Studies on Host Protection in Early Stage of Tyzzer's Disease of Mice

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CONTENTS

CONTENTS 1

PREFACE 3

PART 1: Effect of Cyclophosphamide on Tyzzer's disease of Mice 5

INTRODUCTION 6

MATERIALS AND METHODS 7

RESULTS 9

DISCUSSION 13

SUMMARY 15

TABLES 16

FIGURES 21

PART 2: Reticuloendothelial System and Tyzzer's Disease of Mice 27

INTRODUCTION 28

MATERIALS AND METHODS 29

RESULTS 31

DISCUSSION 35

SUMMARY 38

TABLES 39

PART3: Precociously Induced Resistance to Tyzzer's Disease in Mice 45

INTRODUCTION 46

MATERIALS AND METHODS 47

RESULTS 50

DISCUSSION 55

SUMMARY 58

TABLES 59

CONCLUSION 70

ACKOWLEDGEMENTS 72

REFERENCES 73

PREFACE

Tyzzer's disease is known to be due to bacterial infection affecting laboratory rodents[4,7-10,20,28,30,31,43,48,55,61, 65,69,73,74,76], rabbits[1,6,21,56,70], dogs[54,58], cats[22,32, 33] and horses[23-25,49,57,68] as well as rhesus monkeys[49]. The disease is characterized by necrotic hepatitis, enteritis and encephalitis and myocardiopathy, which can be experimentally reproduced in rodents[12,17,47,51,64], rabbits[35,64] and cats[33]. The infection was enhanced by either administration of glucocorticoid[18] or irradiation[64]. The production of liver lesions in mice depends upon the protein-level in diet and the inhibitory effect of starvation on the disease production has been evidenced [38].

Protective immunity in Tyzzer's disease can be induced by injection of bacterial antigens with detectable serum antibodies at later stage of infection [13,19,46]. While the target cells in Tyzzer's disease are known to be highly differenciated epithelial and nerve cells of the host, the involvement of the mesenchymal elements in the host protection remains unstudied. The causative organism is assumed to be extremely fragile in vitro[15] and sensitive to activity of host phagocytic cells[13,17,18], but the role of the phagocyte system during the early stage of infection, when antibody was still undetectable, remains unclear.

This paper deals with effect of cyclophosphamide (Part 1) as well as macrophage-blocking agent or splenectomy (Part 2) on experimental infection in mice, suggesting more important role of macrophages during early phase of infection than has been previously believed [11,34]. Also the paper is to describe protective ability induced in mice precociously by treating with sublethal dose of live bacteria and bacterial lysate as well as immunopotentiators such as lipopolysaccharide of Escherichia coli or Mycobacterium bovis BCG (Part 3).

PART 1

Effect of Cyclophosphamide on Tyzzer's Disease of Mice

INTRODUCTION

Tyzzer's disease is known to be dramatically enhanced by administration of glucocorticoids(GC), that is, cortisone, prednisolone or hydrocortisone[18], as well as by irradiation[64], leading to that the enhancing effects might be related to immunosuppression[2,5,44]. Fujiwara et al. [16] suggested that T-lymphocytes had important roles in protecting mice from the infection using congenitally athymic nude mice having received the T-cell transfer from euthymic heterozygotes. However, Waggie et al. [71] suggested that resistance to Tyzzer's disease was related to a B-cell function. On the other hand, it was reported that apparent hepatic lesions failed to be produced in fasted mice or those fed low-protein diet, suggesting protein metabolism in the host liver would be of importance in the growth of this strictly intracellular parasite[38].

The enhancing effect of GC on Tyzzer's disease might result from a complicated effect both immunosuppressive and metabolic [2,5,37,44]. In this study, a more simple immunosuppressant, cyclophosphamide[2], was administered to infected mice, and their survival time, production of liver lesions and changes of specific antibody titers were compared with those of non-treated or GC-treated animals.

MATERIALS AND METHODS

Animals: Four- to 5-week-old ICR female mice weighing 17 to 23 g (Chales River Japan, Atsugui, Kanagawa) were used. Animals were kept in aluminium cages with a filter cap (Sanki-kagaku, Tokyo) and given commercial pellets (Oriental Yeast, Tokyo) and tap water ad libitum.

Inoculation: Mice were injected intravenously(i.v.) with severely infected mouse liver homogenate in phosphate buffered saline, pH 7.4 (PBS) containing 10^5 to 10^6 organisms of MSK[14] strain of "Bacillus piliformis [69]". Shortly after inoculation they received subcutaneously(s.c.) 2.5 mg of hydrocortisone acetate (HCA)(Scheroson F, Nippon Schering, Osaka). On days 3 to 4 postinoculation (p.i.) moribund animals were killed by ethylether or chloroform, and livers with severe necrotized lesions were used for subsequent inoculations or stored at -80°C.

Inoculation was made i.v. with 0.2 ml of an appropriate dilution of the freshly prepared affected liver homogenate. The number of bacteria was counted as previously described[63]. Two days before or shortly after inoculation mice were treated with either cyclophosphamide (CY) (Endoxan, Shionogi Co., Osaka) (0.04 to 8 mg per mouse i.v.). or HCA (2.5 mg per mouse s.c.).

Anti-MSK immune serum: Ten female ICR mice, 4-week-old, were injected s.c. with a mixture of 0.75 ml of MSK infected mouse liver

homogenate (1:100) and an equal volume of Freund complete adjuvant (Difco Lab., U.S.A.). Ten days after inoculation the mice were injected i.v. with 9.2 x 10^5 MSK organisms and 7 days after the second injection blood was sampled by heart puncture. A pool of sera from 10 mice showing an IFA titer of 1:800 was used for transfer.

Histopathology: Tissue samples from the liver, spleen and thymus of killed or dead animals, were fixed in 10 % neutral buffered formalin. Paraffin sections 2 μm thick were made by a routine procedure and stained by hematoxylin and eosin (HE) , periodic acid Schiff (PAS) and periodic acid methenamin silver (PAM) methods.

Indirect immunofluorescence (IFA): IFA was performed as described previously [14]. Briefly, drops of MSK-infected mouse liver homogenate were placed on a 76 x 26 mm slide glass (Matsunami, Osaka), which was airdried, fixed in methanol and used for target antigen preparation. The target was treated first with serial dilutions of serum samples obtained by heart puncture and then with fluorescein-isothiocyanate(FITC)-conjugated anti-mouse IgG goat serum (Cappel Lab., U.S.A.). The titers of anti-MSK IgG antibody were expressed by the highest serum dilution showing specific fluorescence. The IgM antibody titers were estimated using FITC-conjugated anti-mouse IgM goat serum (Cappel Lab., U.S.A.) in the same manner.

RESULTS

Four-week-old mice were inoculated i.v. with 1.1×10^6 MSK organisms. They were divided into five groups of ten mice and three groups were given i.v. 4 mg CY per mouse. Another group of mice received s.c. injection of 2.5 mg HCA shortly after bacterial inoculation. The remaining one had no treatment.

As shown in Table 1-1, more than a half of animals died before day 8 p.i. in CY-treated groups, while all survived on day 10 p.i. in the non-treated group. In the HCA-treated group most animals died before day 6 p.i.

Next, four groups of 4-week-old mice were injected i.v. with 0.04, 0.4, 4 or 8 mg of CY, and 2 days later they were injected i.v. with the same dose of bacteria as in the foregoing experiment. One group of mice received 2.5 mg HCA immediately after inoculation of the bacteria and another served as a non-treated but inoculated control.

As shown in Table 1-2, all five animals treated with 8 mg CY or 2.5 mg HCA died on days 4 to 6 p.i. and two of five mice treated with 4 mg CY died on days 6 and 7 p.i. Those treated with reduced doses of CY survived for 10 days p.i.

In the next experiment, 4-week-old mice were i.v. injected with 1.2×10^6 MSK organisms and they were divided into three groups. One of them had received i.v. injection of 4 mg CY 2 days before,

while another received s.c. injection of 2.5 mg HCA immediately after inoculation. The remaining one group was non-treated. Two or three animals each were killed on days 1 to 11 p.i., and the thymus, spleen and liver were examined grossly as well as histopathologically. The gross lesions of the liver were graded as described already [19].

