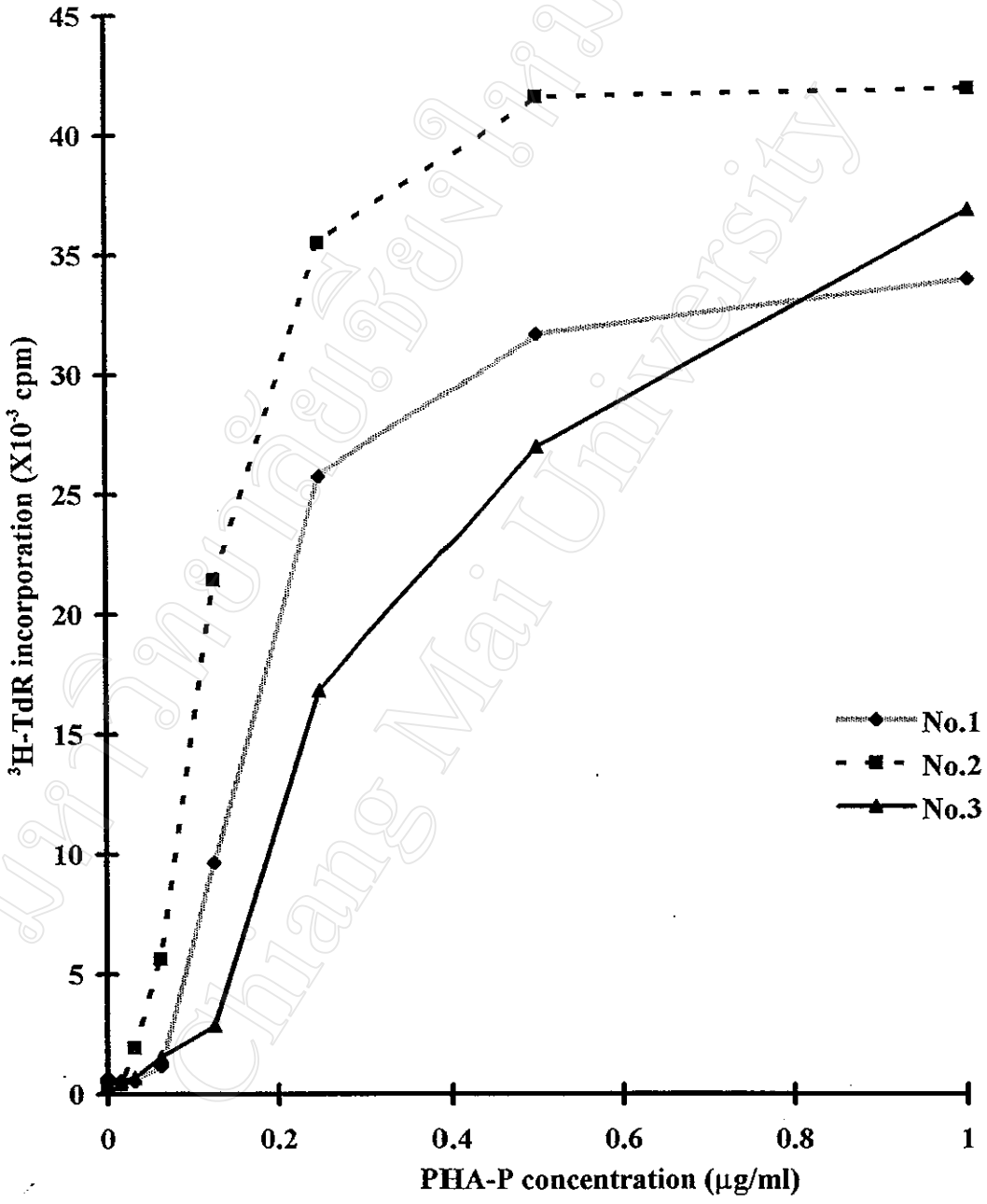

Figure 1b. ^{3}H-thymidine incorporation of PBMC stimulation with various concentrations of PHA-P (0 to 1 µg/ml). The radioactive was expressed in term of Δcpm (cultures without PHA-P were subtracted).

Table 2. ^{3}H-thymidine incorporation by various concentrations of PBMC stimulated with PHA-P.

No. of donor	^{3}H-TdR incorporation of PBMC (Δcpm)					
	concentration of PBMC (X 10^{5} cells/well)					
	0.25	0.5	1	1.5	2	4
1	19,453	25,403	31,029	28,532	28,243	22,213
2	11,157	20,572	29,545	29,705	33,203	25,568
3	12,339	22,977	33,778	44,136	43,670	36,542

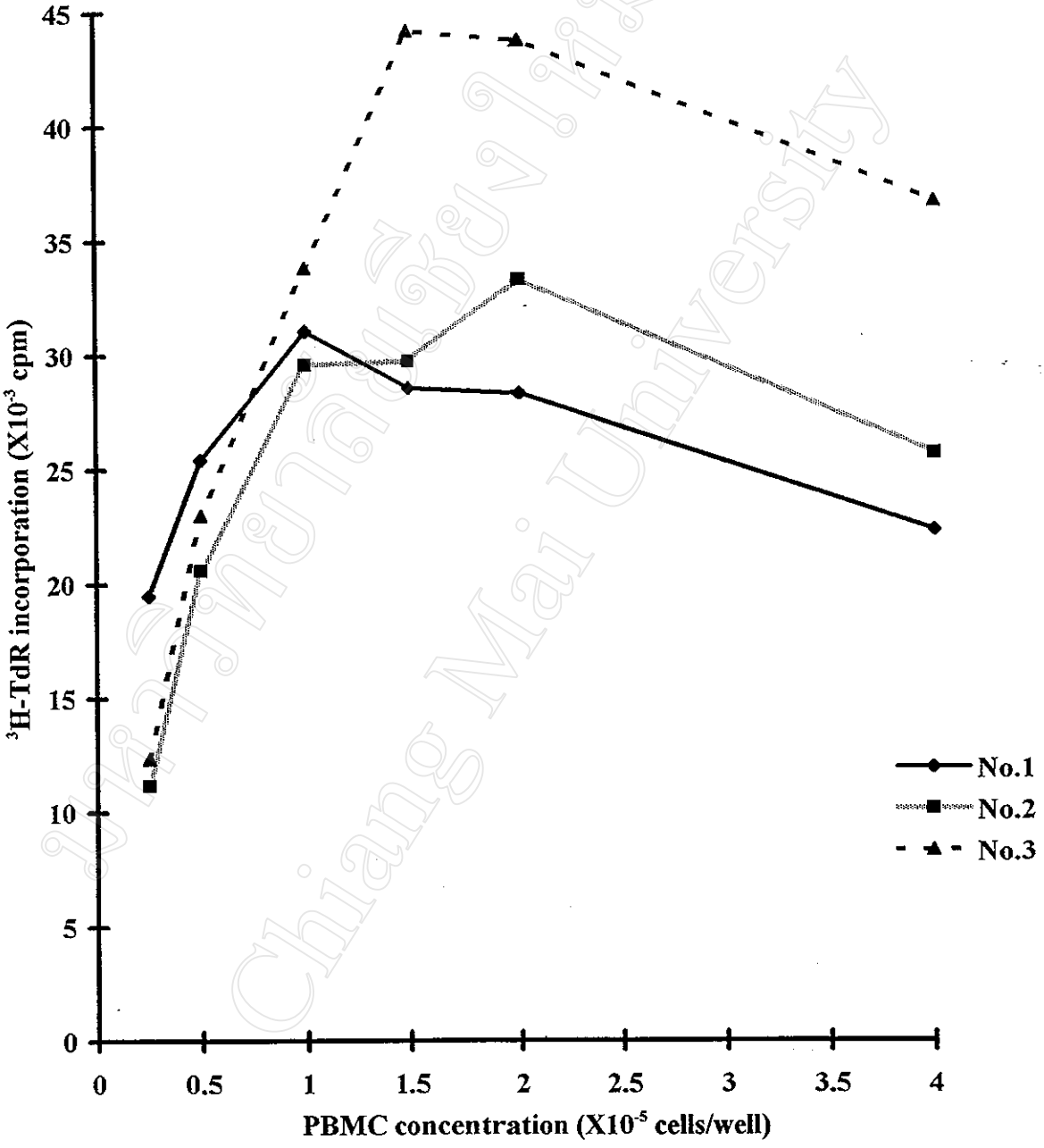

Figure 2. ^{3}H-thymidine incorporation by various concentrations of PBMC stimulated with PHA-P. The radioactive was expressed in term of Δcpm (cultures without PHA-P were subtracted).

3. Determination of the optimal concentration of PHA-P and PBMC by block titration method

In order to confirm the results mentioned above and to substantiate the appropriate concentration of PHA-P and PBMC used, various concentrations of PHA-P (0.25, 1.0 and 2.0 μg/ml) were individually cultured with PBMC (0.5, 1.0 and 1.5 x 10^6 cells/ml). It was found that PBMC at the concentration of 1.5 x 10^6 cells/ml and PHA-P at 1.0 μg/ml provided the best incorporation of the radioactivity (Table 3, Fig. 3). This concentration was used in further assays.

4. Determination of the suboptimal and optimal concentration of crude sonicated *P. marneffei* antigen

To determine the appropriate concentration of crude sonicated *P. marneffei* antigen used in lymphocyte transformation assay, various concentrations of crude sonicated *P. marneffei* antigen (0, 0.32, 0.64, 1.28, 2.56 and 5.12 μg/well) were individually cultured with lymphocytes. It was found that the crude sonicated *P. marneffei* antigen at 2.56 and 5.12 μg/well addressed the maximal incorporation of tritiated-thymidine (Table 4, Fig. 4). According to the result, crude sonicatd *P. marneffei* antigen at the concentration of 0.32 and 2.56 μg/well which was the suboptimal and optimal concentrations were used, respectively.

Table 3. The block titration to show the ability of various concentration of PHA-P to induce PBMC proliferation at different concentration of PBMC.

PHA-P (μg/ml)	No. of donor	^{3}H-TdR incorporation of PBMC (Δcpm) concentration of PBMC (X 10^5cells/well)		
		0.5	1	1.5
	1	10,993	13,943	15,424
	2	5,681	7,531	5,626
0.25	3	11,023	13,148	12,918
	4	10,036	8,251	9,298
	$\bar{X}$	9,433	10,718	10,816
	SD.	2,543	3,293	4,277
	1	13,403	19,554	19,169
	2	9,369	12,078	13,660
1	3	15,997	18,581	16,730
	4	11,396	11,613	11,356
	$\bar{X}$	12,541	15,456	15,228
	SD.	2,831	4,192	3,472
	1	15,150	18,188	17,395
2	2	10,682	13,271	14,588
	3	16,169	17,730	17,371
	4	11,003	10,702	9,969
	$\bar{X}$	13,251	14,972	14,830
	SD.	2,815	3,609	3,498

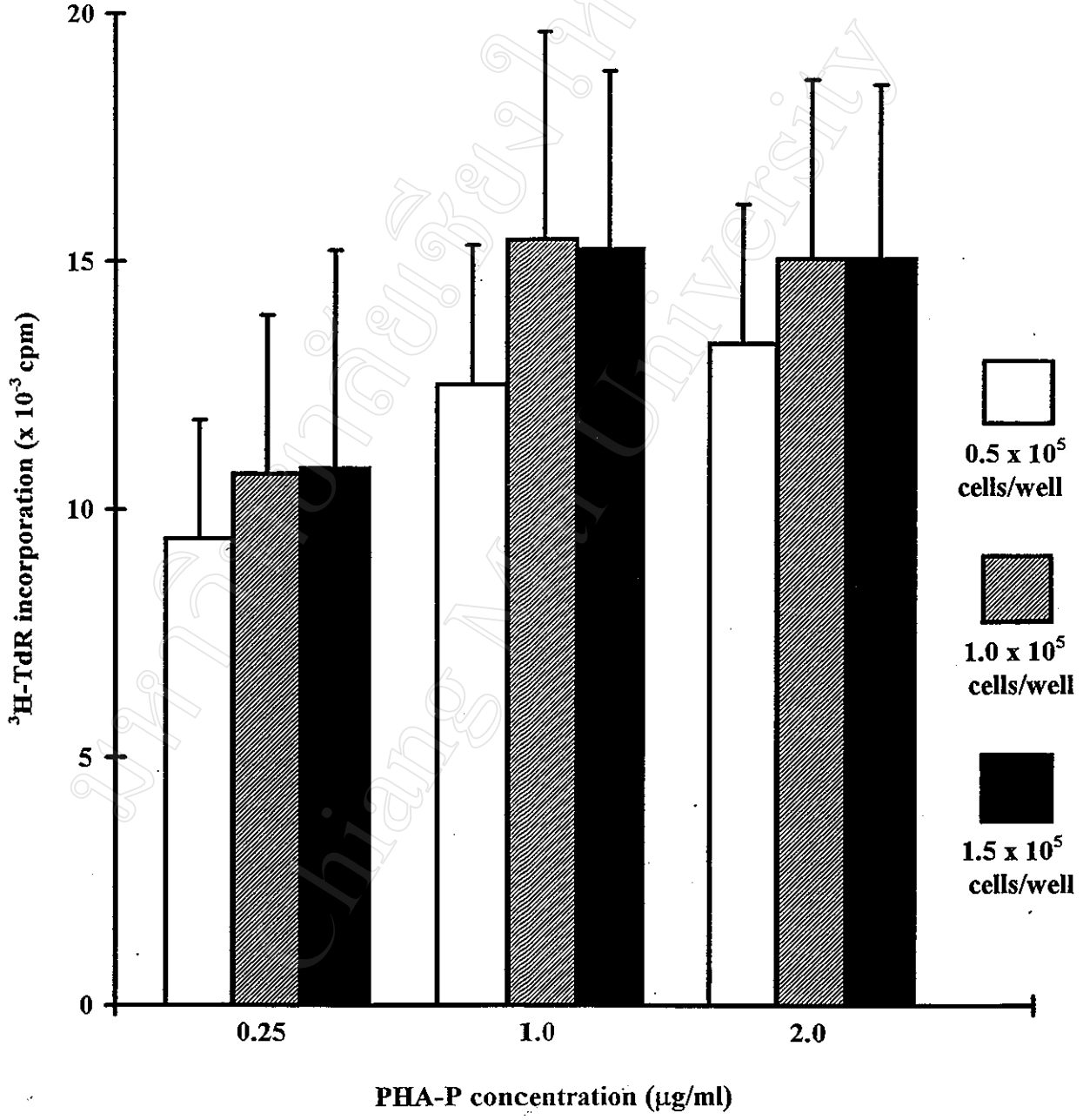

Figure 3. The block titration to show the ability of various concentrations of PHA-P to induce PBMC proliferation at different concentration of PBMC. Data were expressed as mean cpm ± SD derived from four different HIV negative donors.

