CHAPTER IV

RESULTS

1. Lethal Doses for Oral Challenge

To determine the dose of S.typhimurium C_5 for oral challenge in mouse protection test , 50 % lethal dose was performed and calculated by Reed and Muench method . The 50 % lethal dose of S.typhimurium C_5 for Oral challenge was 3.87×10^3 organisms (Appendix) . In mouse protection test 5×10^6 S. typhimurium C_5 was used , which equal to approximately 1000 times of the LD_{50} .

2. Mouse Protection Test

This study was performed to demonstrate the mouse protective capacity of orally administered live S. typhimurium G_{30} BALB/cJ mice used in this experiment was highly susceptible to infection with S. typhimurium C_5 so group of mice was fed one dose of 1×10^{10} S. typhimurium G_{30} compared with the other group which was fed three doses of 1×10^3 S. typhimurium G_{30} on each of three alternate days .

From data presented in Table 1 , it was found that significant protection was achieved against a challenge dose of 5×10^5 S. typhimurium C_5 when ;

- Mice were challenged 21 days after immunization with one dose of 1×10^{10} S. typhimurium G_{30} .

- Mice were challenged 7 days after immunization with three doses of 1×10^{10} S.typhimurium G_{30} on each of three alternate

days .

- Mice were challenged 21 days after immunization with three doses of 1 x10^{10} S. typhimurium G_{30} on each of three alternate days .

On the other hand , oral immunization with one dose of 1 x 10^{10} S. typhimurium G_{30} 7 days before challenge with 5 x 10^{5} S. typhimurium C_5 protected about 25 % of the mice .

All control mice died within 10 days after challenge in this experiment .

Table 1 Mouse protection from S. typhimurium C_5 Infection Afforded by Oral Vaccination with Living Cells of S. typhimurium G_{30}

Immunization Orally with S. typhimurium G_{30}		Days After Immunization					
		7			21		
Organisms/Dose	No. of Doses	Survivors/Totals after oral Challenge with S. typhimurium C_5	% Survival	Significance of Test	Survivors/Totals After Oral Challenge with S. typhimurium C_5	% Survival	Significance of Test
1×10^{10}	1	2/8 (0/10)[b]	25 (0)[b]	> 0.05	13/15 (0/10)[b]	87 (0)[b]	< 0.005
1×10^{10}	3	11/15 (0/10)	73 (0)	< 0.005	6/6 (0/10)	100 (0)	< 0.005

a = Oral challenge with 5×10^6 living S. typhimurium C_5 after orally immunization with either one dose or three doses of living Cells of S. typhimurium G_{30} on each of three alternate days . The survivors or the death were observed within 30 days after Challenge .

b = Control , non - immunized mice .

c = Values of 0.005 indicated highly significant protection . Statistically significance of test was determined by Chi' square test.

3. Immune Serum

Antibodies against S. typhimurium C_s obtained from rabbits that had been immunized by repeated subcutaneous injection of heat - killed S. typhimurium C_s had an agglutination titer of 1:256 .

This specific antibodies were used for preopsonizing S.typhimurium C_s and in vitro intracellular killing assay .

4. Indirect Immunofluorescent Antibody Test

Indirect immunofluorescent antibody test was performed in order to check whether S. typhimurium C_s was preopsonized .

As shown in Figure 6 , the presence of immunoglobulins on the surface of S.typhimurium C_s was evidenced by very bright fluorescence of S. typhimurium C_s .

5. Enzymatic Extraction of Peyer' s Patches cells

Enzymatic extraction of PP yielded 3 - 4 fold higher cell number than conventional mechanical methods of cell preparation (teasing , wire screen) (151) . Yields from 12 seperate experiments using 8 mice per experiments averaged $1.74 \pm 0.33 \times 10^7$ cells per mouse with the enzyme method .Cell viability always exceed 95 % .

Furthermore , by the enzyme method 7.06 ± 0.95 % of these cells were esterase - positive macrophages (Figure 7) and actively phagocytized latex particles (Figure 8) . The other cells which remained in PP except macrophages were lymphocytes .

a

b

Figure 6 The typical fluorescence of preopsonized Salmonella typhimurium C_5 . a) Magnification 500 x b) Magnification 1000 x