On day 2 p.i. all the three groups of mice showed one or several necrotized foci less than 0.5 mm in diameter on the surface of the liver, as presented in Table 1-3. HCA-treated mice had multiple hepatic lesions on day 3 to 4 p.i., and they died on day 4 showing severe confluent necrosis in the liver. The gross findings of the liver in the CY-treated mice, which died on day 5 p.i., were similar to those seen in the HCA group (Fig. 1-1). Without CY nor HCA treating the liver lesions developed much more slowly and comparatively larger foci of necrosis 1 to 2 mm in diameter were produced on days 5 to 7 p.i. (Fig. 1-2). On day 11 many scarred lesions 1 to 5 mm in diameter were distributed in the liver of this group of mice.

The thymus of CY- or HCA-treated animals was atrophied on and after day 1 p.i. (Fig.1-1), while it appeared intact in non-treated ones (Fig. 1-2). The spleen was prominently atrophied in HCA-treated mice but rather enlarged in non-treated ones (Fig. 1-2). In the CY group the spleen was atrophied on days 1 to 4 p.i., but rather

enlarged on and after Day 5 p.i. (Fig. 1-1).

Histopathology revealed focal coagulative necrosis in the liver with neutrophil infiltration on day 4 in all the three groups (Fig. 1-3). In HCA- or CY-treated groups, many slender rods stained with PAS and PAM within seemingly living hepatocytes surrounding the necrotic foci (Fig. 1-3). The atrophied thymus in HCA- or CY-treated mice showed severe cortical destruction without any medullary changes (Fig. 1-4). In the spleen of HCA-treated mice follicular atrophy was prominent (Fig. 1-5). In CY-treated mice splenic follicles were atrophied on days 1 to 4 p.i. but enlarged on and after day 5 p.i.

In infected but non-treated mice surviving on day 5 p.i., macrophages and fibroblasts were present around or in the necrotic foci of the liver and the splenic follicles were markedly enlarged in these animals (Fig. 1-6). On day 7 p.i. necrotic foci were replaced by granulomatous tissue (Fig. 1-7) and scarring was remarkable on day 11 p.i. (Fig. 1-8).

The next experiment was performed to see production of serum IgG or IgM antibody after inoculation. Four- to 5-week-old mice were injected i.v. with 10^5 to 10^6 MSK organisms and they were divided into three groups. Two groups were treated with either 4 mg CY 2 days before inoculation or 2.5 mg HCA shortly after inoculation, respectively. The remaining served as non-treated

control. As shown in Table 1-4, the non-treated group had IgG antibody titers of 1:50 to 1:1,600 on days 5 to 15 p.i. Already on day 4, they were shown to have a titer of 1:10. In this group IgM antibody titers were 1:25 to 1:50 on days 5 to 7. However, HCA- or CY-treated group remained negative for any types of antibody on day 5 p.i. when most animals of the groups died.

Five-week-old female ICR mice received i.v. inoculation with 8.6 x 10^5 MSK organisms. The mice were treated with 4 mg CY or 2.5 mg HCA, 2 days before or shortly after inoculation, respectively. On day 2 p.i. they received i.v. 0.2 ml of a 1:2 dilution of anti-MSK immune serum, and the survival time was recorded. As controls some infected mice were given 0.2 ml of a 1:2 dilution of serum from non-immunized mice and others remained without any serum treatment.

As shown in Table 1-5, most of CY-treated mice with or without normal serum injection died on days 6 to 8 p.i. With antiserum treating most mice survived much longer. Similar results were shown in HCA-treated groups.

DISCUSSION

Tyzzer's disease in mice has been reported to be severer when infected animals received GC treatment or irradiation [18,64]. Using GC the mechanism of the infection seems difficult to analyze because the drug has so complicated effect as mentioned before. On the other hand, CY is thought to have only immunosuppressive effect, and this study revealed that the infection was enhanced also by CY administration.

The enhancing effect of HCA or CY appeared on days 3 to 5 p.i., while liver lesions were produced already on day 2 p.i. irrespective of the drug treatment. In Tyzzer's disease, the bacteria would invade into and propagate in hepatocytes, probably within 5 to 6 hr after i.v. inoculation[19]. Therefore, HCA or CY is not considered to have any effect during bacterial invasion into hepatocytes, but it might promote the development of lesions. The bacterial growth in the liver became prominent in HCA-treated mice later than 48 hr p.i., resulting in the number of bacteria ten times or more than in non-treated ones [63]. The enhancement of bacterial propagation might be due to either depression of immune responses or changes of metabolic activities of hepatocytes[38].

Without HCA or CY treating, the antibody increased in titers on day 5 or later, when most animals treated with HCA or CY died. Therefore, the enhancing effect of GC or CY might not be due to

their action to inhibit antibody production. When infected and HCA- or CY-treated mice were injected with antibody on day 2 p.i., they could survive longer than controls without any treatment. The antibody given after infection has been shown to be ineffective on intrahepatic bacteria[19]. Each necrotic focus of the liver in infected mice without drug treatment became larger on days 5 to 7, probably only by cell-to-cell infection between neighboring cells occurring even in the presence of circulating antibody. The antibody might only inhibit the spreading of bacteria via the blood stream.

Striking atrophy of the thymus and spleen correlated with enhanced infection in HCA- or CY-treated animals suggested important roles of lymphoid[16,71] and/or reticuloendothelial cells in the host defense. Such cell-mediated protection would be suppressed by HCA or CY administration. However, only poor infiltration of these cells was present in liver lesions, suggesting indirect but not direct participation of the cells in the protection from the development of the disease.

SUMMARY

Experimental Tyzzer's disease of mice was enhanced by administration of CY, showing prominent atrophy of the thymus and spleen, while necrotizing lesions were produced in the liver on day 2 after intravenous inoculation irrespective of the drug treatment. CY-treated mice remained antibody-negative on day 7 when most of them died. Without the CY treatment, serum antibody incresed in titers on day 4 or later attaining a maximum level on days 7 to 15. Infected and CY-trated mice given antibody on day 2 survived 3 or more days longer than those without antibody transfer.

Table 1-1. Effect of CY and HCA (1)

Treatment		Mortality	Liver lesion	Days p.i. a)						
				4	5	6	7	8	9	10≤
CY 4mg (i.v.)	Day -2	7/10 b)	9/10 c)		1 d)	4	2			3
	Shortly after inoculation	6/10	6/10			6				4
	Day 2	6/10	6/10				3	3		4
HCA 2.5mg, (s.c.)	Shortly after inoculation	9/10	9/10	1	5	3				1
None		0/10	6/10							10

a) Inoculated i.v. with 1.1×10^6 of MSK organisms.
b) Dead/tested(Day 10 p.i.).
c) Positive/tested.
d) No. of dead mice.

Table 1-2. Effect of CY and HCA (2)

Treatment		Mortality	Liver lesion	Days p.i. a) 4	5	6	7	8	9	10≤
CY (i.v., Day -2)	8 mg	5/5 b)	5/5 c)	2 d)	3					
	4 mg	2/5	3/5			1	1			3
	0.4 mg	0/5	3/5							5
	0.04 mg	0/5	5/5							5
HCA (s.c., Shortly after inoculation)	2.5 mg	4/5	4/5	1	2	1				1
None		0/5	3/5							5

a-d) See Table 1-1.

Table 1-3. Development of liver lesions in infected and CY or HCA treated mice

Treatment	Days p.i. [a]					
	1	2	3	4	5	7
CY (4 mg, i.v., Day -2)	-[b]	+	++	+++	++++(D) [c]	
	-	+	++	+++	++++(D)	
		+				
HCA (2.5 mg, s.c., shortly after inoculation)	-	+	+++	++++,++++(D)		
	-	+	+++	++++,++++(D)		
		+	+++	++++,++++(D)		
None	-	+	++	+++	+++	++++
	-	+	++	+++	+++	++++
		+				

a) Inoculated i.v. with 1.2×10^6 of MSK organisms.
b) Severity of liver lesions.
c) Died.

Table 1-4. Ig G and Ig M antibody titers of infected mice by indirect immunofluorescence

Experiment	Inoculation	Treatment	Antibody class	Days p.i. 1	2	3	4	5	6	7	10	15
I	7.7×10^5 i.v.	HCA[a]	Ig G	25>[b]	25>	25>		25>				
				25>	25>	25>						
			Ig M	25>	25>	25>		25>				
				25>	25>	25>						
		None	Ig G	25>	25>	25>		25		400	100	400
				25>	25>	25>		25		1600	800	800
								25				
			Ig M	25>	25>	25>		25>		25>	25>	25>
				25>	25>	25>		25		25	25>	25>
								50				
II	8.8×10^5 i.v.	None	Ig G			10>,10>		10>	25>	25		
						10>,10>		10	50	50		
						10>,10>		50	100	100		
						10>,10>			200			
						10>,10>						
III	9.0×10^5 i.v.	CY	Ig G			10>	10>	10>		10>		
						10>	10>	10>		10>		
		HCA	Ig G			10>	10>	10>		10>		
						10>	10>	10>				
								10>				
		None	Ig G			10>	10	10		100	400	
						10>	10	25		200	800	

a)See Table 1-3.
b)Titer of individual mice: Reciprocal of the highest dilution of antiserum showing positive reaction.