Table 4. ^{3}H-thymidine incorporation by PBMC from HIV negative donors stimulated with various concentration of crude sonicated *P. marneffei* antigen.

No. of donor	^{3}H-TdR incorporation of PBMC (cpm)					
	concentration of *P. marneffei* antigen (μg/well)					
	0	0.32	0.64	1.28	2.56	5.12
1	966	2,710	2,877	4,124	8,568	8,047
2	727	2,214	2,557	3,567	3,467	4,292
3	1,343	12,825	14,615	15,585	15,141	16,208

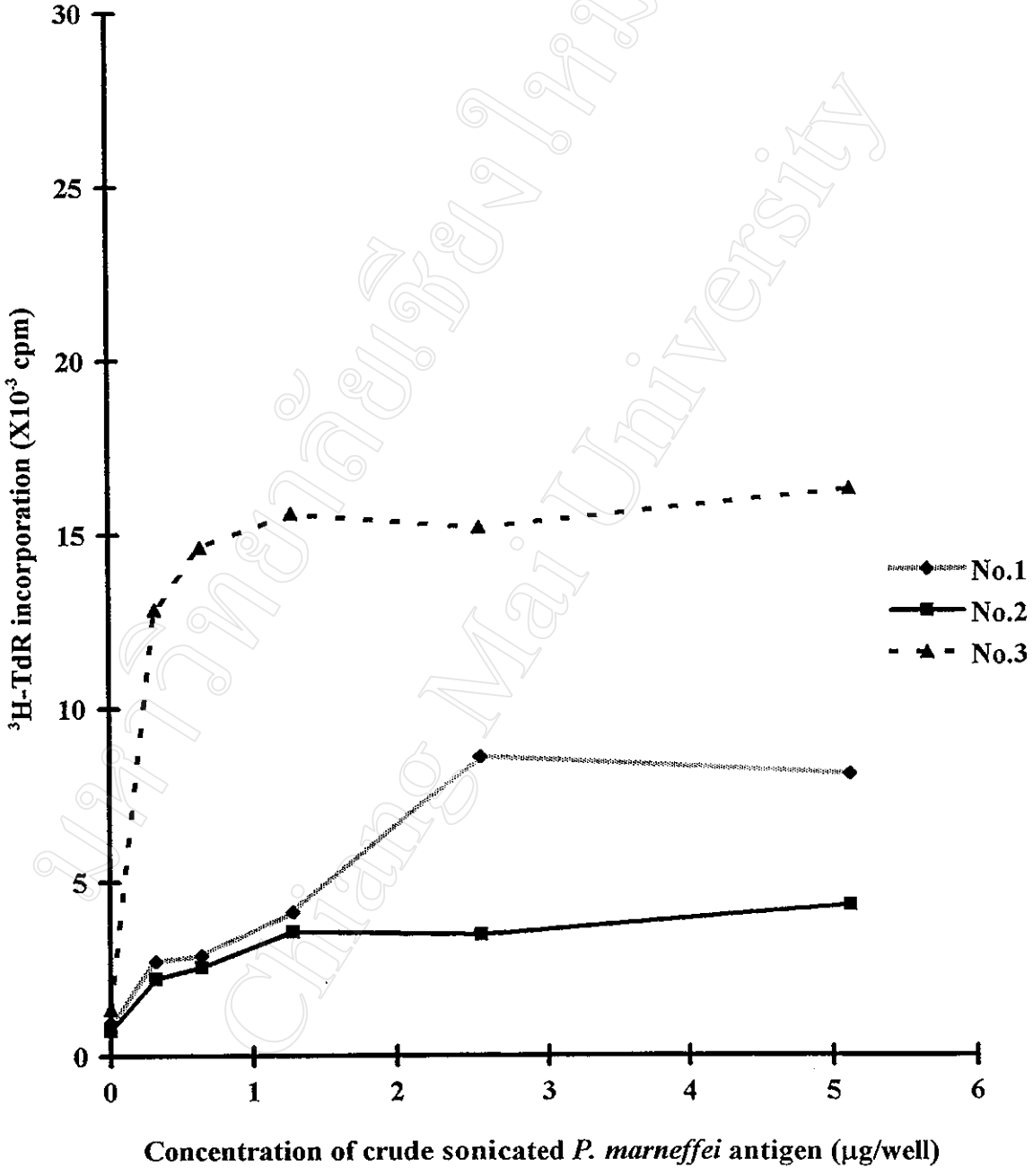

Figure 4. ^{3}H-thymidine incorporation of PBMC stimulation with various concentrations of crude sonicated *P. marneffei* antigen. The radioactive was expressed in term of Δcpm (cultures without crude sonicated *P. marneffei* antigen were subtracted).

5. Determination of the suboptimal and optimal concentration of PPD

To determine the appropriate concentration of PPD used in lymphocyte transformation assay, various concentrations of PPD (0, 0.3125, 0.625, 1.25, 2.5, 5.0, 10.0, and 20.0 µg/ml) were individually cultured with lymphocytes. It was found that PPD at 10 and 20 µg/ml addressed the high incorporation of tritiated-thymidine (Table 5, Fig. 5). According to the result, PPD at the concentration of 0.3125 and 10 µg/ml which was the suboptimal and optimal concentrations were used, respectively.

Table 5. ^{3}H-thymidine incorporation by PBMC from HIV negative donors stimulated with various concentration of PPD.

No. of donor	^{3}H-TdR incorporation of PBMC (cpm)							
	concentration of PPD (μg/ml)							
	0	0.3125	0.625	1.25	2.5	5	10	20
1	1,801	13,371	18,261	18,780	22,214	20,603	22,399	24,056
2	1,933	2,290	3,359	3,109	4,908	5,557	11,600	14,683
3	1,548	5,607	5,786	5,263	5,091	6,612	7,315	13,638

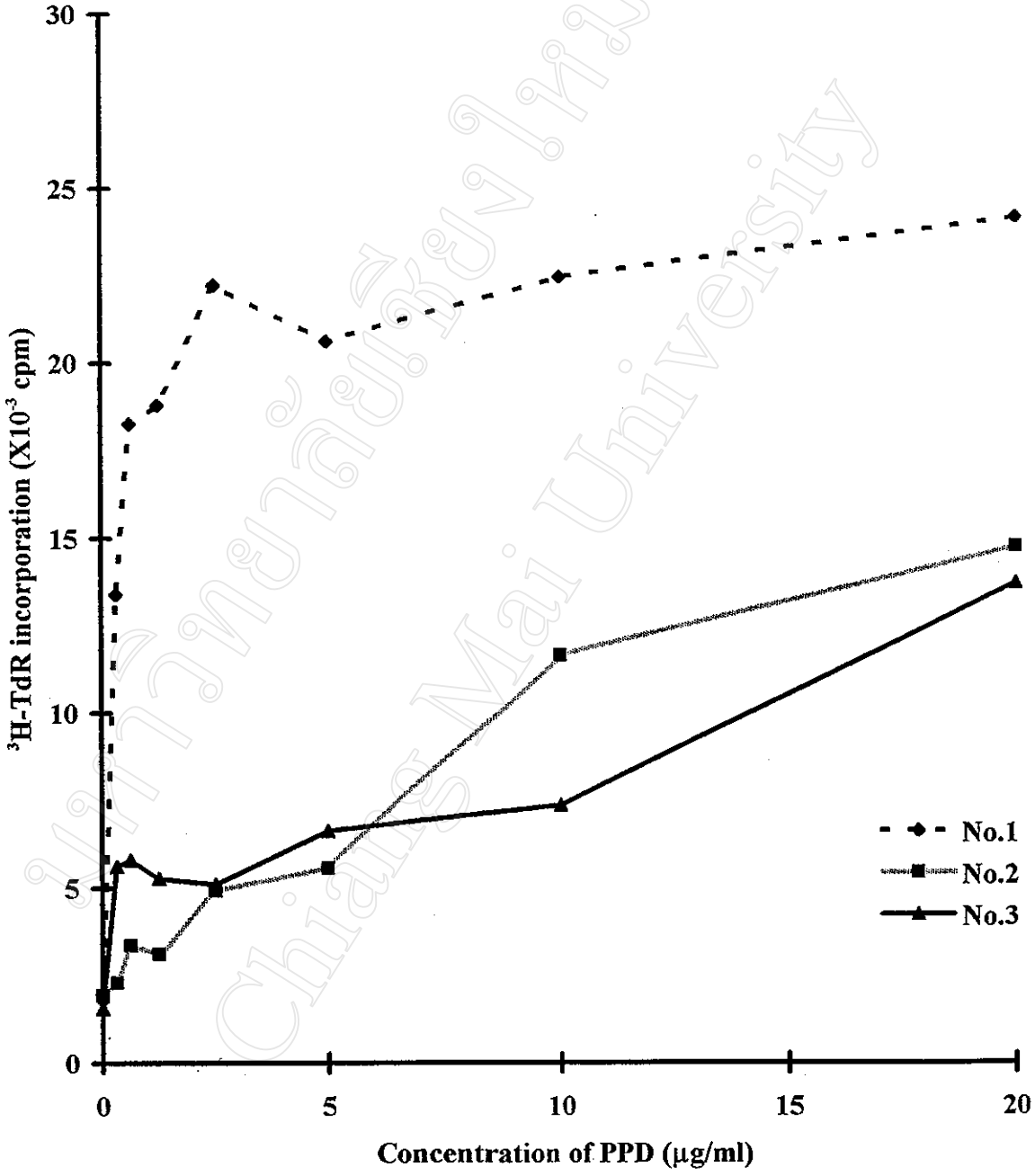

Figure 5. ^{3}H-thymidine incorporation of PBMC stimulation with various concentrations of PPD. The radioactive was expressed in term of Δcpm (cultures without PPD were subtracted).

6. Determination of the suboptimal and optimal concentration of the fraction *P. marneffei* antigen after gel filtration

A sample of concentrate *P. marneffei* cell lysate (2 ml) was separated by Sephadex G-100 gel filtration column, and two distinct protein peaks were observed (Fig. 6). The antigen protein activity of each peak was tested by lymphocyte transformation test. In order to compare the appropriate concentration use, between crude sonicated *P. marneffei* antigen and the fraction protein of *P. marneffei* antigen, various concentrations of each protein antigen (0.16, 0.64, 1.28, 2.56, 5.12 and 8.0 µg/well) were used to stimulate lymphocytes. The protein antigen activity was observed in the crude sonicated and peak 1 protein of eluate filtration (fraction 7 through 12) but not in peak 2 protein (fraction 19 through 23). The concentration of crude sonicated *P. marneffei* antigen and peak 1 protein antigen at 2.56, 5.12 and 8.0 µg/well gave high incorporation of tritiated-thymidine (Table 6, Fig. 7). Therefore, 0.16 and 1.28 µg/well of each protein antigen which were the suboptimal concentration and 5.12 µg/well was the optimal concentration, were used in all subsequent experiments of PBMC proliferation.

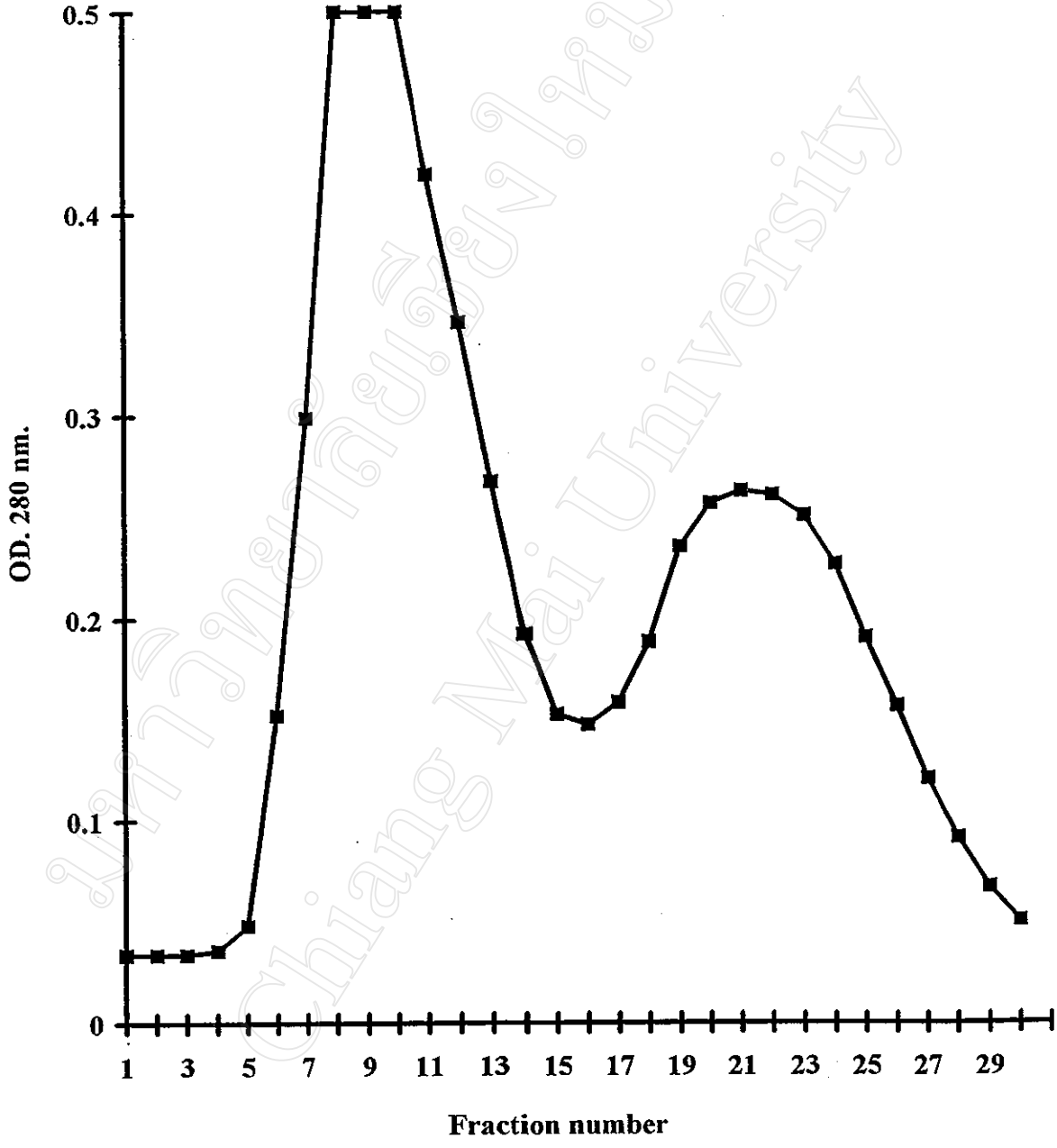

Figure 6. Sephadex G-100 column fraction of *P. marneffei* antigen. The protein concentration of each fraction is measured at OD 280 nm.