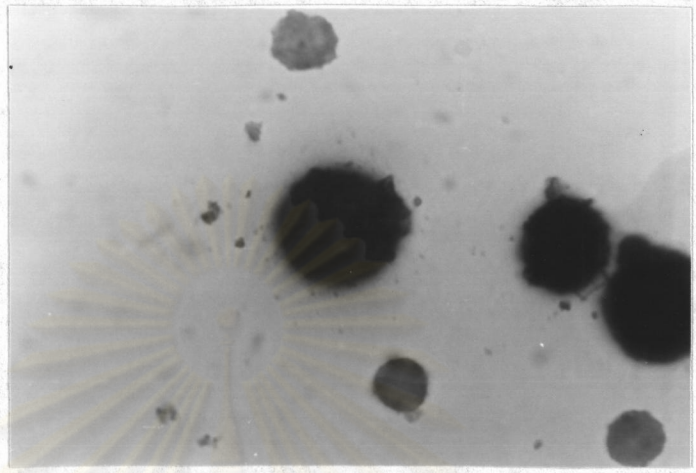

Figure 7 Esterase - staining patterns in Peyer's patch cell suspensions . Magnification 1000 x

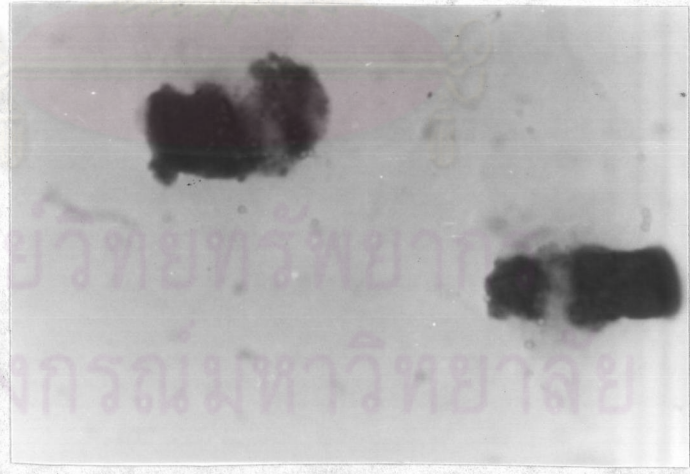

Figure 8 Ingestion latex particles by Peyer's patch macrophages . Magnification 1000 x

6. **Comparison of the Number of Macrophages in the Peyer's Patches of Mice after feeding Salmonella typhimurium G_{30} with Control Group**

Since the ability of S. typhimurium G_{30} to induce resistance should be associated with the ability of this organism to cause an increase in the number of macrophages in the Peyer's patches, it was of interest to determine whether feeding S. typhimurium G_{30} to mice increased in the number of macrophages in the Peyer' s patches cell suspensions .

The results of these studies are summarized in Table 2 .

Table 2 Numbers of Macrophages in Enzymetically Dissociated Peyer's patch Cell Suspensions of Mice Immunized Orally with Salmonella typhimurium G_{30} Compared with Normal Mice .

Source of PP Cells	No.of PP Cells($\times 10^7$)	No.of Macrophages ($\times 10^6$)	Percentage of Macrophages	Significance[a] of Test
	[c]			
Normal Mice	1.72 ± 0.67	1.24 ± 0.29	7.20 ± 0.96	P > 0.05
S.typhimurium G_{30} Immunized Mice (7 days ,1 dose)[b]	1.84 ± 0.13	1.90 ± 0.24	10.40 ± 1.76	P > 0.05
Normal Mice	1.76 ± 0.05	1.22 ± 0.27	6.97 ± 1.50	P > 0.05
S.typhimurium G_{30} Immunized Mice (21 days ,1 dose)	1.91 ± 0.38	1.90 ± 0.75	9.70 ± 1.98	P < 0.001
Normal Mice	1.73 ± 0.08	1.23 ± 0.08	7.13 ± 0.58	P < 0.001
S.typhimurium G_{30} Immunized Mice (7 days ,3 doses)	1.90 ± 0.24	4.07 ± 0.59	21.37 ± 1.16	P < 0.001
Normal Mice	1.76 ± 0.34	1.24 ± 0.34	6.93 ± 1.29	P > 0.05
S.typhimurium G_{30} Immunized Mice (21 days ,3 doses)	1.88 ± 0.36	3.77 ± 0.93	19.97 ± 1.36	P < 0.001

a = Significance of test was determined by student's t - test .

b = Number in parenthesis indicated days in which mice were orally immunized and the number of doses which mice were orally immunized .

c = Data were obtained from three different expressed as mean ± sd

7. Specific Antibody Requirement for Preopsonization and Intracellular Killing Assay

To demonstrate requirement of specific antibody for preopsonization and intracellular killing assay , Intracellular killing assay was performed in the absence of specific antibody and by using unpreopsonized S.typhimurium C_s compared with using preopsonized S. typhimurium C_s and in the presence of specific antibody .

From this experiment , it was found that in the absence of specific antiboby and using unpreopsonized S. typhimurium C_s , S. typhimurium C_s could not be ingested by normal macrophages or activated macrophages . All noningested S. typhimurium C_s were removed by centrifugation at 75 x G for 6 min followed by three washing with gelatin - HBSS and an intracellular killing assay was then performed , the number of viable intracellular bacteria after lysis of the macrophages being determined ; no growth of bacteria was observed . Thus these results indicated that the intracellular killing of S. typhimurium C_s by macrophages depended on the presence of specific antibody .