Table 1-5. Effect of serum transfer

Treatment	Serum [a]	Mortality	Liver lesion	Days p.i.[b] 4	5	6	7	8	9	10≤
CY [c]	Ab(+)	3/10 [e]	9/10 [f]				2 [g]	1		7
	Ab(-)	9/10	10/10			5	3		1	1
	None	9/10	10/10			5	1	2	1	1
HCA [d]	Ab(+)	9/11	10/11		1	3	3	1	1	2
	Ab(-)	10/11	10/11	1	8	1				1
	None	10/10	10/10	2	8					

a)0.2 ml i.v., Day 2 p.i., IFA titer: 1:400(+) or 1:10>(-).
b)i.v. with 8.6×10^5 MSK organisms.
c)4 mg i.v., Day -2. d)2.5 mg s.c., Day 0.
e)Dead/Tested. f)Positive/Tested. g)No. of dead mice.

EXPLANATION OF FIGURES

Fig.1-1 Multiple hepatic necrosis and atrophied thymus and spleen of an infected and CY-treated mouse on day 4 p.i.

Fig.1-2 Multiple hepatic necrosis, intact thymus and swollen spleen of an infected and non-treated mouse on day 7 p.i. Each hepatic lesion was 1 to 2 mm in diameter, being larger than that of HCA- or CY-treated mice.

Fig.1-3 Focal coagulative necrosis with neutrophil infiltration in the liver of an infected and HCA-treated mouse on day 3 p.i. HE. x 175. Inset: Slender rods within the hepatocytes. PAM. x 700.

Fig.1-4 Severe cortical destruction and intact medulla in the thymus of an infected and CY-treated mouse on day 4 p.i. HE. x 175.

Fig.1-5 Prominent atrophy of lymphoid follicles in the atrophied spleen of an infected and HCA-treated mouse on day 3 p.i. HE. x 175.

Fig.1-6 A markedly enlarged follicle in the spleen of an infected and non-treated mouse on day 4 p.i. HE. x 175.

Fig.1-7 Granulomatous tissue replacing necrotized lesions in the liver of an infected and non-treated mouse on day 7 p.i. HE. x 175.

Fig.1-8 Scarring in the liver of an infected and non-treated mouse on day 11 p.i. HE. x 175.