Table 6. ^{3}H-thymidine incorporation by PBMC after stimulation with various concentrations of crude sonicated *P. marneffei* or the fractions of *P. marneffei* antigen from gel filtration.

	No. of donor	^{3}H-TdR incorporation of PBMC (Δcpm)					
		Concentration of protein antigen (μg/ml)					
		0.16	0.64	1.28	2.56	5.12	8
Crude Ag.	1	397	1,089	1,748	7,386	8,918	7,384
	2	442	1,888	1,766	7,947	8,424	3,629
Peak 1 Ag.	1	222	388	2,103	3,750	4,537	8,776
	2	776	1,647	4,240	4,841	7,885	11,153
Peak 2 Ag.	1	97	18	3	295	221	676
	2	0	181	526	0	588	1,141

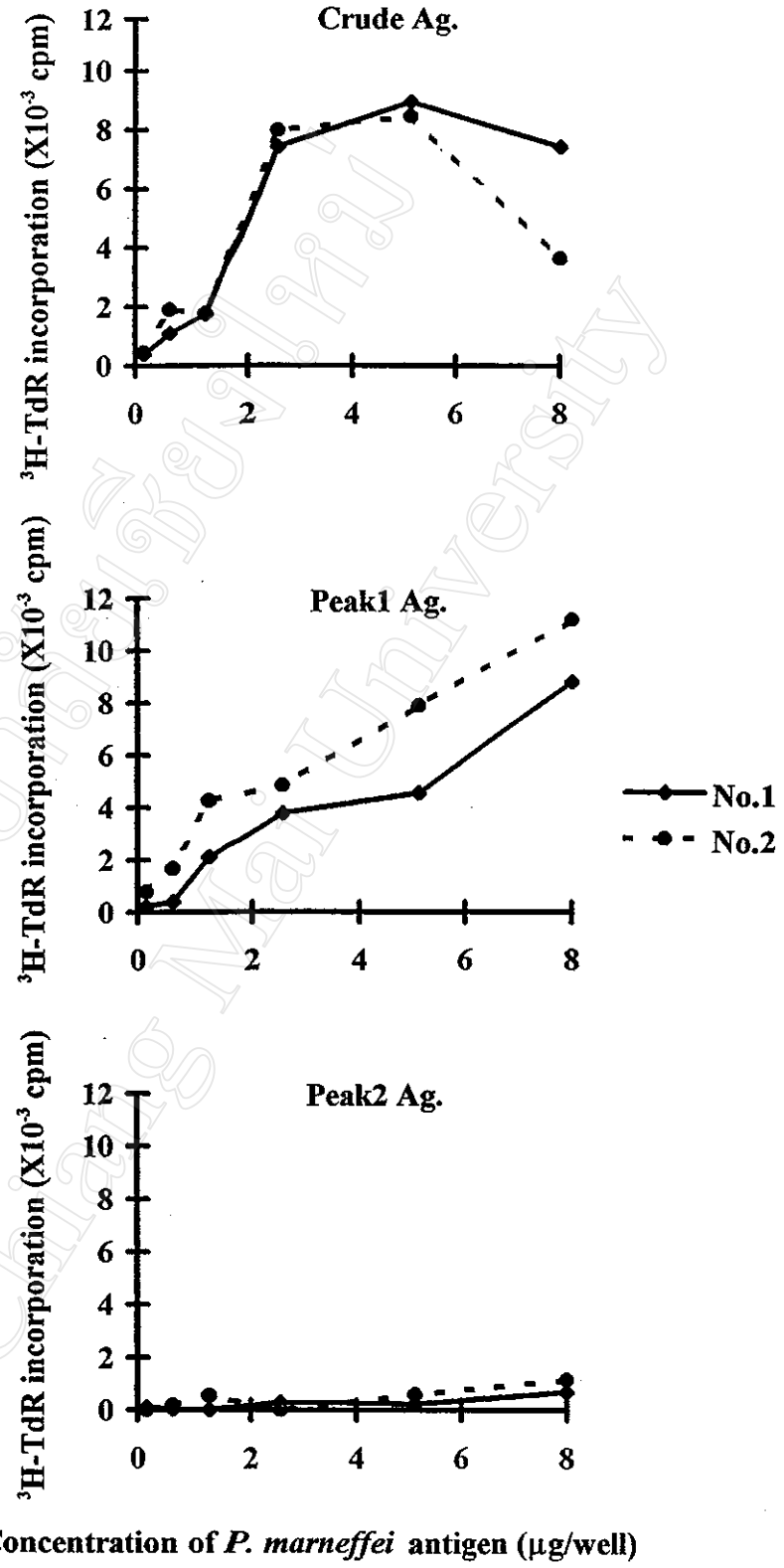

Figure 7. ^{3}H-thymidide incorporation by HIV negative PBMC stimulation with various concentrations of crude sonicated of *P. marneffei* or the fractions of *P. marneffei* antigen from gel filtration.

7. Determination of ability of lymphocytes transformation in AIDS patients stimulated with PHA-P

PBMCs were prepared from 15 asymtomatic HIV-infected individuals, 15 AIDS patients without *P. marneffei* infection, and 16 AIDS patients with *P. marneffei* infection. The mean amount of tritiated-thymidine incorporated by in each group was compared to 22 HIV-negative donors. The mean amount of tritiated-thymidine incorporation of HIV-negative lymphocytes stimulated by suboptimal and optimal concentration of PHA-P was 20,746 ± 7,594 cpm (range, 13,152 to 28,340) and 29,304 ± 7,094 cpm. (range, 22,210 to 36,398), respectively (Table 7). The mean amount of tritiated-thymidine incorporated by the lymphocytes from asymtomatic HIV-infected individuals, AIDS patients without *P. marneffei* infection, and AIDS patients with *P. marneffei* infection stimulated with the suboptimal concentration of PHA-P were 7,223 ± 7,896 cpm (range 0 to 15,119) (Table 8), 2,742 ± 5,074 cpm (range 0 to 7,816) (Table 9), and 596 ± 949 cpm (range, 0 to 1,545) (Table 10), respectively, and with the optimal concentration of PHA-P were 17,744 ± 9,600 cpm (range, 8,144 to 27,344) (Table 8), 12,923 ± 9,137 cpm (range 3,786 to 22,060) (Table 9), and 7,696 ± 8,404 cpm (range, 0 to 16,100) (Table 10), respectively. Lymphocyte transformation of all groups of such patients was less than that of HIV-negative donors ($p < 0.001$), indicating the impairment of response in those patients (Table 11, Fig. 8 and 9).

Table 7. The suboptimal and optimal concentration (0.125 and 1.0 μg/ml, respectively) of PHA-P induced proliferation of PBMC from HIV negative donors.

No. of donor	^{3}H-TdR incorporation (cpm)				
	Unstim.	PHA-P (0.125 μg/ml)	Δcpm	PHA-P (1.0 μg/ml)	Δcpm
1	542	25,761	25,219	35,631	35,089
2	394	21,578	21,184	30,917	30,523
3	317	12,103	11,786	12,103	11,786
4	243	24,314	24,071	38,506	38,263
5	164	5,529	5,365	34,767	34,603
6	134	19,606	19,472	33,333	33,199
7	255	32,099	31,844	36,764	36,509
8	260	30,568	30,308	38,339	38,079
9	169	18,155	17,986	27,700	27,531
10	351	15,219	14,868	23,095	22,744
11	176	22,198	22,022	36,271	36,095
12	814	32,698	31,884	35,660	34,846
13	139	33,580	33,441	33,442	33,303
14	188	17,097	16,909	24,296	24,108
15	182	6,148	5,966	28,755	28,573
16	213	20,143	19,930	25,774	25,561
17	199	21,902	21,703	31,520	31,321
18	521	13,655	13,134	16,961	16,440
19	397	23,750	23,353	32,388	31,991
20	132	22,124	21,992	24,544	24,412
21	107	19,499	19,392	20,399	20,292
22	231	24,811	24,580	29,656	29,425
$\bar{X}$			20,746		29,304
SD.			7,594		7,094

Table 8. The suboptimal and optimal concentration (0.125 and 1.0 μg/ml, respectively) of PHA-P induced proliferation of PBMC from asymptomatic HIV infected individuals.

No. of patient	^{3}H-TdR incorporation (cpm)				
	Unstim.	PHA-P (0.125 μg/ml)	Δcpm	PHA-P (1.0 μg/ml)	Δcpm
1	111	2,648	2,537	20,398	20,287
2	196	4,096	3,900	21,136	20,940
3	229	26,285	26,056	34,770	34,541
4	335	6,096	5,761	20,594	20,259
5	160	19,448	19,288	27,223	27,063
6	188	18,958	18,770	28,438	28,250
7	249	10,759	10,510	30,601	30,352
8	310	3,453	3,143	12,327	12,017
9	142	136	0	4,006	3,864
10	91	3,472	3,381	20,468	20,377
11	760	6,463	5,703	6,330	5,570
12	200	2,862	2,662	9,559	9,359
13	262	486	224	6,351	6,089
14	615	5,440	4,825	16,189	15,574
15	785	2,369	1,584	12,410	11,625
$\bar{X}$			7,223		17,744
SD.			7,896		9,600

Table 9. The suboptimal and optimal concentration (0.125 and 1.0 μg/ml, respectively) of PHA-P induced proliferation of PBMC from AIDS pateints without *P. marneffei* infection.

No. of patient	^{3}H-TdR incorporation (cpm)				
	Unstim.	PHA-P (0.125 μg/ml)	Δcpm	PHA-P (1.0 μg/ml)	Δcpm
1	234	2,374	2,140	19,776	19,542
2	105	165	60	6,351	6,246
3	661	1,318	657	8,561	7,900
4	210	187	0	9,819	9,609
5	582	20,197	19,615	30,832	30,250
6	446	2,345	1,899	10,662	10,216
7	283	3,470	3,187	6,822	6,539
8	147	618	471	20,963	20,816
9	186	5,736	5,550	21,053	20,867
10	51	53	2	1,412	1,361
11	286	6,413	6,127	28,749	28,463
12	193	216	23	13,069	12,876
13	214	1,294	1,080	3,756	3,542
14	541	791	250	3,139	2,598
15	100	182	82	13,123	13,023
$\overline{X}$			2,742		12,923
SD.			5,074		9,137

Table 10. The suboptimal and optimal concentration (0.125 and 1.0 μg/ml, respectively) of PHA-P induced proliferation of PBMC from AIDS pateints with *P. marneffei* infection.

No. of patient	^{3}H-TdR incorporation (cpm)				
	Unstim.	PHA-P (0.125 μg/ml)	Δcpm	PHA-P (1.0 μg/ml)	Δcpm
1	384	2,158	1,774	2,323	1,939
2	117	145	28	5,236	5,119
3	478	635	157	13,075	12,597
4	224	133	0	5,814	5,590
5	618	1,123	505	16,330	15,712
6	914	431	0	3,186	2,272
7	433	1,808	1,375	29,651	29,218
8	418	164	0	3,058	2,640
9	459	701	242	3,714	3,255
10	379	1,008	629	4,101	3,722
11	544	4,074	3,530	21,584	21,040
12	545	624	79	4,119	3,574
13	20	93	73	1,327	1,307
14	49	24	0	821	772
15	53	234	181	574	521
16	484	1,457	973	14,353	13,869
$\bar{X}$			596		7,696
SD.			949		8,404

Table 11. The suboptimal and optimal concentration (0.125 and 1.0 μg/ml, respectively) of PHA-P induced proliferation of PBMC from HIV negative donors, asymptomatic HIV infected individuals, AIDS patients without and with *P. maneffei* infection.