8. Control Studies to Demonstrate the Intracellular Presence of Salmonella typhimurium C_s during the Killing Assay

To be certain that the number of bacteria measured by the assays represented intracellular bacteria , various control experiments were performed . To determine bacteria were removed by centrifugation at 75 x G for 6 min and three washes with gelatin - HBSS , the effect of centrifugation of suspensions of bacteria was

investigated first . After centrifugation of 5 x 10^6 S. typhimurium C_s /ml for 6 min at 75 x G followed by three washing with gelatin - HBSS , 96.26 ± 1.45 % of the initial number of bacteria could be recovered in the supernatant plus washing fluids .

To make certain thet three washing of the macrophages was sufficient to remove the extracellular bacteria , the efficiency of the washing of the macrophages was investigated by incubating 5 x 10^5 macrophages with 5 x 10^5 preopsonized S. typhimurium /ml and three washes with 1 ml of ice cold gelatin - HBSS . The number of bacteria present in the last washing fluid was approximately 3.85 ± 0.48 % .

9. Optimal Concentration of Immune Serum for in Vitro Intracellular Killing Assay

To determine optimal concentration of immune serum for in vitro intracellular killing assay , preopsonized S.typhimurium C_s with various concentrations of immune serum were incubated with 5 x 10^5 macrophages/ml at a bacteria - to - macrophages ratio of 1:1 in the presence of corresponding serum concentration at 37° C .

The results in Figure 9 - 12 showed that at serum concentrations of 10 % was the optimal concentration to obtain maximum intracellular killing of S. typhimurium C_s .