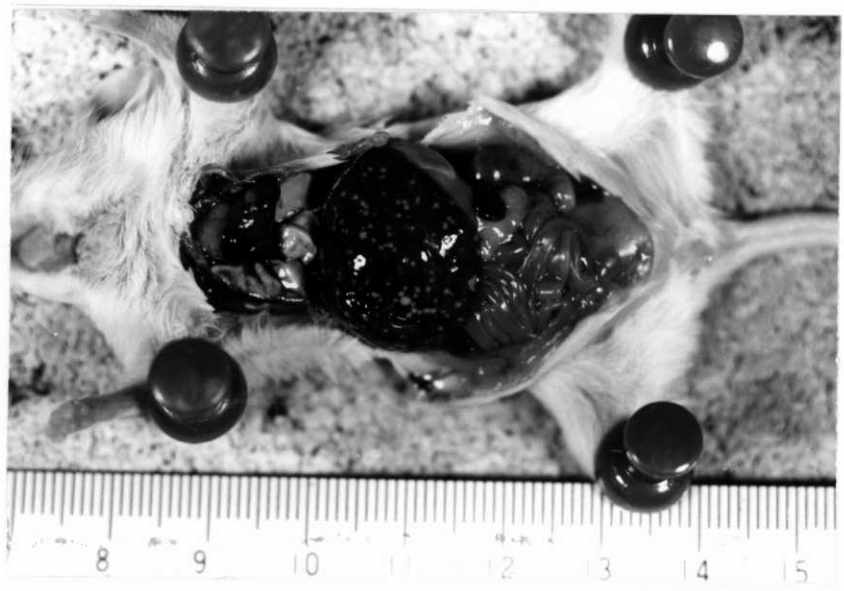


Fig.1-1

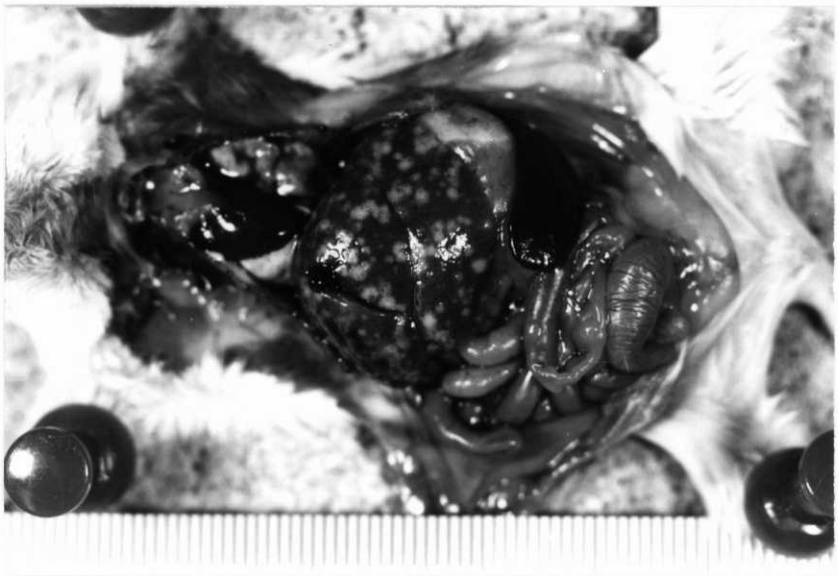


Fig.1-2

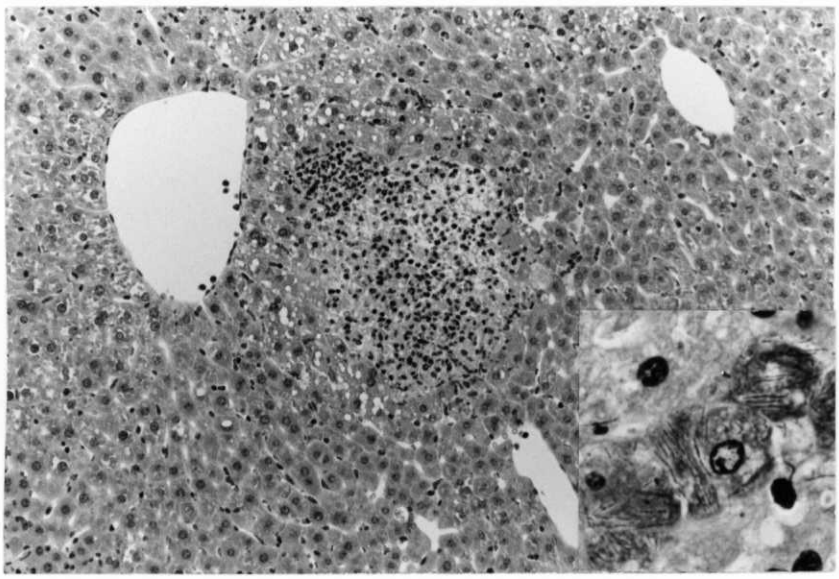


Fig.1-3

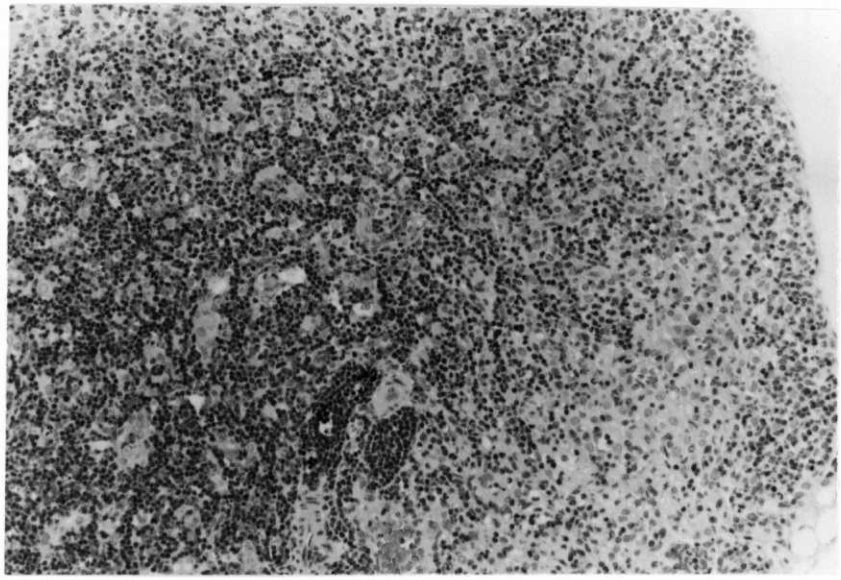
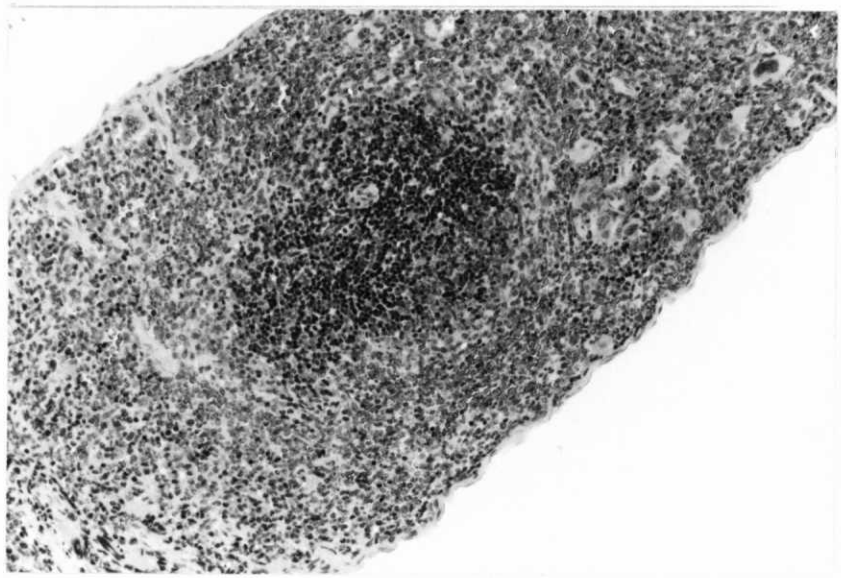


Fig.1-4



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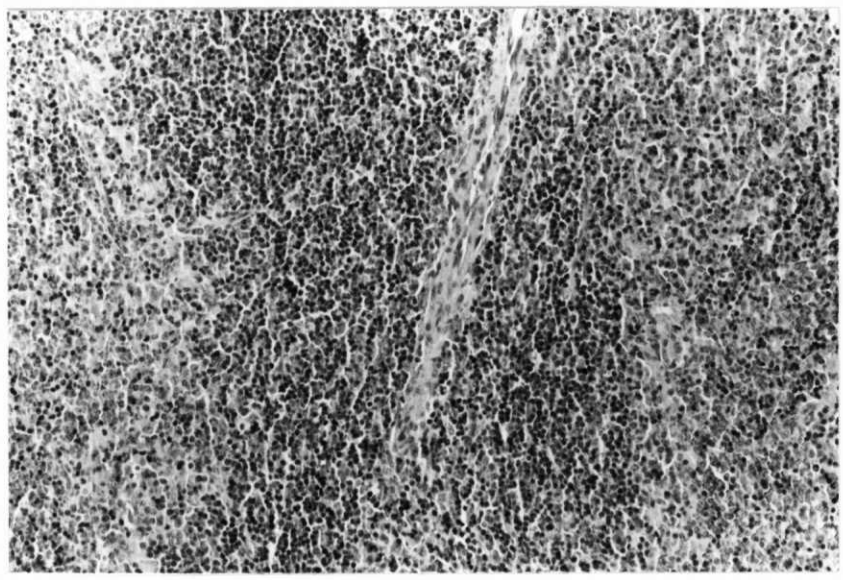

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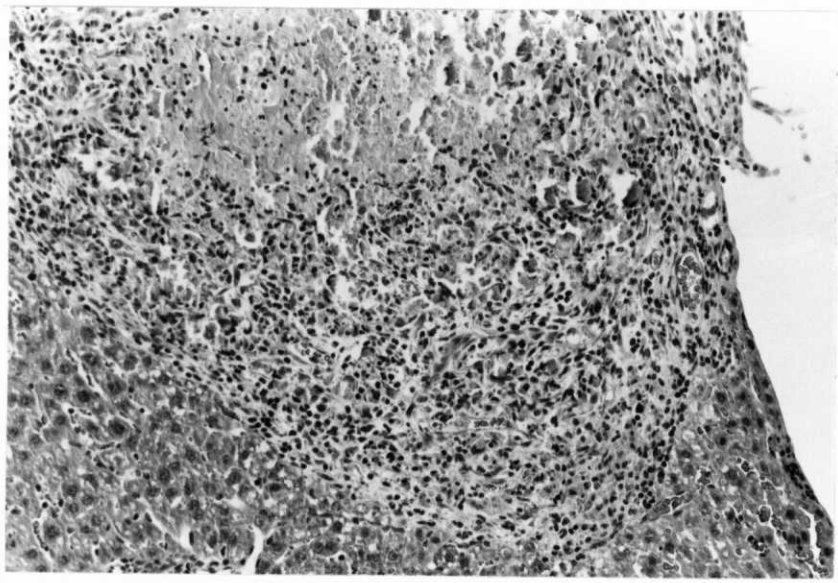

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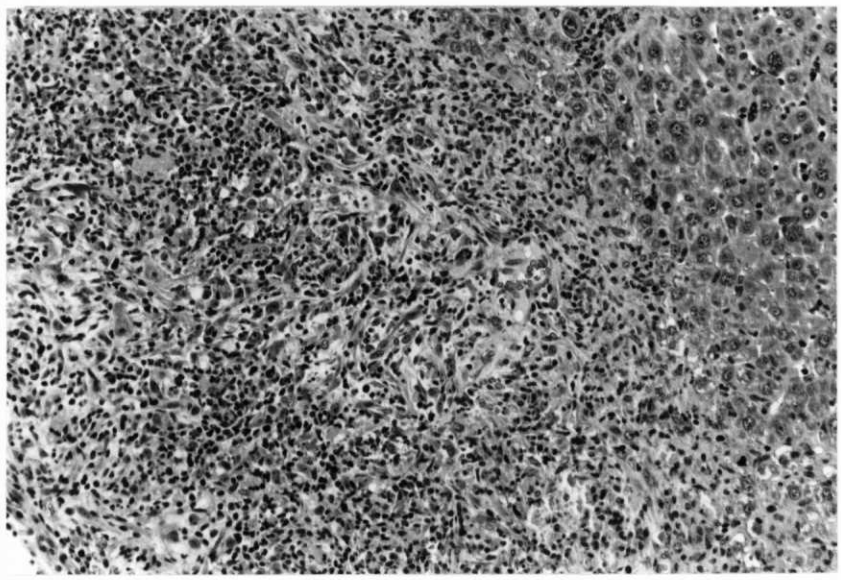

Fig.1-8

PART 2

Reticuloendothelial System and Tyzzer's Disease of Mice

INTRODUCTION

Severity of experimental Tyzzer's disease in mice was enhanced by irradiation [64], administration of glucocorticoids (GC)[18] or cyclophosphamide(CY) (See Part 1). The last two treatments are known to cause immunodificiency by killing or suppressing lymphocytes in the spleen and thymus. Based on their observation with mice genetically deficient in B- or T-cells, Waggie et al. [71] stated that only B-cells were responsible for protection against Tyzzer's disease. On the other hand, Fujiwara et al. [16] observed that athymic nude mice did not develop protective immunity after vaccination and that the immune potential against the infection was reconstituted after receiving spleen cells from euthymic mice. These observations suggest an important role of T-cells in producing protective antibody. However, most of infected mice died within 7 days p.i. and no antibody might play any effective role in protection at early stage of the disease (Part 1). Then, in the protection during such early phase, other immunological factors than T-cell or antibody would also have an important role.

In this part, were described effects of splenectomy as well as blocking of the reticuloendothelial system(RES) on the host response in the early stage of Tyzzer's disease in mice.