PHA-P (0.125μg/ml)	^{3}H-TdR incorporation (Δcpm)			
	HIV neg.	Asymp. HIV	AIDS s̄ PM.	AIDS c̄ PM.
$\bar{X}$	20,746	7,223	2,742	596
SD.	7,594	7,896	5,074	949
p*(& normal)		< 0.001	< 0.001	< 0.001
p*(& asymp.)			< 0.025	< 0.01
p*(& AIDS)				< 0.05
PHA-P (1.0 μg/ml)				
$\bar{X}$	29,304	17,744	12,923	7,696
SD.	7,094	9,600	9,137	8,404
p*(& normal)		< 0.001	< 0.001	< 0.001
p*(& asymp.)			> 0.1	< 0.005
p*(& AIDS)				< 0.05

p* = The Mann-Whitney U test

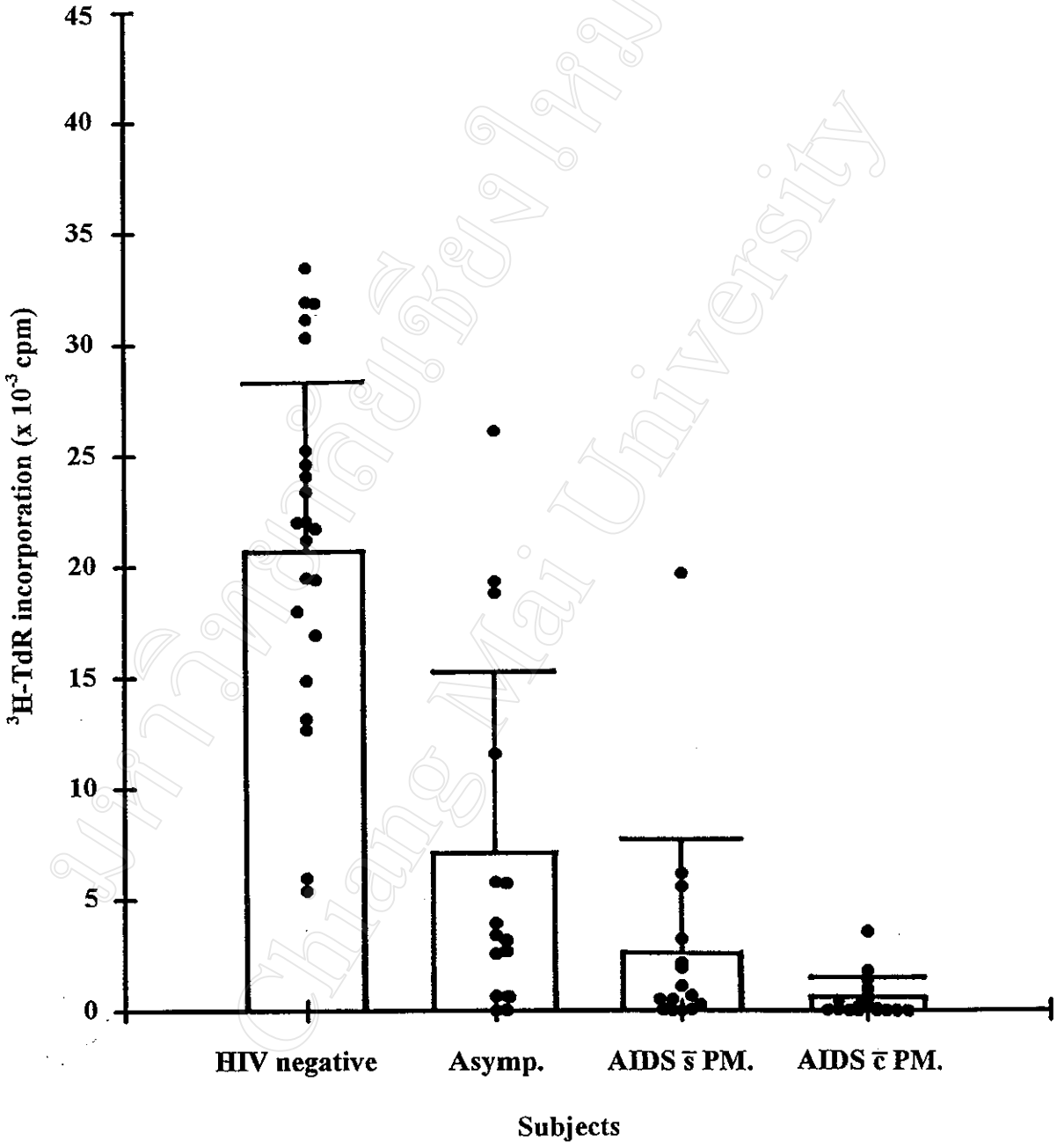

Figure 8. The suboptimal concentration of PHA-P (0.125 µg/ml) induced proliferation of PBMC from HIV negative donors, asymptomatic HIV infected individuals, AIDS patients without and with *P. marneffei* infection in the presence of fetal bovine serum.

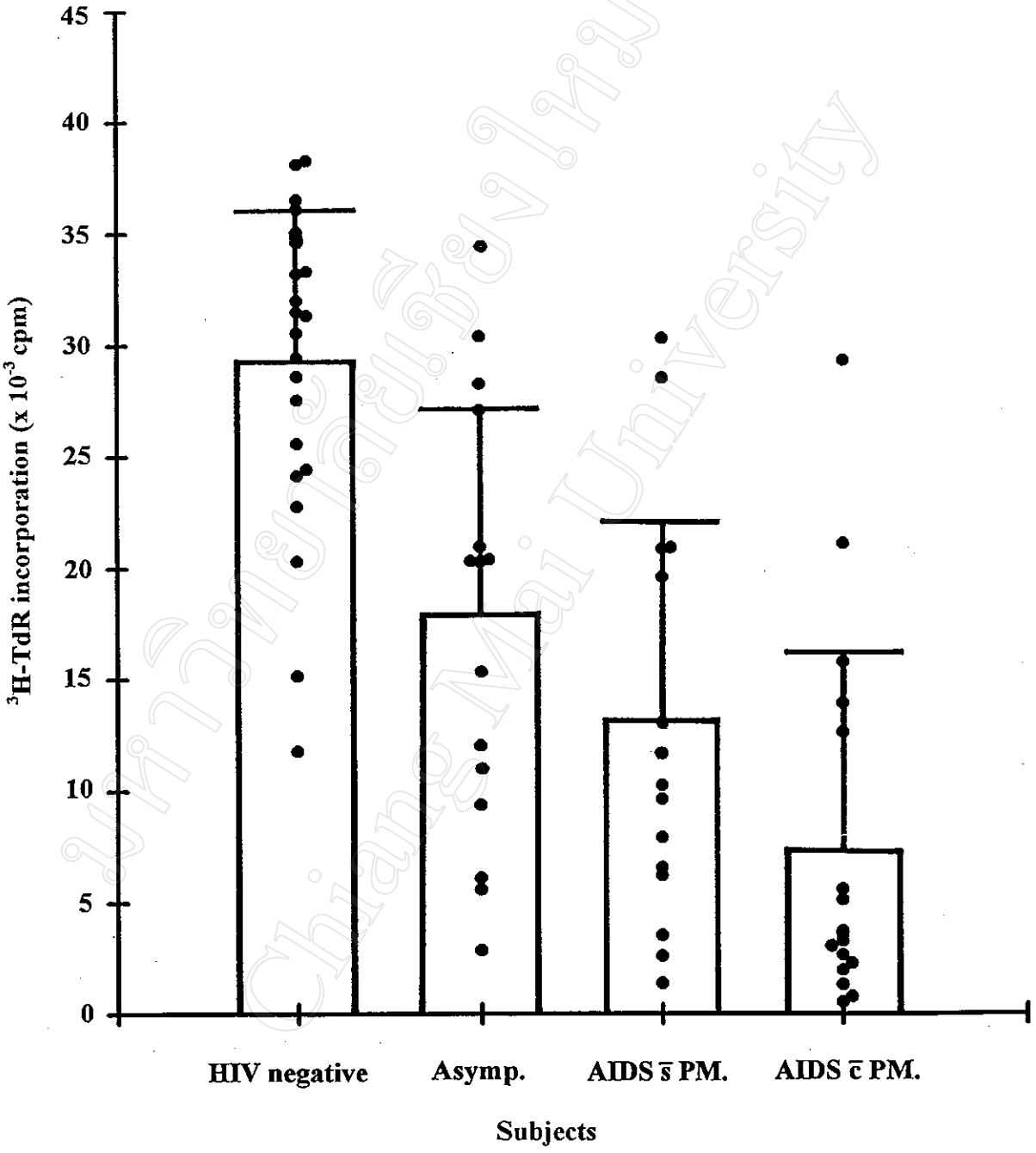

Figure 9. The optimal concentration of PHA-P (1.0 µg/ml) induced proliferation of PBMC from HIV negative donors, asymptomatic HIV infected individuals, AIDS patients without and with *P. marneffei* infection in the presence of fetal bovine serum.

8. Determination of the ability of lymphocyte transformation in AIDS patients stimulated with crude sonicated *P. marneffei* antigen

The PBMCs were prepared from 15 asymtomatic HIV-infected individuals, 15 AIDS patients without *P. marneffei* infection, and 16 AIDS patients with *P. marneffei* infection. The mean amount of tritiated-thymidine incorporated by each group of lymphocytes was compared to that of 22 HIV-negative donors. The mean amount of tritiated-thymidine incorporated by HIV-negative lymphocytes stimulated with crude sonicated *P. marneffei* antigen at the suboptimal and optimal concentration was 10,016 ± 6,922 cpm (range, 3,094 to 16,938), and 12,280 ± 7,206 cpm (range, 5,074 to 19,486), respectively (Table 12). The mean amount of tritiated-thymidine incorporated by lymphocytes from asymtomatic HIV-infected individuals, from AIDS patients without and with *P. marneffei* infection stimulated with the suboptimal concentration of crude sonicated *P. marneffei* antigen was 2,129 ± 5,354 cpm (range, 0 to 7,488) (Table 13), 129 ± 219 cpm (range, 0 to 348) (Table 14), and 142 ± 347 cpm. (range, 0 to 489) (Table 15), respectively. In addition, stimulation with the optimal concentration of crude sonicated *P. marneffei* antigen resulted in 2,232 ± 6,247 cpm (range, 0 to 8,479) (Table 13), 193 ± 469 cpm (range, 0 to 662) (Table 14), and 110 ± 229 cpm (range, 0 to 339) (Table 15), respectively. The lymphocyte transformation in HIV-infected patient stimulated with the suboptimal and optimal concentration of crude sonicated *P. marneffei* antigen was also compared to HIV negative donors. It was found that the average actual amount of tritiated-thymidine incorporated by lymphocytes from

Table 12. The suboptimal and optimal concentration (0.32 and 2.56 μg/well, respectively) of crude sonicated *P. marneffei* antigen induced proliferation of PBMC from HIV negative donors.

No. of donor	^{3}H-TdR incorporation (cpm)				
	Unstim.	PM. antigen (0.32 μg/well)	Δcpm	PM. antigen (2.56 μg/well)	Δcpm
1	1,742	20,511	18,769	27,015	25,273
2	1,507	9,008	7,501	9,646	8,139
3	3,802	10,275	6,473	10,492	6,690
4	3,434	15,319	11,885	17,883	14,449
5	1,951	6,216	4,265	10,100	8,149
6	582	5,884	5,302	10,230	9,648
7	2,665	17,087	14,422	21,210	18,545
8	2,685	18,541	15,856	22,791	20,106
9	250	1,296	1,046	2,751	2,501
10	2,470	25,346	22,876	26,242	23,772
11	1,530	17,769	16,239	16,854	15,324
12	685	21,255	20,570	21,853	21,168
13	1,274	11,081	9,807	15,455	14,181
14	1,146	19,404	18,258	19,550	18,404
15	790	1,159	367	2,113	2,113
16	1,127	16,318	15,191	18,829	17,702
17	453	5,545	5,092	8,617	8,164
18	283	10,769	10,486	12,908	12,625
19	362	9,575	9,213	8,934	8,572
20	454	597	115	787	333
21	943	3,366	2,423	4,345	3,402
22	531	4,739	4,208	11,444	10,913
$\bar{X}$			10,016		12,280
SD.			6,922		7,206

Table 13. The suboptimal and optimal concentration (0.32 and 2.56 μg/well, respectively) of crude sonicated *P. marneffei* antigen induced proliferation of PBMC from asymptomatic HIV infected individuals.

No. of patient	^{3}H-TdR incorporation (cpm)				
	Unstim.	PM. antigen (0.32 μg/well)	Δcpm	PM. antigen (2.56 μg/well)	Δcpm
1	131	91	0	80	0
2	39	239	200	1,146	1,107
3	1,004	21,656	20,652	25,267	24,263
4	141	330	189	447	305
5	114	1,478	1,364	2,726	2,612
6	1,068	3,312	2,244	264	0
7	483	1,232	749	346	0
8	175	554	379	290	115
9	101	115	14	64	0
10	408	503	95	535	127
11	436	516	80	442	6
12	150	185	35	140	0
13	249	100	0	170	0
14	96	6,026	5,930	5,027	4,931
15	45	43	0	66	21
$\bar{X}$			2,129		2,232
SD.			5,354		6,247

Table 14. The suboptimal and optimal concentration (0.32 and 2.56 μg/well, respectively) of crude sonicated *P. marneffei* antigen induced proliferation of PBMC from AIDS patients without *P. marneffei* infection.

No. of patient	^{3}H-TdR incorporation (cpm)				
	Unstim.	PM. antigen (0.32 μg/well)	Δcpm	PM. antigen (2.56 μg/well)	Δcpm
1	322	353	31	435	113
2	84	53	0	109	25
3	294	327	33	378	84
4	42	101	59	36	0
5	106	751	645	1,916	1,810
6	134	709	575	154	20
7	891	1,165	274	1,174	283
8	86	78	0	28	0
9	539	857	318	1,060	521
10	85	61	0	61	0
11	56	40	0	70	14
12	228	235	10	121	17
13	373	173	0	185	0
14	385	310	0	397	12
15	98	96	0	46	0
$\overline{X}$			129		193
SD.			219		469

Table 15. The suboptimal and optimal concentration (0.32 and 2.56 μg/well, respectively) of *P. marneffei* antigen induced proliferation of PBMC from AIDS patients with *P. marneffei* infection.