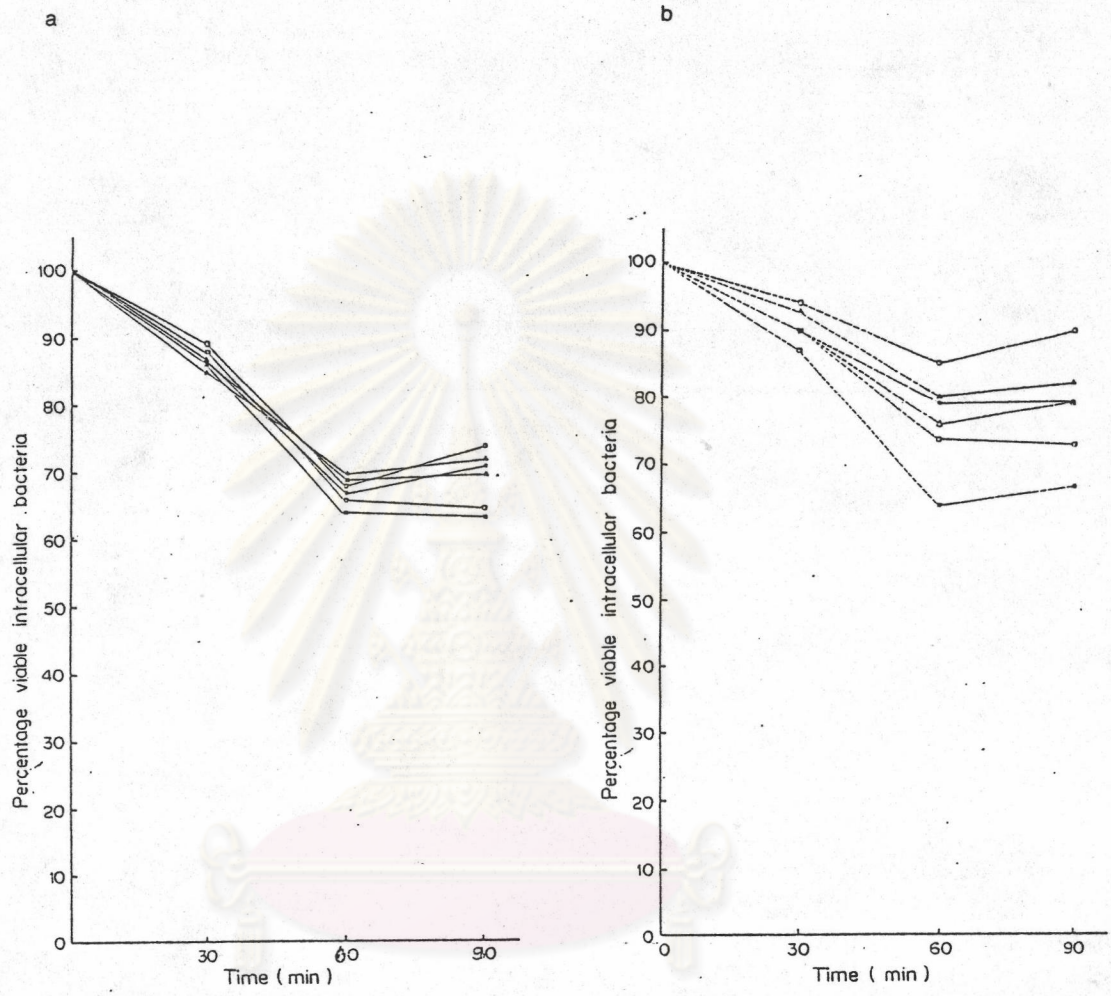

Figure 9 Effect of the immune serum concentration on the intracellular killing . Intracellular killing was performed with a bacteria - to - macrophage ratio of 1:1 at 37° C with immune serum concentration of 1 % (○), 5 % (●), 7 % (▫), 10 % (■), 15 % (△) and 20 % (▲). a) Macrophages were collected from PP of mice orally immunized 7 days earlier with one dose of 1 x 10^{10} S. typhimurium G_{30} (——) . b) Macrophages were collected from PP of normal mice (----). Each time point represents the mean of 3 experiments .