MATERIALS AND METHODS

Animals: Four- to five-week-old ICR female mice (17-21 g), female athymic nude(nu/nu) mice (13-18 g) and their euthymic littermates(nu/+ and +/+) (18-22 g) with the ICR background (Charles River Japan, Atsugui, Kanagawa) were used. Animals were kept in aluminium cages with a filter cap (Sanki-Kagaku, Tokyo) and given autoclaved commercial pellets (Oriental Yeast, Tokyo) and tap water ad libitum.

Splenectomy: Mice were anesthetized with ethylether. An incision was made on the left abdomen and the spleen was removed after ligating the splenic artery and vein. After suturing the peritoneum and abdominal muscles, the skin opening was clipped. For the sham operation the spleen was once taken out of and then put back into the peritoneal cavity.

Inoculation: Mice were injected intravenously(i.v.) with 0.2 ml of severely infected mouse liver homogenate in phosphate buffered saline (PBS) containing 10^5 to 10^6 organisms of the MSK strain [14]. Shortly after the inoculation they received subcutaneously(s.c.) with 2.5 mg hydrocortisone acetate (HCA) (Scheroson F, Nippon Schering, Osaka). On days 3 to 4 postinoculation (p.i.) moribund animals were killed by ethylether or chloroform, and livers with severe necrotized lesions were collected and stored at -80°C. For inoculation the frozen livers were thawed

and homogenized in PBS and a dilution of the homogenate was made so as to contain 10^6 organisms per 0.2 ml.

RES-blockers: One group of mice received s.c. injection with 2.5 mg HCA while another group recieved intraperitoneal (i.p.) injection with 1 mg carrageenan(Wako-Junyaku Ltd., Osaka) in 0.25 ml warm PBS or with 30 mg silica (Sigma Chemical, St. Luis, U.S.A.) in 0.2 ml PBS. Some mice were injected i.v. with 10 % India-ink (Pelikan, Hannover, West Germany) in 0.2 ml PBS.

Bacterial counts: Infected mouse liver homogenate was placed on a 76 x 26 slide glass (Matsunami, Osaka), air-dried, fixed in methanol and stained with 1 % thionine solution. Visible bacteria on a 1 cm^2 area were counted under light microscope and the number of bacteria per g-liver was calculated [63].

Disease indexes (DI): In each experiment, animals dead on day 7 p.i. were given a disease score of 5. Those that died "n" days earlier than day 7 p.i. were given a score of 5 plus "n". Surviving mice were killed on day 7 p.i. by chloroform, and disease scores of 0 to 4 were given according to the grades of liver gross lesions[18]. The disease index of each group was calculated by dividing the total of scores by the number of mice included, and the difference between groups was analyzed using the Student's t-test.

RESULTS

The ICR mice were distributed to six groups, 8 to 11 mice per group. Mice of one group were splenectomized on day -3, and another group of mice on day -7. Mice in other two groups were sham-operated. All the mice received i.v. inoculation with 1.1×10^6 organisms. Shortly after inoculation one of the remaining two groups was treated with HCA, while the other remained without any treatment. Time-to-death was observed and mice surviving on day 7 p.i. were killed and examined for the liver disease indexes.

As shown in Table 2-1, mice splenectomized on day -3 showed a significantly higher DI (2.9) than either sham-operated(1.3) ($P<0.02$) or non-operated control without HCA(1.1) ($P<0.02$). No difference was observed among those splenectomized on day -7 (2.1), sham-operated(0.8) and non-operated control without HCA(1.1). Non-operated and HCA-treated mice (4.0) showed a significant difference from mice without any treatment($P<0.01$).

For the next experiment, ICR mice were distributed to three groups, 9 to 20 mice per group. Mice of one group were splenectomized and received i.v. inoculation with 1.2×10^6 organisms 6 days after the operation. Of other two groups without operation, one group was inoculated with the same number of organisms with HCA treatment while the other without HCA. Two to 3 mice were killed daily thereafter and examined for necrotic lesions and the

number of bacilli in the liver.

As presented in Table 2-2, splenectomized mice showed severe hepatic lesions on day 5 or 6 p.i. and some of them died on day 6. Organisms in the liver increased in number as the necrotic lesions became more severe. In non-operated and non-HCA-treated mice bacteria slowly increased in number during a period between day 3 and 5 p.i. followed by a decrease. Liver lesions developed more slowly in this group as compared with splenectomized mice. Scar formation was observed on day 8 p.i.

For the third experiment, ICR mice were distributed to 6 groups, 9 to 10 mice per group, and were injected i.v. with 1.7×10^6 organisms. Each of two groups received carrageenan i.p. on day -1 or -3 while the other two shortly after inoculation or on day 1. One of the remaining two groups was injected with HCA shortly after inoculation and the other was non-treated. Death was recorded daily and survivors on day 7 p.i. were killed and examined for liver lesions.

As presented in Table 2-3, mice given carrageenan on day 1 p.i. showed a DI of 1.8, being significantly different from that of the non-treated control($P<0.01$). However, no significant difference was observed among the other three carrageenan-treated groups. The group of mice given HCA showed a score of 6.1 which was significantly higher than that of the non-treated control

($P<0.01$).

Carbon and silica, phagocytes blockers in vivo, were examined for effects on Tyzzer's disease. Each of the four groups received either agent 1 day prior to or after the i.v. inoculation of 2.9×10^6 organisms. The other two groups were subjected to non-treated and HCA-treated controls as in the foregoing experiment.

As shown in Table 2-4, mice treated with either carbon or silica on day 1 p.i. showed indexes of 2.7 and 3.0, respectively, both significantly different from the index of the non-treated control ($P<0.1$ and $P<0.01$, respectively). In contrast, those treated with either agent 1 day before the inoculation showed no significant difference as compared with the non-treated control.

Athymic nu/nu, euthymic nu/+ and another euthymic +/+ mice were employed to investigate the role of T-cells in the resistance against the disease. They were inoculated i.v. with 2.6×10^6 organisms, and a half of the mice in each group were given HCA shortly after inoculation.

There were no significant differences in the disease indexes among the non-treated nu/nu, nu/+ and +/+ mice(Table 2-5). There was also no difference in the enhancing effect of HCA on the infection among these three groups of mice.

In the following experiment, the combined effect of splenec-

tomy and treatment with carrageenan was examined in nu/nu mice. Mice nu/nu were splenectomized and 3 and 4 days later, they were inoculated i.v. with 2.3×10^6 organisms and i.p. with 1 mg carrageenan, respectively. One of the two infected groups of +/+ mice was treated with HCA while the other remained non-treated.

All nu/nu mice and HCA-treated +/+ mice died within 6 days p.i. with indexes of 6.4 and 6.8, respectively, being significantly different ($P<0.01$) from the value of non-treated +/+ mice (Table 2-6).

DISCUSSION

In the present study, the severity of infection in mice was estimated by the disease index that was calculated from data on time-to-death and grades of liver lesions [13,18] for 7 days after the infection. Liver lesions of both dead and surviving mice can be included in the evaluation of the severity of infection with the index. Since antibody which was shown to be protective[19] increased in titers on day 7 p.i. or later and lead to the recovery (Part 1), observation of time-to-death until day 7 p.i. was reasonable for estimation of the severity of the acute phase of disease. The proposed scoring is simple and objective, and would be useful to evaluate the severity of infection.

The enhancing effect of splenectomy may be attributed to the decrease of both lymphoid and RES cells. The enhancement was most remarkable when mice were splenectomized 3 days than 7 days before inoculation. Since compensatory hyperplasia of both lymphoid organs and RES may immediately occur, the resistance to infection could develop rather rapidly after the operation.

Enhancement of the severity was also observed with RES blocking with carrageenan, carbon or silica. This indicates an important role of RES in the host resistance against the infection possibly by inhibiting the growth of organisms. The RES blocking was only effective after inoculation and not before the inocula-

tion of the organisms. The blocking effect of these treatments probably lasts for a relatively short period. In murine viral hepatitis also, the protective role of RES at the early stage of infection was indicated[62,66]. In experimental Tyzzer's disease, however, Kupffer cells were shown to remain histopathologically inactive [11,17,34] carrying little amount of bacterial antigen through the course of infection.

Glucocorticoids given shortly after inoculation markedly enhanced infection, but much less when given before inoculation [18]. Both GC and carrageenan induces a decrease in both the number and function of RES cells [53,67,75], probably resulting in the propagation of organisms within hepatocytes. In addition, GC is known to modify carbohydrate as well as protein metabolism in hepatocytes, which might induce the intracellular propagation of organisms [18,38]. Cyclophosphamide, on the other hand, has been known to enhance the infection when it was used either before or after infection (Part 1).