No. of patient	^{3}H-TdR incorporation (cpm)				
	Unstim.	PM. antigen (0.32 μg/well)	Δcpm	PM. antigen (2.56 μg/well)	Δcpm
1	336	291	0	171	0
2	95	152	57	169	74
3	859	430	0	669	0
4	136	151	15	227	91
5	228	274	46	180	0
6	629	1,983	1,354	629	0
7	1,557	725	0	805	0
8	346	258	0	554	208
9	449	697	248	401	0
10	501	987	485	1,358	857
11	2,215	2,261	46	2,640	425
12	202	166	0	310	108
13	103	93	0	40	0
14	139	123	0	57	0
15	104	96	0	139	0
16	390	414	24	357	0
$\overline{X}$			142		110
SD.			347		229

Table 16. The suboptimal and optimal concentration (0.32 and 2.56 μg/well, respectively) of crude sonicated *P. marneffei* antigen induced proliferation of PBMC from HIV negative donors, asymptomatic HIV infected individuals, AIDS patients without and with *P. maneffei* infection.

PM. Ag. (0.32μg/well)	^{3}H-TdR incorporation (Δcpm)			
	HIV neg.	Asymp. HIV	AIDS s̄ PM.	AIDS c̄ PM.
$\bar{X}$	10,016	2,129	129	142
SD.	6,922	5,354	219	347
p*(& normal)		< 0.001	< 0.001	< 0.001
p*(& asymp.)			< 0.025	< 0.025
p*(& AIDS)				> 0.1
PM. Ag. (2.56 μg/well)				
$\bar{X}$	12,280	2,232	193	110
SD.	7,206	6,247	469	229
p*(& normal)		< 0.001	< 0.001	< 0.001
p*(& asymp.)			> 0.1	> 0.1
p*(& AIDS)				> 0.05

p* = The Mann-Whitney U test

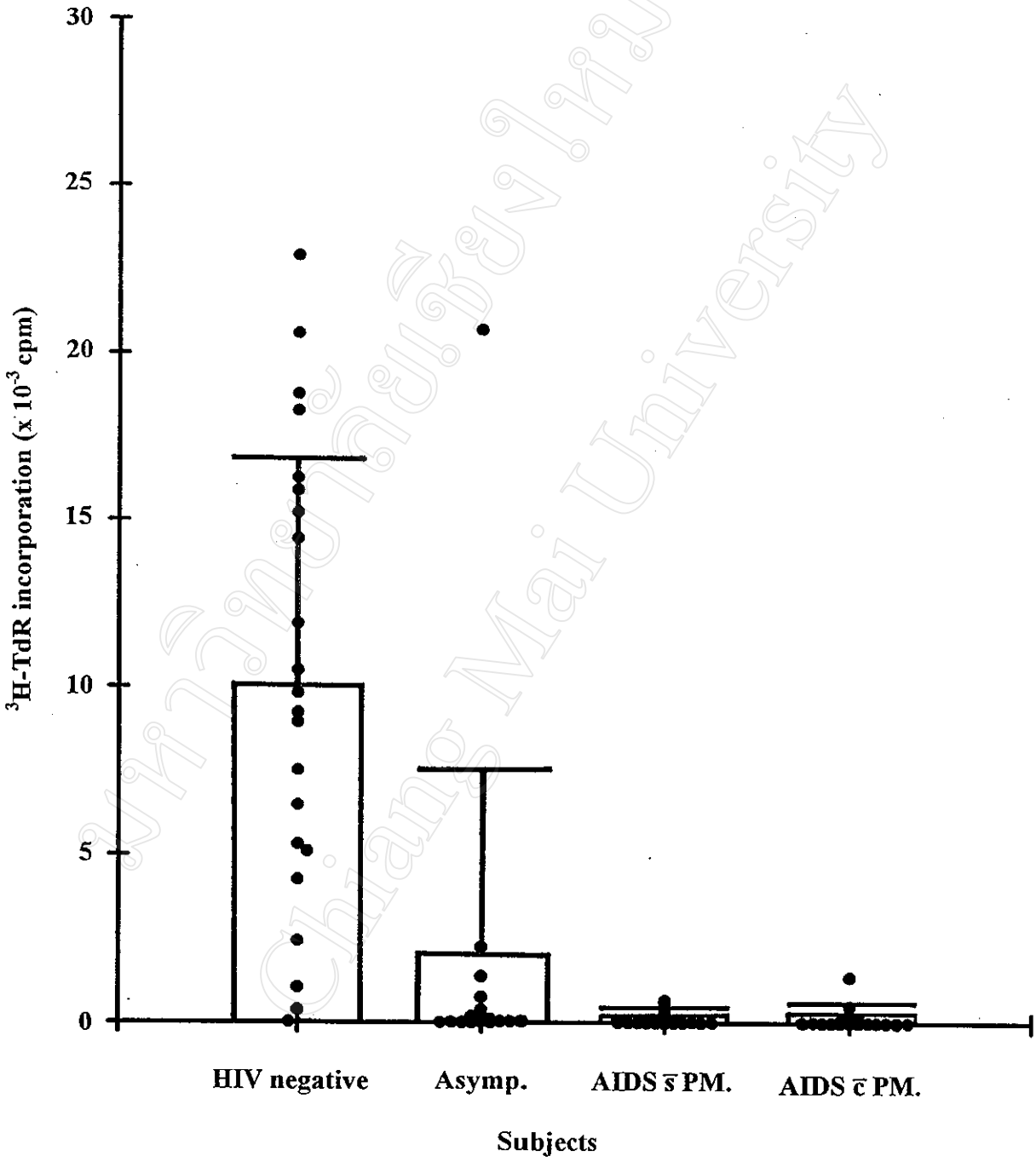

Figure 10. The suboptimal concentration of crude sonicated *P. marneffei* antigen (0.32 µg/well) induced proliferation of PBMC from HIV negative donors, asymptomatic HIV infected individuals, AIDS patients without and with *P. marneffei* infection in the presence of fetal bovine serum.

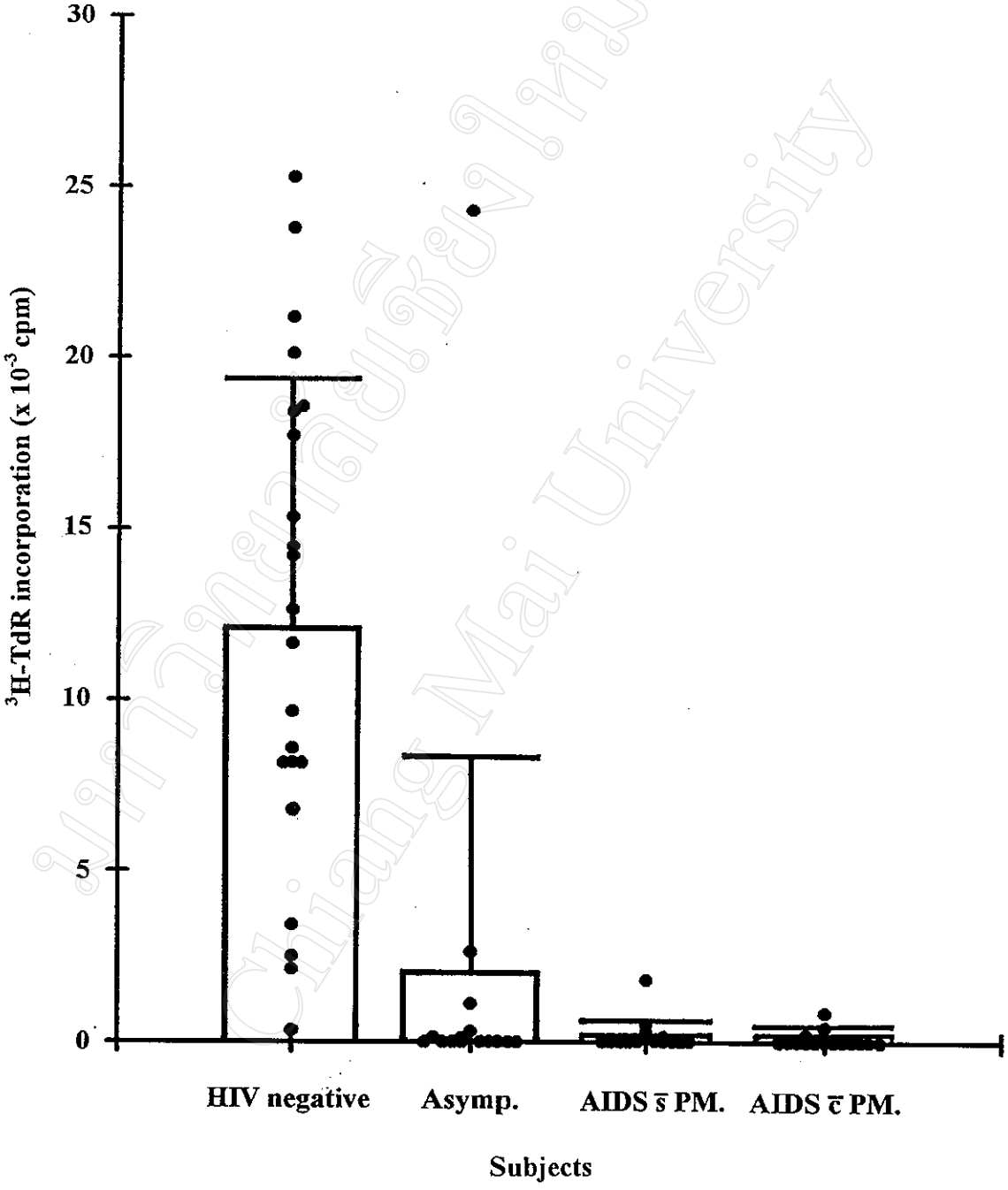

Figure 11. The optimal concentration of crude sonicated *P. marneffei* antigen (2.56 μg/well) induced proliferation of PBMC from HIV negative donors, asymptomatic HIV infected individuals, AIDS patients wihtout and with *P. marneffei* infection in the presence of fetal bovine serum.

all groups of patients were less than that by lymphocytes from HIV-negative donors ($p < 0.001$) (Table 16, Fig. 10 and 11).

9. Determination of the ability of lymphocyte transformation in AIDS patients stimulated with PPD

Lymphocyte transformation of 14 asymtomatic HIV-infected individuals, 6 AIDS patients without *P. marneffei* infection, and 5 AIDS patients with *P. marneffei* infection was assayed and compared to 15 HIV-negative donors. The mean amount of tritiated-thymidine incorporated by HIV-negative lymphocytes stimulated with the suboptimal and optimal concentration was 5,333 ± 5,849 cpm (range, 0 to 11,182) and 8,808 ± 6,544 cpm (range, 2,264 to 15,352) (Table 17), respectively, whereas the tritiated-thymidine incorporated by lymphocytes from asymptomatic HIV-infected individuals, AIDS patients without and with *P. marneffei* infection stimulated with the suboptimal concentration of PPD were 1,009 ± 2,612 cpm (range , 0 to 3,621) (Table 18), 8 ± 12 cpm (range, 0 to 20) (Table 19), and 145 ± 240 cpm (range 0 to 385) (Table 20), respectively, These results were less than the cpm. derived from HIV-negative donors ($p < 0.001$). Moreover, the stimulation effect with the optimal concentration were 2,089 ± 5,268 cpm (range, 0 to 7,357) (Table 18), 0.5 ± 1 cpm (range, 0 to 1.5) (Table 19), and 111 ± 187 (range, 0 to 298) (Table 20), respectively. These results were also significantly decreased when compared to HIV-negative donors ($p < 0.001$) (Table 21, Fig. 12 and 13).

Table 17. The suboptimal and optimal concentration (0.3125 and 10.0 μg/ml, respectively) of PPD induced proliferation of PBMC from HIV negative donors.

No. of donor	^{3}H-TdR incorporation (cpm)				
	Unstim.	PPD (0.3125 μg/ml)	Δcpm	PPD (10.0 μg/ml)	Δcpm
1	1,233	8,624	7,391	20,621	19,388
2	197	504	307	3,823	3,626
3	2,388	16,794	14,406	18,564	16,176
4	576	10,600	10,024	12,331	11,755
5	524	6,066	5,542	16,838	16,314
6	402	3,671	3,269	12,063	11,661
7	750	20,962	20,212	20,665	19,915
8	190	4,685	4,495	7,160	6,970
9	551	1,858	1,307	5,512	4,961
10	314	1,027	713	1,712	1,398
11	137	2,046	1,909	7,381	7,244
12	224	8,186	7,962	5,117	4,893
13	145	545	400	2,889	2,744
14	156	399	243	1,344	1,188
15	422	2,235	1,813	4,311	3,889
$\bar{X}$			5,333		8,808
SD.			5,849		6,544

Table 18. The suboptimal and optimal concentration (0.3125 and 10.0 μg/ml, respectively) of PPD induced proliferation of PBMC from asymptomatic HIV infected individuals.

No. of patient	^{3}H-TdR incorporation (cpm)				
	Unstim.	PPD (0.3125 μg/ml)	Δcpm	PPD (10.0 μg/ml)	Δcpm
1	162	73	0	80	0
2	42	22	0	25	0
3	1,083	6,689	5,606	11,354	10,271
4	86	120	34	97	11
5	207	8,663	8,456	17,967	17,760
6	104	97	0	1,073	969
7	112	130	8	208	96
8	186	140	0	217	31
9	49	36	0	33	0
10	70	106	0	90	20
11	98	114	16	139	41
12	404	400	0	411	7
13	80	90	10	113	33
14	37	23	0	42	5
$\bar{X}$			1,009		2,089
SD.			2,612		5,268

Table 19. The suboptimal and optimal concentration (0.3125 and 10.0 μg/ml, respectively) of PPD induced proliferation of PBMC from AIDS patients without *P. marneffei* infection.

No. of patient	^{3}H-TdR incorporation (cpm)				
	Unstim.	PPD (0.3125 μg/ml)	Δcpm	PPD (10.0 μg/ml)	Δcpm
1	1,545	739	0	1,101	0
2	15	22	7	12	0
3	136	71	0	81	0
4	178	165	0	181	3
5	278	310	32	242	0
6	42	52	10	29	0
$\bar{X}$			8		0.5
SD.			12		1

Table 20. The suboptimal and optimal concentration (0.3125 and 10.0 μg/ml, respectively) of PPD induced proliferation of PBMC from AIDS patients with *P. marneffei* infection.