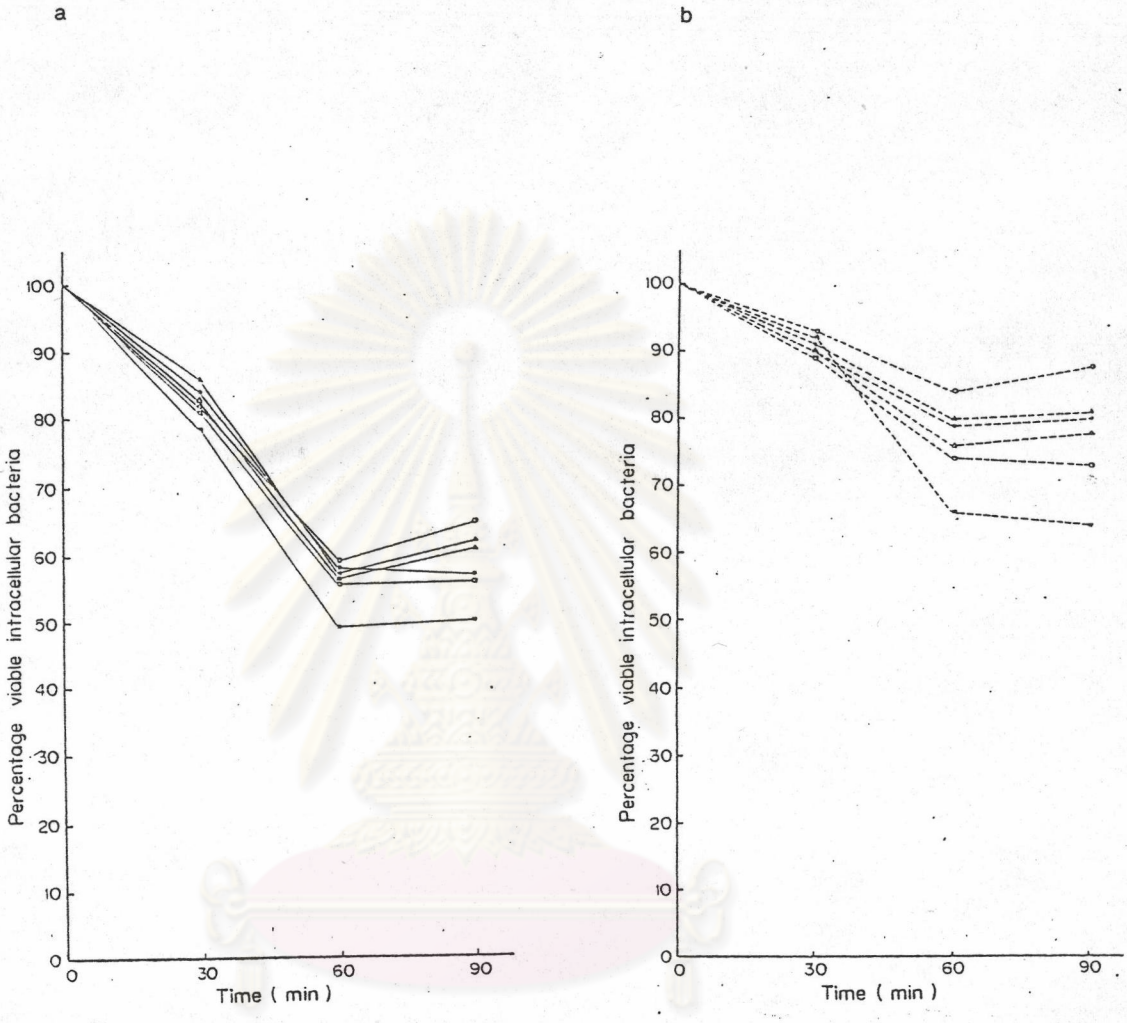

Figure 10 Effect of the immune serum concentration on the intracellular killing . Intracellular killing was performed with a bacteria - to - macrophage ratio of 1:1 at 37° C with immune serum concentration of 1 % (○) , 5 % (●) , 7 % (□) , 10 % (■) , 15 % (Δ) and 20 % (▲) . a) Macrophages were collected from PP of mice orally immunized 21 days earlier with one dose of 1×10^{10} S. typhimurium G_{30} (——) . b) Macrophages were collected from PP of normal mice (----). Each time point represents the mean of 3 experiments .

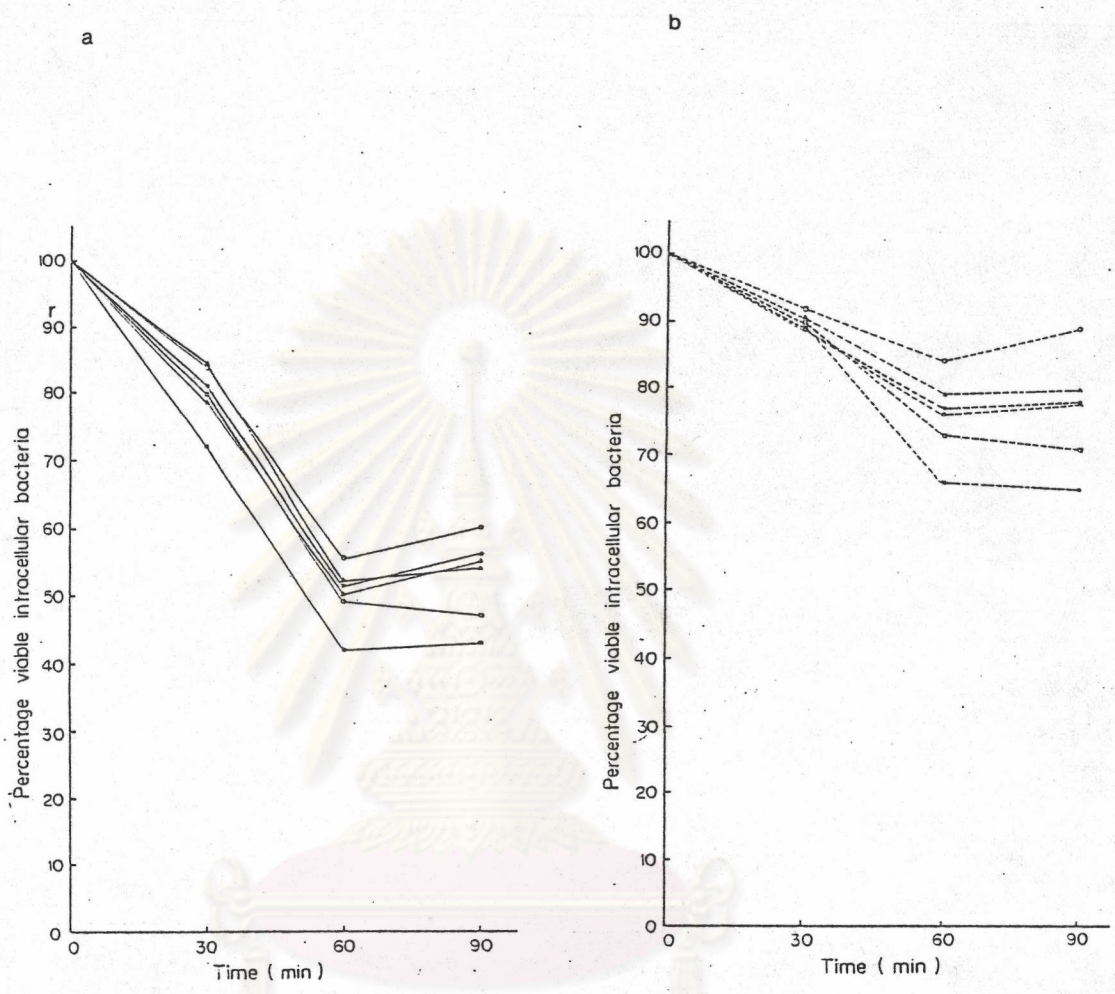

Figure 11 Effect of the immune serum concentration on the intracellular killing . Intracellular killing was performed with a bacteria - to - macrophages ratio of 1:1 at 37° C with immune serum concentration of 1 % (o), 5 % (•), 7 % (□) , 10 % (■) , 15 % (△) and 20 % (▲) . a) Macrophages were collected from PP of mice orally immunized 7 days earlier with three doses of 1×10^{10} S. typhimurium G_{30} on each of three alternate days (——) . b) Macrophages were collected from PP of normal mice (----) . Each time point represents the mean of 3 experiments .