A previous study with athymic mice demonstrated that T-cells are needed for antibody production against Tyzzer's disease [16]. The thymus was revealed to be severely atrophied in mice treated with either HCA or CY (Part 1). In this study, the disease scores of nu/nu and nu/+ mice were not significantly higher than that of +/+ mice, suggesting that T-cells might not be concerned

in the early phase of resistance against Tyzzer's disease [71]. In murine viral hepatitis no T-cells seem to participate during early stage of infection [29].

The present results indicate that the RES plays an important role in the host resistance in the acute phase of Tyzzer's disease.

SUMMARY

Tyzzer's disease in euthymic mice was aggravated by splenectomy and treatment with carrageenan, India ink or silica. The aggravating effect was more prominent when splenectomy was performed 3 days rather than 7 days before infection. Blocking of the RES was most effective in increasing the severity of infection when made on the day following the inoculation. Athymic nude mice did not show severer infection as compared with euthymic mice. In nude mice splenectomized on day -6 and treated with carrageenan on 1 day p.i. induced hepatic lesions as severe as seen in cortisone-treated euthymic mice. These data suggested that macrophages had important role(s) in early protection against Tyzzer's disease in mice.

Table 2-1 The effect of splenectomy on the severity of Tyzzer's disease

Treatment		No. tested	Disease score a)								DI b)
			0	1	2	3	4	5	6	7	
day -7	Splenectomized	11	4 c)		2	2	2	1			2.1
	Sham	8	4	2	2						0.8
day -3	Splenectomized	10	1	1	2	2	3		1		2.9*
	Sham	10	1	6	2	1					1.3
----------		9	4		5						1.1
Shorly after inoculation HCA 2.5 mg s.c.		10	3				1		6		4.0**

a) Scored on day 7 p.i. according to time-to-death or liver lesions (See the text).
b) Disease index (DI). See the text. c) No. of cases.
* $P<0.02$ compared with non-treated control and sham-operated on the same day.
** $P<0.01$ compared with non-treated control.

Table 2-2 The effect of splenectomy on the severity of Tyzzer's disease and bacterial counts in the liver

Treatment	Disease score and No. of bacteria in parenthesis (day p.i.)						
	1	3	5	6	7	8	10
	a) b)						
Splenectomized on day -6	0(4>)	2(4>)	4(6.1)	4(8.2),4(8.2),D[c]			
	0(4>)	2(4>)	4(5.8)	4(8.9),4(5.0),D			
			4(5.8)	4(6.3),4(4.9),D			
--- ---	0(4>)	2(5.3)	3(5.4)	2(5.1)	1(4>)	1(4>)*[d]	1(4.9)*
	0(4>)	3(4>)	3(4>)	3(5.1)	3(5.4)	2(4>)	2(4>)*
		3(4.9)	3(5.2)	3(5.6)	3(4>)	3(4>)	3(4>)*
HCA 2.5 mg s.c., shortly after inoculation	0(4>)	2(6.1)	D				
	0(4>)	3(7.3)	D				
	0(4>)	3(7.8)	D				

a) Score of each mouse. See Table 2-1. b) Number of bacteria, $\log_{10}$/g liver. c) Dead. d) Scar lesion.

Table 2-3. The effect of carrageenan treatment on the outcome of Tyzzer's disease

Treatment		No. tested	Disease Score a)								DI b)
			0	1	2	3	4	5	6	7	
Carrageenan 1 mg i.p.	day -3	10	8 c)			1	1				0.7
	day -1	10	7	3							0.3
	Shortly after inoculation	10	6		2	1	1				1.1
	day 1	9	2		3	4					1.8*
----------		10	9		1						0.2
HCA 2.5 mg s.c., Shortly after		10					1		6	3	6.1*

a) - c) See Table 2-1.
* $P<0.01$ compared with non-treated control.

Table 2-4. The effect of carbon or silica treatment on the outcome of Tyzzer's disease

Treatment	No. tested	Disease Score a)								DI b)
		0	1	2	3	4	5	6	7	
Carbon 10 % i.v. day -1	9	2 c)	2	3	2					1.4
Carbon 10 % i.v. day 1	10			5	4		1			2.7*
Silica 30 mg i.p. day -1	10	3		3	3	1				1.9
Silica 30 mg i.p. day 1	10			1	8	1				3.0**
----------	10	2		6	2					1.8
HCA 2.5 mg s.c. shortly after inoculation	10	1					2	7		5.2**

a) - c) See Table 2-1.
* $P<0.1$ and ** $P<0.01$ compared with non-treated control.

Table 2-5. Infection in nu/nu mice (1)

Mice	Treatment	No. tested	Disease score [a] 0	1	2	3	4	5	6	7	8	DI [b]
nu/nu	------	9				1[c]	4	4				4.3
	HCA [d]	8							1		7	7.8*
nu/+	------	9				2	3	2	2			4.4
	HCA	8							1		7	7.8*
+/+	------	10				3	6		1			3.9
	HCA	10									10	8.0*

a) - c) See Table 2-1.
d) HCA 2.5 mg/mouse was injected s.c. shortly after the infection.
* $P<0.01$ compared with non-treated mice.

Table 2-6. Infection in nu/nu mice (2)

Mice	Treatment	No.tested	Disease score a) 0	1	2	3	4	5	6	7	DI b)
nu/nu	Splenectomy on day -3 and Carrageenan 1 mg i.p. on day 1	10							6 c)	4	6.4*
+/+	----------	10			3	3	4				3.1
+/+	HCA 2.5 mg s.c. shortly after inoculation	10							2	8	6.8*

a) - c) See Table 2-1.
* $P<0.01$ compared with non-treated +/+ mice.

PART 3

Precociously Induced Resistance to Tyzzer's Disease in Mice

INTRODUCTION

Tyzzer's disease in mice is known to be enhanced by macrophage blocking(Part 2), splenectomy(Part 2), and treating with glucocorticoids(GC)[18] or cyclophosphamide(CY)(Part 1), suggesting the importance of macrophage activities in early phase of the infection(Part 2). However, no evidences have been obtained with alteration in morphology and phagocytic activity of Kupffer cells in the liver, the main target organ in this disease[11,17,50].

In mouse hepatitis virus (MHV) infection, in which the target cells are also hepatocytes, macrophage-related resistance can be induced against challenge with high-virulent virus shortly after pretreating with low-virulent virus[62]. This part deals with precocious induction of resistance to challenge infection by either specific or non-specific pretreatment probably due to macrophage activation [42,59].

MATERIALS AND METHODS

Animals: Four-week-old ICR female mice weighing 17 to 21 g (Charles River Japan, Atsugui, Kanagawa) were used. Animals were kept in aluminium cages with a filter cap (Sanki-Kagaku, Tokyo) and given autoclaved commercial pellets (Oriental Yeast, Tokyo) and tap water ad libitum.

Drugs and vaccines: Freund complete adjuvant (FCA)(Difco Lab., U.S.A.), Escherichia coli 055:B5 lipopolysaccharide (LPS)(Difco Lab., U.S.A.) and lyophilized viable BCG vaccine (Japan BCG Lab., Tokyo) were used as immunopotentiators [3,24,32], and poly-inosinic-poly-cytidylic acid (poly[I]:poly[C])(Sigma Chemical Co., U.S.A.) were used as an interferon inducer [26].

Tyzzer's organisms and formalin vaccine: Challenge inoculation was made intravenously(i.v.) with 0.2 ml infected mouse liver homogenate in phosphate buffered saline, pH 7.4 (PBS) containing 10^6 MSK strain[14] of organisms. For bacterial pretreating, 10^2 to 10^5 organisms in 0.2 ml were given i.v. on day -1 to -4 of challenge inoculation. As control, 1:2,000 homogenate of normal mouse liver was given i.v.

Also a 1:200 homogenate of the same infected mouse liver was made in distilled water and allowed to stand for 60 min for bacteriolysis[15]. The homogenate was centrifuged at 1,000 rpm for 10 min, and the supernatant was exposed to ultraviolet at 15 W at

a distance of 30 cm and then filtered through a 0.8 μm-membrane filter (Millipore, U.S.A.) to eliminate the remaining organisms and spores. The filtrate was used as a bacterial lysate, and injected i.v. into mice. The control lysate from a normal mouse liver was prepared by the same manner.