No. of patient	^{3}H-TdR incorporation (cpm)				
	Unstim.	PPD (0.3125 μg/ml)	Δcpm	PPD (10.0 μg/ml)	Δcpm
1	1,343	639	0	791	0
2	2,544	3,098	554	2,977	433
3	3,778	356	0	325	0
4	136	24	0	21	0
5	226	401	175	352	126
$\bar{X}$			145		111
SD.			240		127

Table 21. The suboptimal and optimal concentration (0.3125 and 10.0 μg/ml, respectively) of PPD induced proliferation of PBMC from HIV negative donors, asymptomatic HIV infected individuals, AIDS patients without and with *P. maneffei* infection.

PPD (0.3125μg/ml)	^{3}H-TdR incorporation (Δcpm)			
	HIV neg.	Asymp. HIV	AIDS s̄ PM.	AIDS c̄ PM.
$\bar{X}$	5,333	1,009	8	145
SD.	5,849	2,612	12	240
p*(& normal)		< 0.001	< 0.001	< 0.001
p*(& asymp.)			> 0.1	> 0.1
p*(& AIDS)				> 0.1
PPD (10.0 μg/ml)				
$\bar{X}$	8,808	2,089	0.5	111
SD.	6,544	5,268	1	127
p*(& normal)		< 0.001	< 0.001	< 0.001
p*(& asymp.)			< 0.001	> 0.1
p*(& AIDS)				> 0.1

p* = The Mann-Whitney U test

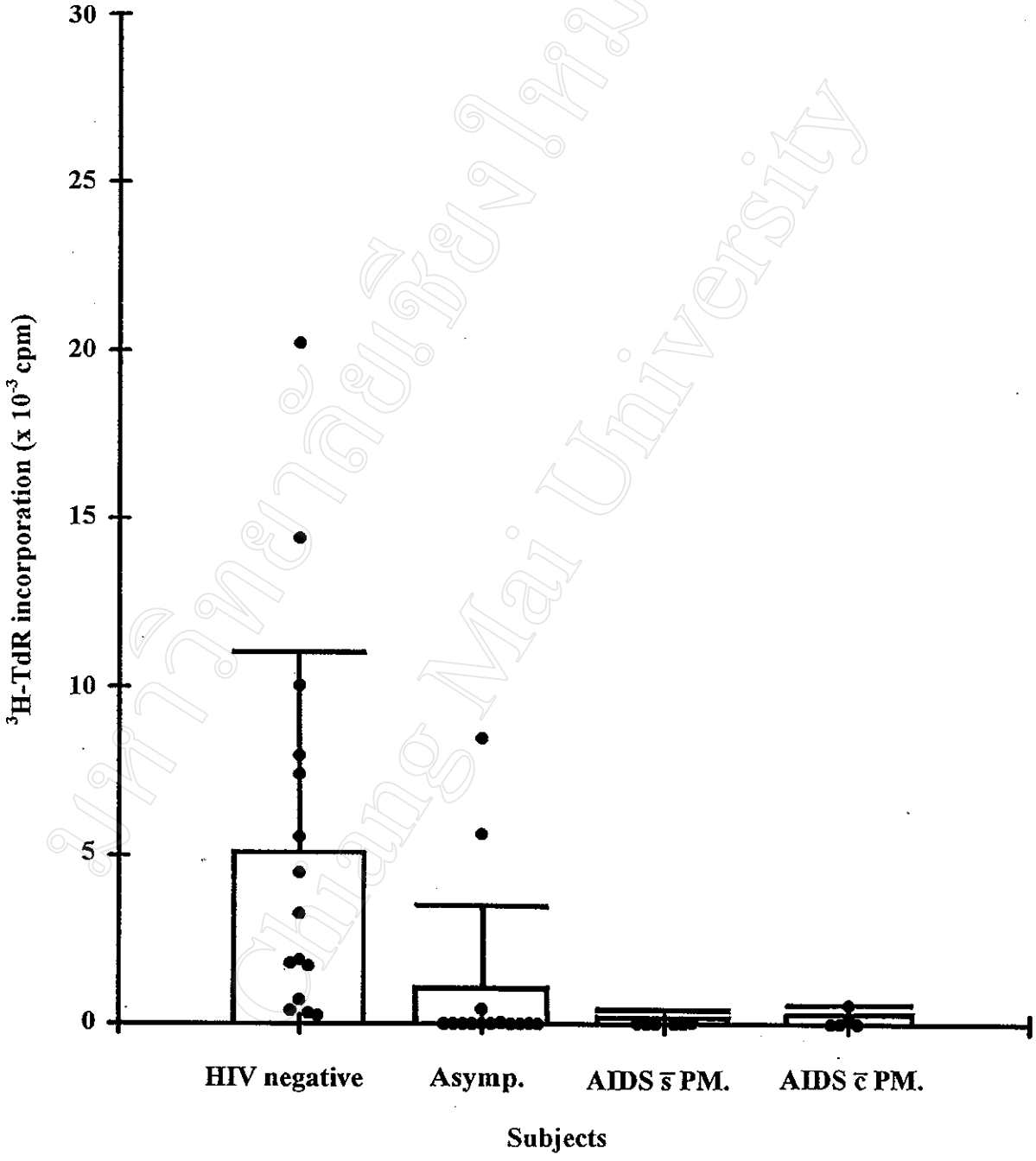

Figure 12. The suboptimal concentration of PPD (0.3125 µg/ml) induced proliferation of PBMC from HIV negative donors, asymptomatic HIV infected individuals, AIDS patients wihtout and with *P. marneffei* infection in the presence of fetal bovine serum.

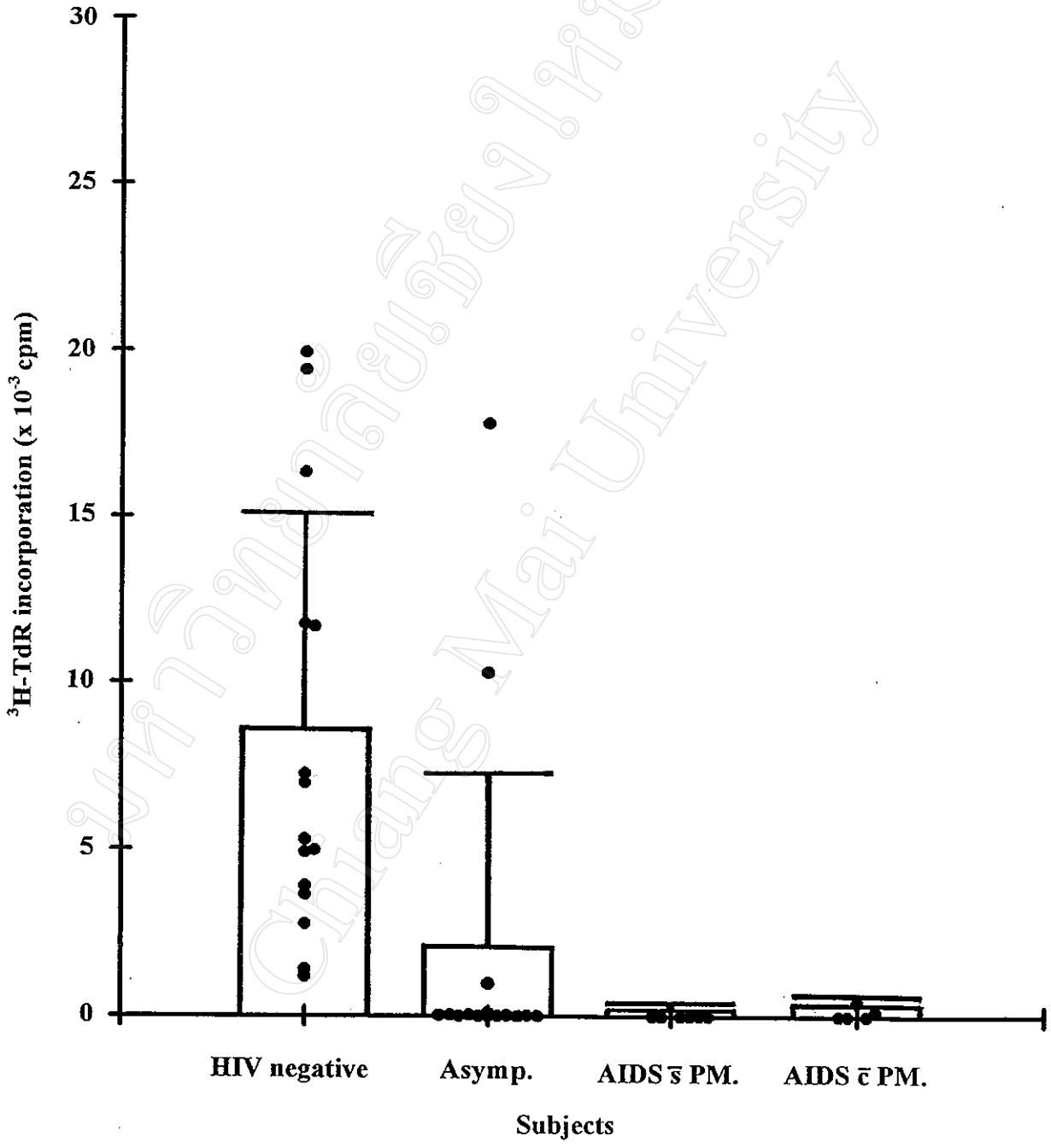

Figure 13. The optimal concentration of PPD (10.0 μg/ml) induced proliferation of PBMC from HIV negative donors, asymptomatic HIV infected individuals, AIDS patients without and with *P. marneffei* infection in the presence of fetal bovine serum.

10. Identification of active fraction of *P. marneffei* antigen after gel filtration

In order to demonstrate the stimulation effect of crude sonicated *P. marneffei* antigen and the fraction protein antigen of *P. marneffei*. Lymphocyte transformation of 8 HIV-negative donors were performed. The mean amount of tritiated-thymidine incorporation of normal lymphocytes stimulated with crude sonicated *P. marneffei* antigen at the suboptimal concentration (0.16 and 1.28 µg/well) and optimal concentration (5.12 µg/well) were 979 ± 1,219 cpm (range to 2,198), 2,952 ± 679 cpm (range 2,273 to 3,631), and 5,435 ± 1,879 cpm (range 3,556 to 7,314), respectively. The mean amount of tritiated-thymidine incorporation of normal lymphocytes stimulated with peak 1 protein antigen at the same concentrations were 1,228 ± 893 cpm (range 335 to 2,116), 3,636 ± 2,974 cpm (range 662 to 6,610), and 7,577 ± 4,806 cpm (range 2,771 to 12,383), respectively. The lymphocyte transformation assay of peak 2 antigen with the same concentration was also evaluated, and it was shown that the mean amount of tritiated-thymidine incorporation were 53 ± 70 cpm (range 0 to 123), 266 ± 336 cpm (range 0 to 602), and 652 ± 474 cpm (range 178 to 1,126), respectively (Table 22 ,Fig. 14). The results altogether indicated that the protein antigen activity was only in peak 1 (fraction 7 through 12).

Table 22. ^{3}H-thymidine incorporation by PBMC from HIV negative donors stimulation with fraction protein of *P. marneffei* antigen.

*No. of test	^{3}H-TdR incorporation of PBMC (Δcpm)								
	crude Ag. (μg/well)			peak 1Ag. (μg/well)			peak 2 Ag. (μg/well)		
	0.16	1.28	5.12	0.16	1.28	5.12	0.16	1.28	5.12
1	205	2,499	3,189	38	2,036	7,942	46	57	233
2	0	2,119	4,739	580	2,087	7,769	149	419	458
3	1,419	3,075	5,217	1,732	4,046	5,408	15	574	926
4	41	2,423	9,373	2,047	10,700	18,870	15	41	1,168
5	2,793	2,699	5,157	1,376	2,514	3,520	0	10	85
6	302	3,916	6,793	1,203	2,127	6,651	0	20	965
7	231	3,973	4,610	256	2,157	4,535	19	91	146
8	2,842	2,913	4,409	2,593	3,424	5,926	180	916	1,241
$\bar{X}$	979	2,952	5,435	1,228	3,636	7,577	53	266	652
SD.	1,219	679	1,879	893	2,974	4,806	70	336	474