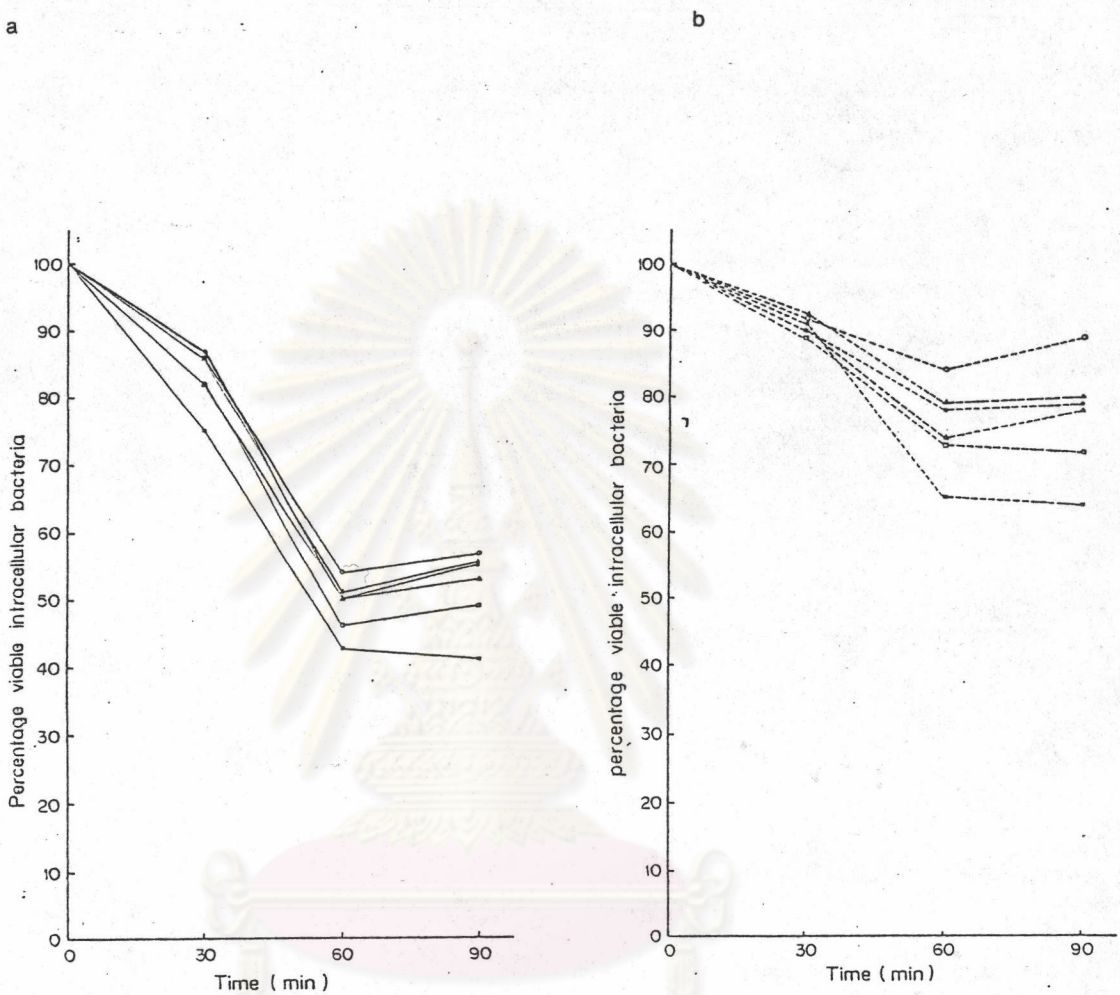

Figure 12 Effect of the immune serum concentration on the intracellular killing . Intracellular killing was performed with a bacteria - to - macrophages ratio of 1:1 at 37° C with immune serum concentration of 1 % (○), 5 % (●), 7 % (□) , 10 % (■) , 15 % (△) and 20 % (▲) . a) Macrophages were collected from PP of mice orally immunized 21 days earlier with three doses of 1 x 10^{10} S. typhimurium G_{30} on each of three alternate days (——) . b) Macrophages were collected from PP of normal mice (----) . Each time point represents the mean of 3 experiments .