To produce bacterial vaccine[13], formalin was added to the infected liver homogenate at a final concentration of 1 %. After incubation at 4°C for 3 days, the formalinized homogenate was centrifuged at 500 rpm for 30 min, and the supernatant was subjected to centrifugation at 10,000 rpm for 20 min and sediment was washed twice in PBS by centrifugation. The final sediment was suspended in PBS and 0.01 ml phenol was added to the suspension. The formalin vaccine was stored at 4°C and its dilutions in PBS containing 10^4 to 10^6 bacteria in 0.2 ml were given i.v.

Mouse hepatitis virus (MHV): Mouse liver infected with JHM strain of MHV was homogenized at 1:10 in PBS. The homogenate showing a virus titer of 250 PFU/ml on DBT cells[27] was heated at 56°C for 30 min to inactivate the virus and mice were injected i.v. with 1:10 dilution of the homogenate.

Indirect immunofluorescence (IF)[14]: About 0.01 ml of MSK-infected mouse liver homogenate was placed on a 76 x 26 mm slide glass (Matsunami, Osaka), and the slide was air-dried, fixed in methanol and used as target antigen preparation. The target was

treated first with serial dilutions of test serum samples obtained by heart puncture and then with fluorescein-isothiocyanate(FITC)-conjugated anti-mouse IgG goat serum (Cappel Lab., U.S.A.). The titer of anti-MSK IgG antibody was expressed by the highest dilution showing specific fluorescence.

Interferon(IFN) assay[36]: The L929 cells were grown in 12 well-plates with Eagle's minimal essential medium (MEM, Nissui-Seiyaku, Tokyo) containing 10 % calf serum (CS). Dilutions of serum samples in MEM containing 4 % CS were placed on the cell layers at day 1 or 2 of culture. After incubation at 37°C for 20 hr, the cell cultures were inoculated with 200 PFU/ml of vesicular stomatitis virus, New Jersey strain. After incubation for 60 min, the first overlay was made with 0.8 % Bacto agar (Difco, U.S.A.) in MEM containing 5 % CS followed by the second overlay made 36 to 48 hr later. Plaques were counted 8 to 14 hr after the second overlay.

Disease index (DI): An index was calculated as previously described(See Part 2). After observation for 7 days postinoculation (p.i.) each animal was given a score according to time-to-death as well as severity of liver lesions as described in Part 2 of this paper. DI was given by dividing the total scores of each group by the number of mice included in the group. The difference of DI between groups was analyzed using Student's t-test.

RESULTS

Mice were divided into 3 groups of 10 mice each and two of them were given i.v. 6.0×10^4 organisms. Two days later, one of the pretreated as well as non-pretreated control were challenged i.v. with 1.0×10^6 organisms and given subcutaneous(s.c.) administration of 2.5 mg hydrocortisone acetate(HCA) (Scheroson F, Nippon Schering, Osaka). In mice pretreated with 6.0×10^4 organisms time-to-death was significantly prolonged when exposed to HCA-associated challenge (Table 3-1). In another experiment, mice were divided into 3 groups of 10 to 11 mice each. Mice of the two groups were injected i.v. with 1.0×10^5 organisms and 2 days later, one of the two groups as well as control without pretreatment were challenged i.v. with 7.6×10^5 organisms. All mice which survived for 7 days postchallenge were examined for liver lesions, revealing that the pretreated mice resisted to challenge infection, as shown in Table 3-2.

Next, 3 groups of 10 mice received i.v. 7.4×10^2 to 7.4×10^4 organisms and 2 days later, these mice as well as controls either non-treated or given 0.2 ml PBS were challenged with 1.7×10^6 organisms. As shown in Table 3-3, with any pretreatment dose protective effect was evident ($P<0.05$) showing DI of 0.7, 1.2 and 1.1, in cases of pretreatment with 7.4×10^4, 7.4×10^3 and 7.4×10^2, respectively, as compared with non-pretreated control group

(DI=2.3). The DI of PBS-pretreated group (1.7), however, showed no significant difference as compared with that of non-pretreated control.

To see time period required for the production of precocious resistance to challenge, 4 groups of 9 to 10 mice each were pretreated i.v. with 8.1 to 9.9 x 10^4 organisms and 1 to 4 days later, they were challenged with 1.4 x 10^6 organisms. Another group of 10 mice was given 7.9 x 10^4 organisms 1 day after challenge. As shown in Table 3-4, the DI of mice having been pretreated on days -4, -3, -2 and -1 were 0.3, 0.2, 0.4 and 0.3, respectively, being significantly smaller than the values of non-pretreated control ($P<0.05$), while postchallenge treating (DI=1.1) was not effective.

An experiment was done to see relatedness among severity of liver lesions, serum antibody titers and IFN values in pretreated and challenged mice. Two groups of 18 mice each received i.v. inoculation with 1.3 x 10^5 organisms and 2 days later, one of them and non-pretreated control mice were inoculated i.v. with 2.6 x 10^6 organisms. Three mice of each group were killed each day after inoculation, and sera were collected from all cases and tested for anti-MSK IgG antibody titers and IFN. They were examined for liver lesions.

As presented in Table 3-5, non-pretreated and challenged mice

were shown to have liver lesions only on day 2 or later postchallenge, but the lesions developed very fast and became very severe on days 4 to 5 postchallenge. In these non-pretreated and challenged mice antibody was detected on day 7 postchallenge. In pretreated mice with or without challenge, however, necrotized lesions in the liver appeared already on the next day of challenge inoculation and slowly developed with time. They were undetectable on day 7 postchallenge in some cases. Anti-MSK IgG antibody was detected on day 4 postchallenge in these pretreated and challenged mice. IFN remained undetectable in all cases tested.

The following experiment was done to see whether pretreatment with formalin vaccine was effective. Three groups of 8 to 11 mice received s.c. or i.v. injection of 8.2×10^5, 8.2×10^4 and 8.2×10^3 formalin-killed organisms, respectively. Another group of 10 mice was inoculated i.v. with 2.4×10^4 live organisms. Two days later all these mice and those of non-treated controls received i.v. challenge with 1.5×10^6 organisms. As presented in Table 3-6, those pretreated with formalin vaccine as well as live organisms showed a significantly lower DI as compared with non-pretreated control ($P<0.01$).

The next experiment was performed to see effects of either supernatant or sediment of the infected mouse liver homogenate. Three groups of 10 mice each received i.v. injection with 0.2 ml

of: (1) infected mouse liver homogenate in PBS (1:2,000) containing 1.5 x 10^5 organisms, (2) supernatant after centrifugation at 10,000 rpm for 20 min from the same homogenate, or (3) normal mouse liver homogenate (1:2,000). Two days later all these mice and another non-pretreated control mice received i.v. challenge of 2.0 x 10^6 organisms. As shown in Table 3-7, the DI was significantly smaller in mice pretreated with the infected mouse liver homogenate than that of non-pretreated control ($P<0.01$). Those pretreated with either the liver supernatant of infected mice or the homogenate of uninfected mice showed no significant difference from non-pretreated controls. Protective effect was remarkable in mice pretreated with bacterial lysate (DI=0.8), as compared with those pretreated with control normal liver lysate (DI=3.1) or those non-pretreated (DI=3.0) ($P<0.01$), as shown in Table 3-8.

The next experiment was done to see effects of non-specific immunopotentiators such as LPS and BCG. Two groups of 10 to 11 mice received s.c. or i.p. injection of 16 μg live BCG vaccine while another group s.c. injection of 20 μg LPS. One of other 2 groups of 8 or 10 mice were either injected i.v. with 1.1 x 10^5 MSK while the other remained non-treated. Two days later, all these mice were exposed to i.v. challenge of 2.6 x 10^6 organisms. As shown in Table 3-9, LPS- and BCG-treated mice as well as those pretreated with viable organisms showed a significantly smaller DI

as compaered with that of non-pretreated control ($P<0.05$).

To see effect of either IFN-inducer or immunostimulator, 4 groups of 10 mice each were given (1) 8μg poly[I]:poly[C] in 0.2 ml PBS i.p., (2) 0.25 ml FCA s.c., (3) 0.2 ml PBS i.p. or (4) 7.6×10^4 MSK organisms i.v. Two days later, these mice and non-treated contols received i.v. challenge with 3.5×10^6 organisms. As shown in Table 3-10, except for those pretreated with 7.4×10^4 bacteria, no significant protective effect was observed. As presented in Table 3-11, any protective effect was not shown by pre-teating with MHV, which is known to be an IFN-inducer[29] as well as a macrophage activator[62,66].