*** No. of test = No. of donor**

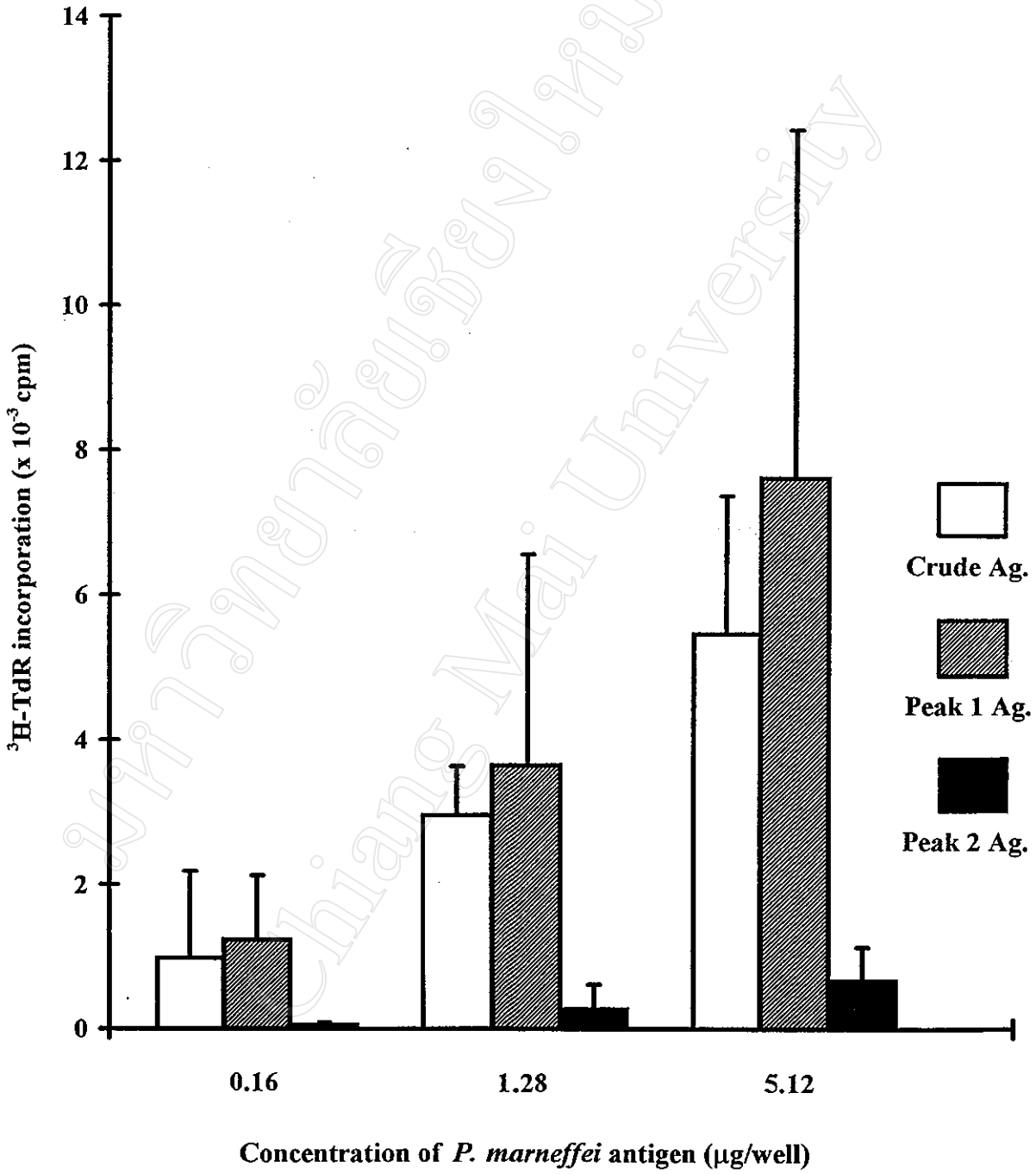

Figure 14. ^{3}H-thymidine incorporation by PBMC from HIV negative donors stimulation with crude sonicated of *P. marneffei* or the fractions of *P. marneffei* antigen from gel filtration.

11. Determination of plasma from AIDS patient with *P. marneffei* infection inhibit proliferration of normal T cell

Plasma from 16 asymtomatic HIV-infected individuals, 16 AIDS patients without *P. marneffei* infection, 16 AIDS patients with *P. marneffei* infection, and autologous plasma was assayed for its effects on proliferation of normal PBMC, at a total concentration of 10%. The mean of percent inhibition ± SD of normal PBMC proliferation individually stimulated with the suboptimal concentration of PHA-P in the presence of 10% plasma from asymtomatic HIV-infected individuals, AIDS patients without and with *P. marneffei* infection compared to those culture with autologous plasma were 23 ± 22% (range, 1 to 53), 68 ± 22% (range 46 to 90), and 57 ± 27% (range, 30 to 84), respectively (Table 23, Fig. 15). These results indicate that transformation was diminished when lymphocytes were cultured with 10% plasma from AIDS patient without and with *P. marneffei* infection when compared to transformation when lymphocytes were cultured with the plasma obtained from asymtomatic HIV-infected individuals ($p < 0.001$). The difference was not observed in culturing with 10% plasma obtained form AIDS patient without *P. marneffei* infection when compared to the plasma form AIDS patient with *P. marneffei* infection ($p > 0.2$). Proliferation of normal PBMC stimulated with the optimal concentration of PHA-P in the presence of 10% plasma from asymtomatic HIV-infected individuals, AIDS patients without *P. marneffei* infection, and AIDS patients with *P. marneffei* infection, compared to those cultured in the presence of autologous plasma. The mean percent inhibition ± SD was 5 ± 7% (range, 0 to 12), 20 ± 17%

(range, 3 to 37), and 27 ± 19% (range, 8 to 46), respectively. In addition when compared to the normal PBMC cultured with 10% plasma obtained from asymtomatic HIV-infected individual, the result showed that there was significant decrease in lymphocyte transformation cultured with 10% plasma obtain form AIDS patients without and with *P. marneffei* infection ($p < 0.001$). There was no significant difference in percent inhibition between cultures with the plasma obtained from AIDS patient without *P. marneffei* infection and cultures with the plasma from AIDS patient with *P. marneffei* infection ($p > 0.2$) (Table 24, Fig. 16).

12. Determination of the concentration of plasma from AIDS patients to inhibit proliferation of normal T cell

The inhibitory effect of the plasma obtained from 6 asymtomatic HIV-infected individuals, 6 AIDS patients without *P. marneffei* infection, and 6 AIDS patients with *P. marneffei* infection on the proliferation of normal healthy PBMC was investigated. Healthy donor plasma or patient plasma (total concentration of 5, 10, 20 and 30%) were individually cultured with PBMC (1.5×10^5 cells/well) in presence of PHA-P. Cells were harvested and analyzed for a final increase or decrease in the level of DNA synthesis. Inhibition of tritiated-thymidine incorporation were observed in all experiments supplemented with the plasma from AIDS patient wihtout and with *P. marneffei* infection. In the presence of a suboptimal concentration of PHA-P (0.125 μg/ml), the inhibition effect of plasma obtained form asymtomatic HIV-infected individuals was 15, 15, 28, and 32% in 5, 10, 20, and 30 percent of plasma concentration,

Table 23. Inhibitory effect of the individual plasma (10%) from asymptomatic HIV infected individuals, AIDS patients without and with *P. marneffei* infection on ^{3}H-thymidine incorporation by suboptimal concentration of PHA-P (0.125 μg/ml) stimulated normal PBMC. Percent inhibition was compared to the proliferation in the presence of 10% autologous plasma.

No. of normal PBMC	% Inhibition by plasma from		
	asymptomatic HIV	AIDS s̄ PM.	AIDS c̄ PM.
1	0	76	82
2	0	26	70
3	0	92	70
4	57	73	52
5	51	80	78
6	0	68	85
7	35	68	0
8	18	92	70
9	14	77	68
10	16	57	24
11	31	57	6
12	40	57	74
13	66	22	29
14	21	59	70
15	0	85	81
16	14	99	62
$\bar{X}$	23	68	57
SD.	22	22	27
p*(&Asymp.)		< 0.001	< 0.001
p*(&AIDS)			> 0.2

p* = Student t-test

Table 24. Inhibitory effect of the individual plasma (10%) from asymptomatic HIV infected individuals, AIDS patients without and with *P. marneffei* infection on ^{3}H-thymidine incorporation by suboptimal concentration of PHA-P (1.0 μg/ml) stimulated normal PBMC. Percent inhibition was compared to the proliferation in the presence of 10% autologous plasma.

No. of nomal PBMC	% Inhibition by plasma from		
	asymptomatic HIV	AIDS $\bar{s}$ PM.	AIDS $\bar{c}$ PM.
1	19	17	17
2	8	17	39
3	5	29	18
4	16	6	12
5	13	0	12
6	0	7	18
7	0	11	0
8	0	29	0
9	0	29	34
10	0	13	23
11	0	17	67
12	0	0	45
13	17	14	55
14	0	28	29
15	0	41	47
16	8	68	24
$\overline{X}$	5	20	27
SD	7	17	19
p*(&Asymp.)		< 0.001	< 0.001
p*(&AIDS)			> 0.2

p* = Student t-test

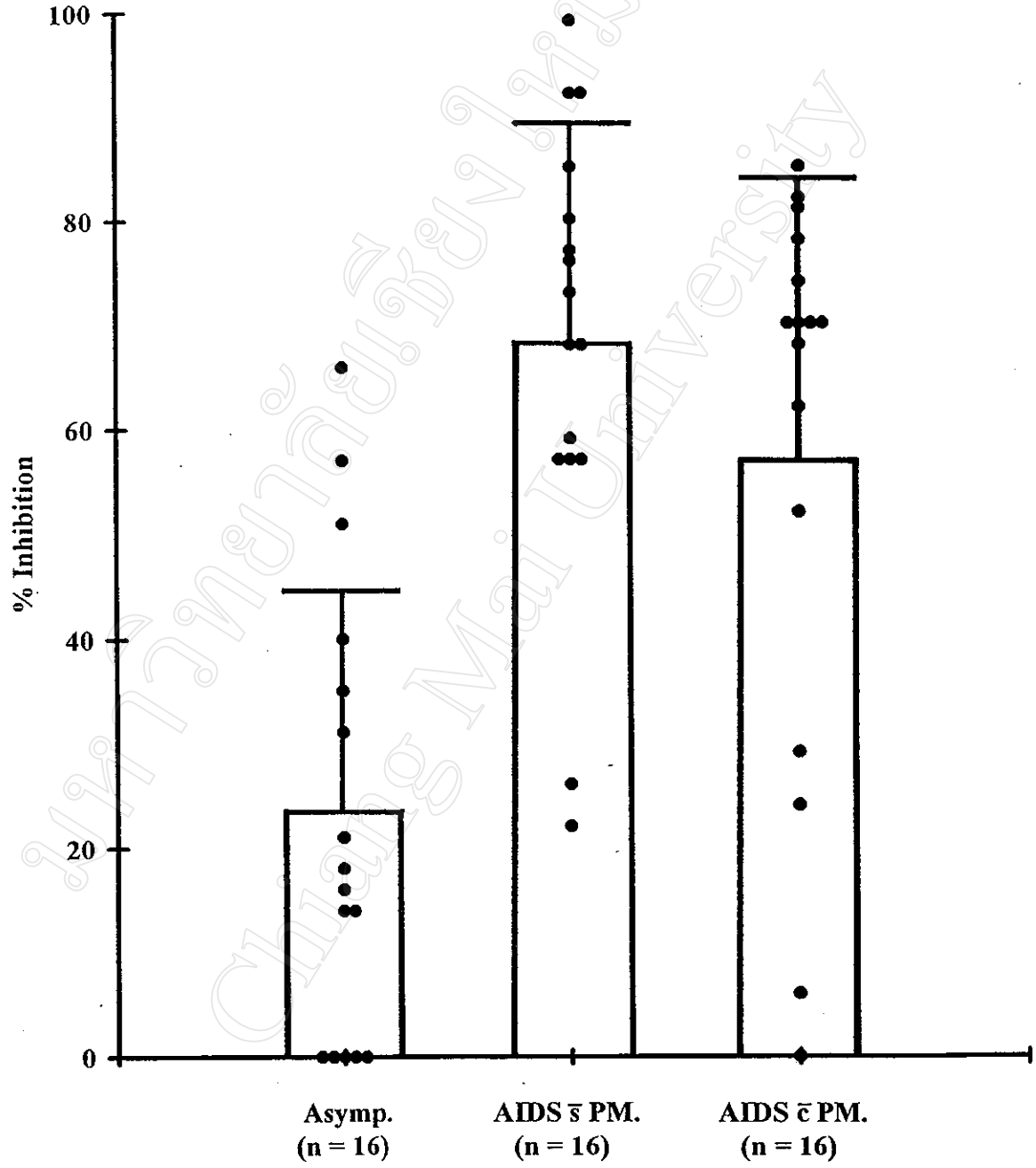

Figure 15. Inhibitory effect of the individual plasma (10%) from asymptomatic HIV infected individuals, AIDS patients without and with *P. marneffei* infection. ^{3}H-thymidine incorporation by suboptimal concentration of PHA-P (0.125 µg/ml) stimulated of normal PBMC. Percent inhibition was compared to proliferation in presence of 10% autologous plasma.

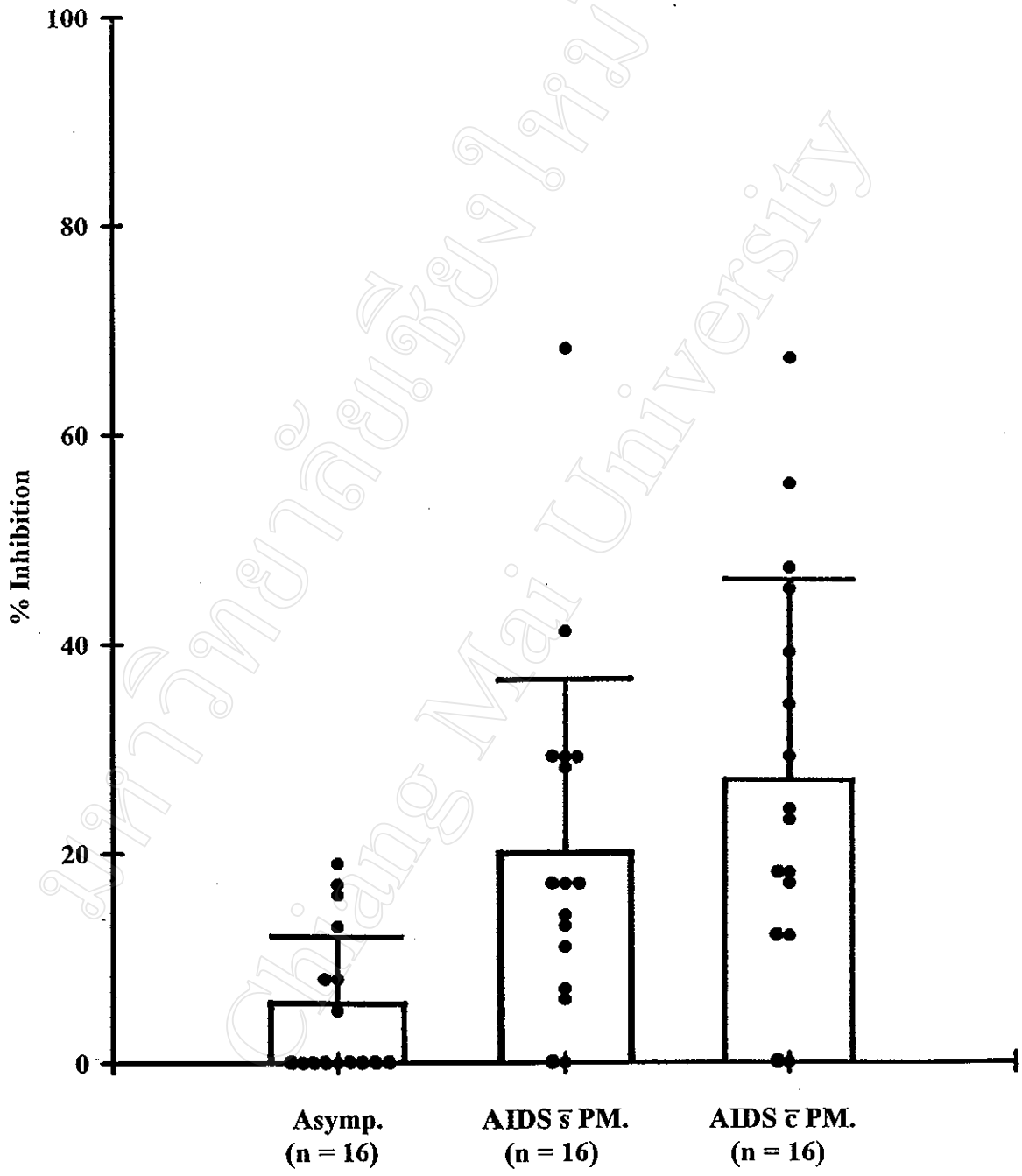

Figure 16. Inhibitory effect of the individual plasma (10%) from asymptomatic HIV infected individuals, AIDS patients without and with *P. marneffei* infection. ^{3}H-thymidine incorporation by optimal concentration of PHA-P (1.0 µg/ml) stimulated of normal PBMC. Percent inhibition was compared to proliferation in presence of 10% autologous plasma.