10. Intracellular Killing of Salmonella typhimurium C_s in Vitro by Macrophages in the Peyer's Patches of Mice

Macrophages were allowed to ingest S.typhimurium C_s preopsonized with 10 % heat - inactivated rabbit immune serum in vitro for 20 min at 37° C , after which the extracellular bacteria were removed and these macrophages for 90 min at 37° C in the presence of 10 % heat - inactivated rabbit immune serum. The results of in vitro intracellular killing assay were summarized in Figure 13 and values of killing indices were shown in Table 3 . The PP macrophages from normal mice killed 9.92 ± 3.47 % of S. typhimurium C_s in 30 min and 34.46 ± 2.19 % in 60 min . No further killing was seen during the follow 30 min (Figure 13) . The PP macrophages from S. typhimurium G_{30} immunized mice (7 days , 1 dose) killed S. typhimurium C_s with an efficient equal to that seen with PP macrophages from normal mice . No significant differences in intracellular killing were observed ($P>0.05$) .

On the other hand , PP macrophages from S. typhimurium G_{30} immunized mice (21 days ,1 dose) , (7 days , 3 doses) and (21 days , 3 doses) killed S. typhimurium C_s with considerably greater than the PP macrophages from normal mice . These differences were significant ($P<0.001$) (Table 3) .

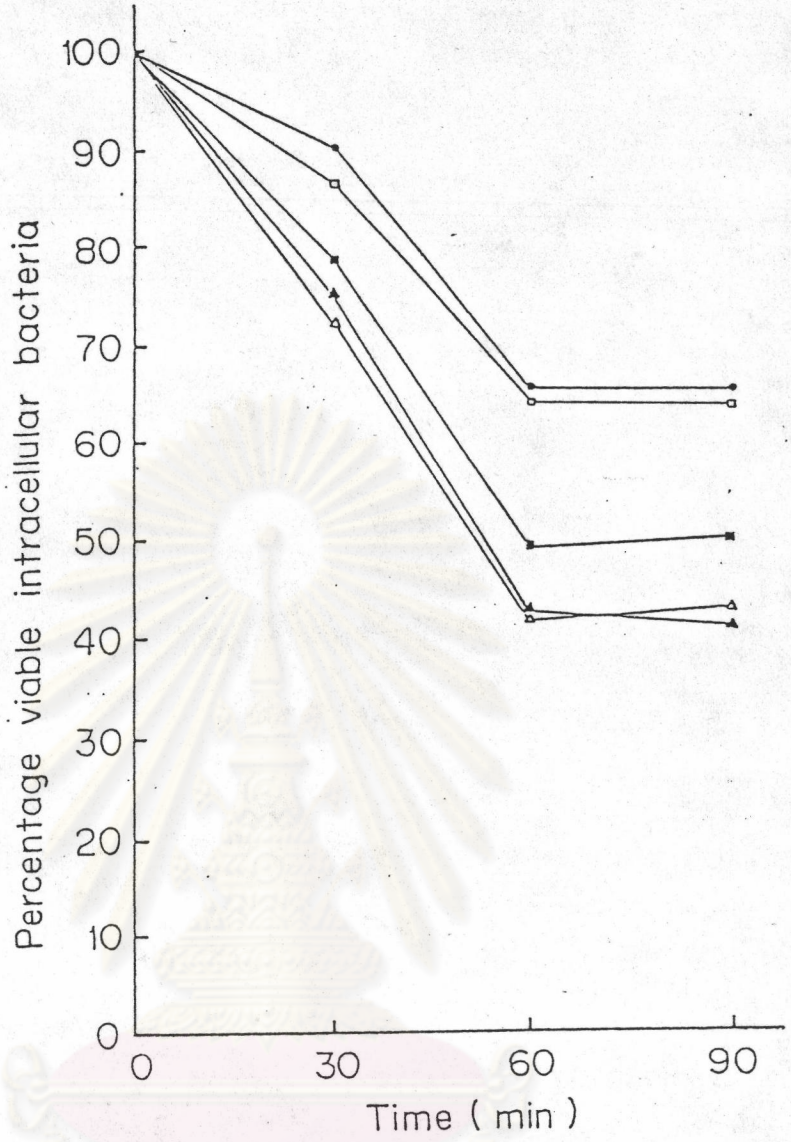

Figure 13 Comparison of intracellular Killing of S. typhimurium C_5 in vitro by PP macrophages of mice orally immunized 7 days earlier with one dose of 10^{10} S. typhimurium G_{30} (□) , 21 days earlier with one dose of 1×10^{10} S. typhimurium G_{30} (■) , 7 days earlier with three doses of 1×10^{10} S. typhimurium G_{30} on each of three alternate days (Δ) , 21 days earlier with three doses of S. typhimurium G_{30} on each of three alternate days (▲) and control group (non immunized) (o) . Bacteria , preopsonized in vitro with 10 % heat - inactivated rabbit immune serum for 30 min , were phagocytized . Macrophages (5×10^5 / ml) containing bacteria were reincubated in the presence of 10 % heat - inactivated rabbit immune serum . Each time point represents the mean of 3 experiments .