respectively. The inhibitory effect of plasma obtained form AIDS patients without *P. marneffei* infection was 54, 62, 85, and 89% in 5, 10, 20, and 30 percent of plasma concentration, respectively. The inhibitory effect of plasma obtained from AIDS patients with *P. marneffei* infection was 28, 35, 79, and 87 in 5, 10, 20, and 30 percent of plasma concentration, respectively (Table 25, Fig. 17). In addition, in the presence of PHA-P at the optimal concentration (1.0 μg/ml), the inhibitory effect of plasma obtained from asymtomatic HIV-infected individual was 5, 7, 12, and 18% in 5,10, 20, and 30 percent of plasma concentration, respectively. The inhibitory effect of plasma obtain from AIDS patients without *P. marneffei* infection was 32, 36, 44, and 41% in 5, 10, 20, and 30 percent of plasma concentration, respectively. The inhibitory effect of plasma obtained from AIDS patients with *P. marneffei* infection was 16, 16, 28, and 44% in 5, 10, 20, and 30 percent of plasma concentration respectively (Table 26, Fig.18). In contrast, in the presence PHA-P of both concentration, the inhibitory effect of homologous plasma was less than 10% in every concentration of plasma (Table 25 and 26)

Table 25. The correlation of the concentration and inhibitory effect of plasma from asymptomatic HIV infected individuals, AIDS patients without and with *P. marneffei* infection on ^{3}H-thymidine incorporation by suboptimal concentration (0.125 μg/ml) stimulated normal PBMC. Percent inhibition was compared to the proliferation in presence of autologous plasma.

*No. of test	% Inhibition by plasma from Concentration of plasma (%)															
	Homologous				Asymp. HIV				AIDS s̄ PM.				AIDS c̄ PM.			
	5	10	20	30	5	10	20	30	5	10	20	30	5	10	20	30
1	0	0	0	0	12	0	72	76	15	26	68	75	28	35	74	76
2	0	0	0	0	16	20	47	32	88	29	70	86	29	26	54	83
3	0	0	0	0	0	22	6	5	84	99	99	99	0	0	78	84
4	0	9	7	36	0	0	0	0	92	99	99	99	20	37	85	95
5					25	32	32	43	3	47	82	81	40	56	56	86
6					36	16	13	35	42	76	92	96	52	60	98	98
$\bar{X}$	0	2.2	1.7	9	15	15	28	32	54	62	85	89	28	35	79	87
SD.	0	4.5	3.5	1.8	14	13	27	28	39	33	13	10	17	21	14	8

* No. of test = No. of normal PBMC

Table 26. The correlation of the concentration and inhibitory effect of plasma from asymptomatic HIV infected individuals, AIDS patients without and with *P. marneffei* infection on ^{3}H-thymidine incorporation by optimal concentration (1.0 μg/ml) stimulated normal PBMC. Percent inhibition was compared to the proliferation in presence of autologous plasma.

*No. of test	% Inhibition by plasma from Concentration of plasma (%)															
	Homologous				Asymp. HIV				AIDS s̄ PM.				AIDS c̄ PM.			
	5	10	20	30	5	10	20	30	5	10	20	30	5	10	20	30
1	0	0	0	0	0	0	13	0	23	11	49	26	27	0	29	73
2	27	0	0	0	9	13	14	19	49	16	5	40	6	27	0	50
3	0	0	0	0	0	1	13	19	48	68	80	75	10	13	37	26
4	0	9	8	2	6	0	5	26	39	82	80	77	14	23	42	44
5					8	13	11	24	18	19	30	7	14	12	33	31
6					6	12	15	20	15	21	24	22	25	21	31	39
$\bar{X}$	7	2	2	0.5	5	7	12	18	32	36	44	41	16	16	28	44
SD.	13	5	4	1	4	7	4	9	15	30	30	29	9	10	15	17

* No. of test = No. of normal PBMC

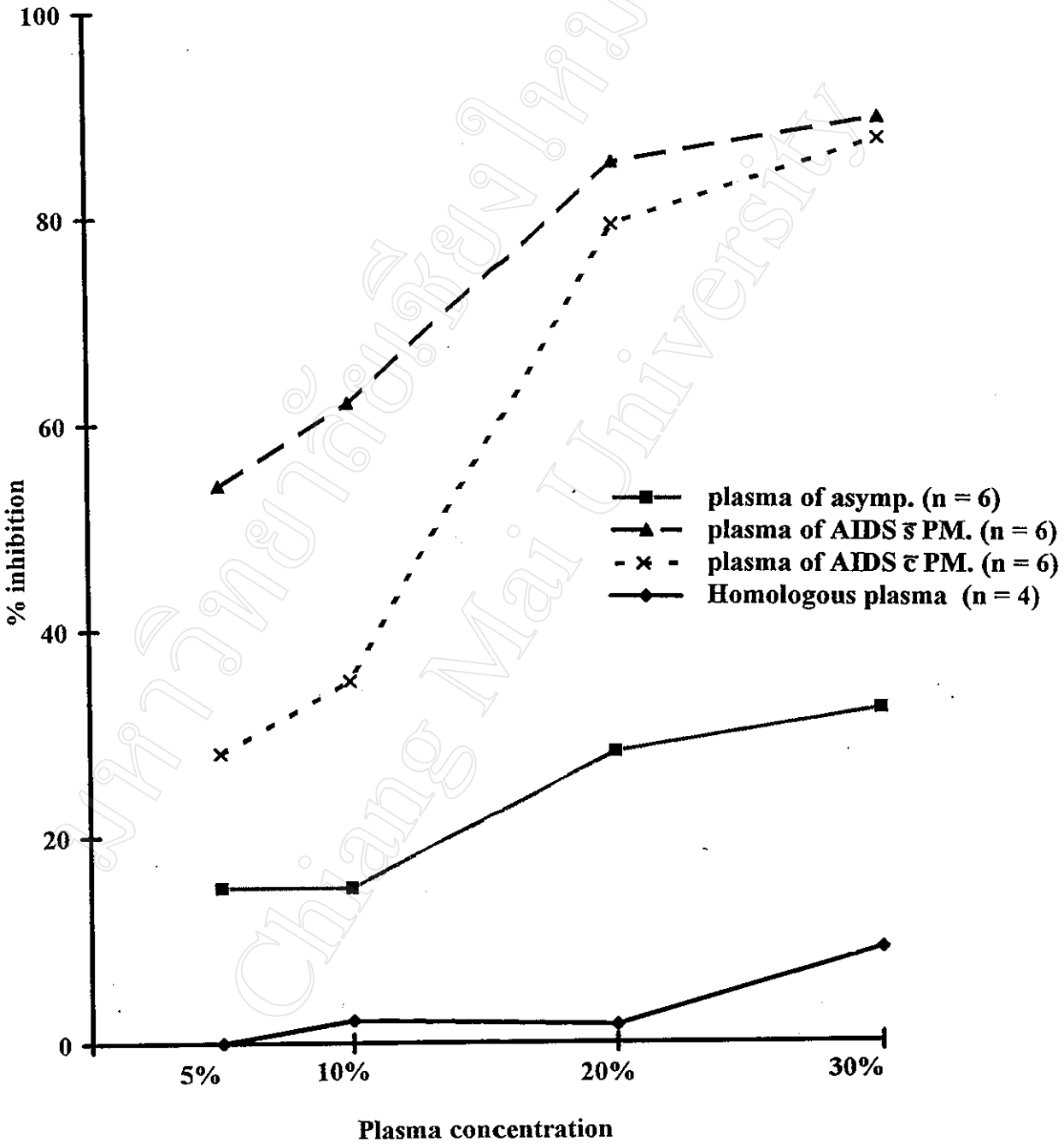

Figure 17. The correlation of the concentration and inhibitory effect of plasma from asymptomatic HIV infected individuals, AIDS patients without and with *P. marneffei* infection on ^{3}H-thymidine incoporation of suboptimal concentration (0.125 µg/ml) stimulated normal PBMC.

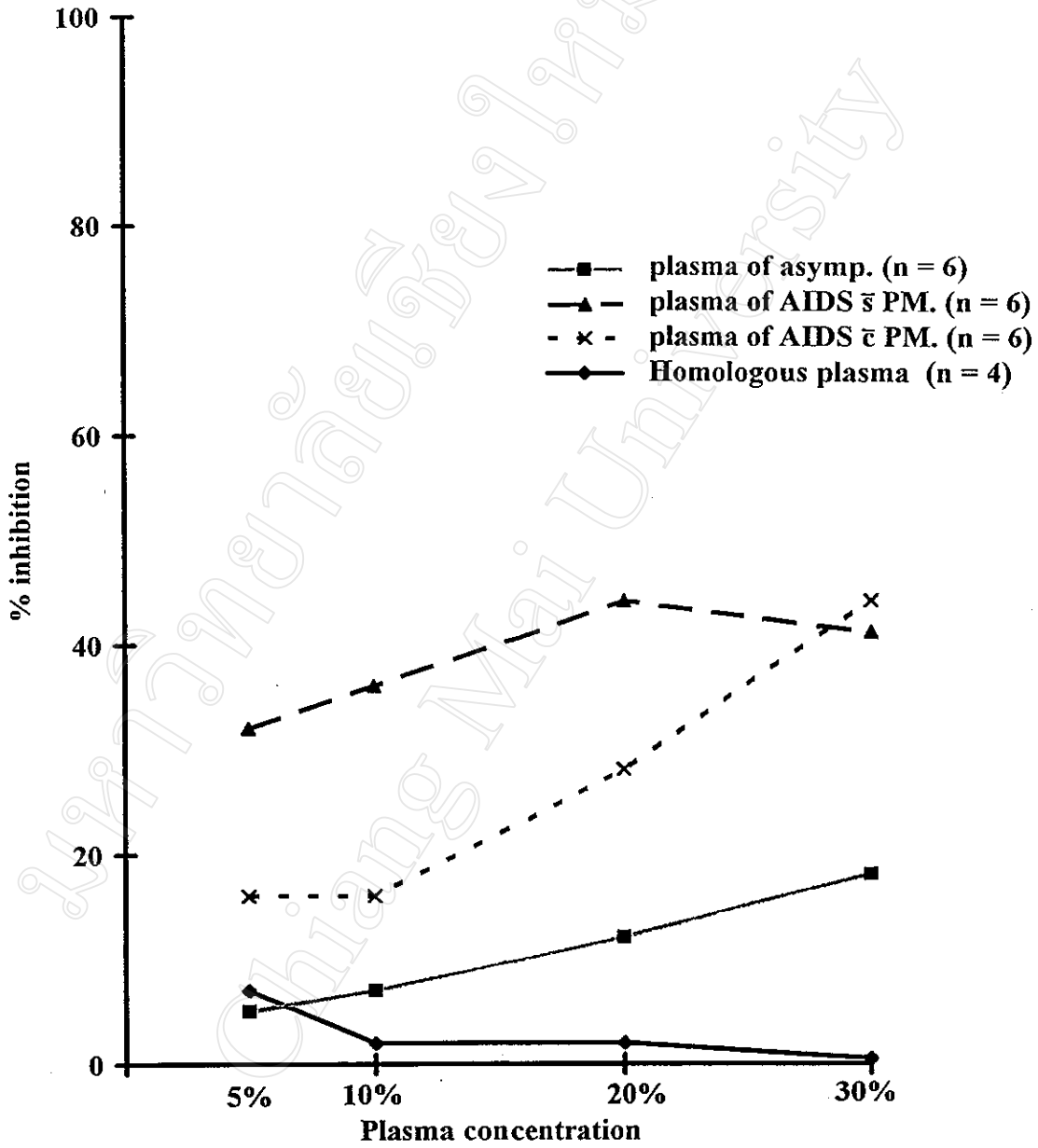

Figure 18. The correlation of the concentration and inhibitory effect of plasma from asymptomatic HIV infected individuals, AIDS patients without and with *P. marneffei* infection on ^{3}H-thymidine incoporation of optimal concentration (1.0 µg/ml) stimulated normal PBMC.