Table 3 Intracellular Killing[a] of *Salmonella typhimurium* C_5 by Peyer's Patch Macrophages

Source of PP Macrophages	No. of experiments	Intracellular Killing[b] (%) 30 min	Significance[c] of Test	60 min	Significance[c] of Test	90 min	Significance[c] of Test
Normal Mice	12	9.92 ± 3.47		34.46 ± 2.19		34.80 ± 2.34	
S. typhimurium G_{30} - Immunized Mice (7 days , 1 dose)[d]	3	13.27 ± 1.46	> 0.05	35.97 ± 3.25	> 0.05	36.20 ± 2.13	> 0.05
S. typhimurium G_{30} - Immunized Mice (21 days , 1 dose)[d]	3	21.30 ± 2.72	< 0.025	50.80 ± 2.26	< 0.005	49.70 ± 2.21	< 0.005
S. typhimurium G_{30} - Immunized Mice (7 days , 3 doses)[e]	3	27.73 ± 3.46	< 0.025	58.17 ± 2.94	< 0.005	57.13 ± 3.75	< 0.005
S. typhimurium G_{30} - Immunized Mice (21 days , 3 doses)[e]	3	25.00 ± 2.82	< 0.01	57.27 ± 4.21	< 0.005	58.87 ± 4.05	< 0.005

a = After phagocytosis at a bacteria : macrophage ratio of 1:1 for 20 min at 37°C . Macrophages containing bacteria were reincubated in the presence of 10 % heat - inactivated immune serum .

b = Expressed as K(t) = (1 - Nt / No) x 100 % (See methods) . Data expressed as means ± SD .

c = Statically significance of test was determined by student's t test compared with normal mice .

d = Numbers in parenthesis indicated days in which mice were orally immunized and the number of doses which were orally immunized

e = Number in parenthesis indicated days after the last oral immunized and the number of doses which mice were orally immunized . Each dose is on each of three alternate days .

Table 4 Comparison of Percentage of Peyer's patch Macrophages from Normal Mice and Peyer's patch Macrophages from *S. typhimurium* G_{30} Immunized Mice

Criterion	Percentage (Mean ± SD)[a]				
	PP Macrophages from Normal Mice	PP Macrophages from *S. typhimurium* G_{30} - Immunized Mice (7 days , 1 dose)[b]	PP Macrophages from *S. typhimurium* G_{30} - Immunized Mice (21 days , 1 dose)[b]	PP Macrophages from *S. typhimurium* G_{30} - Immunized Mice (7 days , 3 doses)[c]	PP Macrophages from *S. typhimurium* G_{30} - Immunized Mice (21 days , 3 doses)[c]
Esterase Positive Cells	7.23 ± 0.38	10.53 ± 0.21	9.87 ± 0.25	22.0 ± 1.75	20.3 ± 1.23
Latex Ingestion	7.06 ± 0.95	10.40 ± 1.76	9.70 ± 1.98	21.37 ± 1.16	19.97 ± 1.36

a = Mean percentage ± SD of 3 experiments

b = Numbers in parenthesis indicated days after which mice were orally immunized and the number of doses which mice were orally immunized .

c = Number in parenthesis indicated days after the last oral immunized and the number of doses which mice were orally immunized . Each dose is on each of three alternate days .

Table 5 Comparison of Rate of in Vitro Intracellular Killing of *S. typhimurium* C_5 at 60 min by Peyer's Patch Macrophages of Mice orally Immunized *S. typhimurium* G_{30} with Normal Mice (Non - Immunized) , and Van Dissel Index

Source of PP Macrophages	Rate of Intracellular Killing at 60 min (X)	$\frac{X}{\text{Van Dissel Index}^a}$
Normal Mice	1	
S. typhimurium G_{30} - Immunized Mice (7 days , 1 dose)[b]	1.04	0.61
S. typhimurium G_{30} - Immunized Mice (21 days , 1 dose)[b]	1.47	0.86
S. typhimurium G_{30} - Immunized Mice (7 days , doses)[c]	1.69	0.99
S. typhimurium G_{30} - Immunized Mice (21 days , 3 doses)[c]	1.66	0.98

a = Van Dissel Index = 1.7 (The rate of intracellular killing by normal macrophages of Salmonella - resistant CBA mice amounted to about 1.7 times the value found for macrophages of susceptible C57BL/10 mice (145)

b = Number in parenthesis indicated days after which mice wer orally immunized and the number of doses which mice were orally immunized

c = Number in parenthesis indicated days after the last oral immunized and the number of doses which mice were orally immunized . Each dose is on each of three alternate days .