DISCUSSION

The present study revealed that mice were protected from challenge infection which was made 2 or 3 days after the foregoing inoculation with a lower dose of bacteria. Serum antibody, which was produced on day 4 or later postchallenge when liver lesions became maximum, seemed to play no significant role in the precocious protection, as already described in Part 1. Pretreating with 10^2 to 10^4 bacteria was effective enough, though the higher dose of bacteria was more effective.

Similar precociously induced resistance was also seen after treating with formalin vaccine as well as bacterial lysate, indicating the induced resistance might be due to some bacterial components stimulating the macrophage system of the host. The supernatant of fresh infected mouse liver homogenate was not effective. BCG vaccine[42], LPS[59] or muramyl dipeptide (MDP), a synthetized adjuvant[41,52] were shown to enhance the activities of macrophages and polymorphonuclear cells inducing protection against bacterial infections. In this study, the pretreatment of LPS or BCG was effective on Tyzzer's disease of mice while FCA or inactivated MHV was not effective. The enhanced non-specific resistance due to macrophage activation induced by pretreating with protozoa Tetrahymena[39,40] and Latobacillus casei[45,60] was also reported in toxoplasmal and pseudomonadal or listerial infec-

tions. In MHV infection in mice the enhancement of antiviral activity of macrophages was shown to be of importance in resistance induced by a low-virulent strain virus [62]. In this study, however, no protection was observed after pretreating with MHV-infected liver.

In our previous studies, Tyzzer's disease in mice was shown to be enhanced by macrophage blocking using carrageenan, silica or carbon particles, indicating the importance of macrophages during the early phase of infection and possibility of indirect inhibition of bacterial growth within hepatocytes by macrophages during early phase of infection was proposed (See Part 2). On the other hand, the severity of Tyzzer's disease was found to colsely depend upon protein metabolism of host hepatocytes[38]. West et al. [72] suggested a close relatedness between protein metabolism within hepatocytes and macrophage activities, since mouse hepatocytes showed decreased ^{3}H-leucine incorporation when cocultured with peritoneal cells from mice treated with either gentamycine-killed Escherichia coli, LPS or MDP. After pretreating with bacterial components of Tyzzer's organisms as well as LPS or BCG, murine macrophages might be activated and produce a certain humoral factor(s) such as interleukin-1, which might affect protein metabolism of hepatocytes resulting in inhibition of intracellular bacterial growth.

Because of strict intracellular parasitism in Tyzzer's disease[69], the host protective mechanism might be comparable to that in viral infections. At early stage of infection the role of IFN produced after the primary infection should be also taken in consideration. In this study, however, IFN remained undetactable in sera from pretreated mice which resisted to challenge infection. The pretreatment with IFN inducers, poly[I]:poly[C][26] or also MHV[29] did not induce any protective effect to challenge infection.

SUMMARY

By pretreating mice with sublethal dose of infected mouse liver homogenate, resistance was precociously induced to fatal challenge inoculation made days 1 to 4 post-treatment. Serum antibody became detectable on day 4 postchallenge in both pretreated and challenged mice but serum interferon remained undetectable. Similar early resistance could be induced by pretreating with formol vaccine or lysate of the bacteria, LPS or BCG but not with Freund complete adjuvant, poly[I]:poly[C] or inactivated mouse hepatitis virus.

Table 3-1. Effect of bacterial pretreatment on time-to-death postchallenge

Inoculation (i.v.)		No. of mice	Time-to-death in days p.i.						
Day -2	Day 0		4	5	6	7	8	9	10≤
6.0×10^4	1.0×10^6 + HCA[a)]	10			1[b)]		1	2	6
6.0×10^4	-	10							10
-	1.0×10^6 + HCA	10	4	2	1	1			2

a) 2.5 mg s.c. b) No. of cases showing indicated time-to-death.

Table 3-2. Effect of bacterial pretreatment on severity of liver lesions produced by challenge

Inoculation (i.v.)		No. of mice	Liver lesion a)				
Day -2	Day 0		-	+	++	+++	++++
1.0×10^5	7.6×10^5	11	7 b)	4			
1.0×10^5	-	10	8		1	1	
-	7.6×10^5	10		1	3	6	

a)On day 7 postchallenge.
b)No. of cases showing indicated severity.

Table 3-3. Effect of various dose of bacterial pretreatment

Pretreatment[a] (i.v., on day -2)	No. of mice	Disease score 0	1	2	3	4	5	DI[b]
7.4×10^4	10	3[c]	7					0.7*
7.4×10^3	10	1	6	3				1.2**
7.4×10^2	10	1	7	2				1.1***
PBS	10	3		4	3			1.7
-------	10	2		2	5	1		2.3

a) Before i.v. challenge with 1.7×10^6 organisms. b) Disease index(DI). See the text. c) No. of cases showing indicated score.
*, **, *** Significantly different at $P<0.01$, $P<0.05$ and $P<0.02$, respectively, as compared with nonpretreated control.

Table 3-4. Effect of bacterial pretreatment made before or after challenge

Pretreatment(i.v.)[a]	No. of mice	Diease score						DI[b]
		0	1	2	3	4	5	
Day -4; 9.2×10^4	9	6[c]	3					0.3**
Day -3; 9.9×10^4	10	8	2					0.2***
Day -2; 9.0×10^4	10	8	1		1			0.4*
Day -1; 8.1×10^4	10	8	1	1				0.3**
Day 1; 7.9×10^4	10	5	1	2	2			1.1
------	11	4		3	4			1.6

a) Before i.v. challenge with 1.4×10^6 organisms.
b) and c) See Table 3-3.
*, **, *** Significantly different at $P<0.05$, $P<0.02$ and $P<0.01$, respectively, as compared with nonpretreated control.

Table 3-5. Relationship between liver lesion and antibody titer in mice pretreated and challenged

Inoculation(i.v.)		Day p.i.					
Day -2	Day 0	1	2	3	4	5	7
		a) b)					
1.3×10^5	2.6×10^6	0(25>)	1(25>)	1(25>)	2(25>)	1(100)	0(100)
		1(25>)	2(25>)	1(25>)	2(25>)	1(200)	0(200)
		1(25>)	2(25>)	1(25>)	2(100)	1(400)	2(200)
1.3×10^5	-	0(25>)	0(25>)	1(25>)	1(25>)	1(25>)	0(25>)
		1(25>)	2(25>)	1(25>)	1(25>)	2(25>)	1(50)
		1(25>)	2(25>)	2(25>)	2(25>)	2(25>)	2(100)
-	2.6×10^6	0(25>)	1(25>)	2(25>)	3(25>)	3(25>)	3(50)
		0(25>)	2(25>)	3(25>)	4(25>)	4(25>)	3(200)
		0(25>)	2(25>)	3(25>)	4(25>)	4(25>)	3(400)

a) Score of each case. b) Reciprocal of serum anti-MSK Ig G antibody titers.

Table 3-6. Effect of formol vaccine pretreatment

Pretreatment [a] (on day -2)	No. of mice	Disease score 0	1	2	3	4	5	DI [b]
Formol vaccine								
8.2×10^5, s.c.	8			2 [c]	3	3		2.1*
8.2×10^4, i.v.	11	1	3	7				1.5*
8.2×10^3, i.v.	10	1	1	5	3			2.0*
Infected liver homogenate, 2.4×10^4 i.v.	10	1	6	2		1		1.4*
----------	10			1	4	5		3.4

a) Before i.v. challenge with 1.5×10^6 organisms. b) and c) See Table 3-3.
* Significantly different at $P<0.01$ as compared with nonpretreated control.

Table 3-7. Effect of pretreatment of supernatant from infected liver homogenate

Pretreatment [a] (0.2 ml, i.v., on day -2)	No. of mice	Disease score 0	1	2	3	4	5	DI [b]
Infected liver homogenate(1.5×10^5)								
Whole	10	1 [c]	3	2	4			1.9*
Supernatant	10	1	1	3	3	2		2.4
Normal liver homogenate, whole	10		1	3	5	1		2.6
-------	10			1	7	1	1	3.2

a) Before i.v. challenge with 2.0×10^6 organisms. b) and c) See Table 3-3.
* Significantly different at $P<0.01$ as compared with nonpretreated control.

Table 3-8. Effect of pretreatment of infected liver lysate

Pretreatment a) (0.2 ml, i.v., on day -2)	No. of mice	Disease score						DI b)
		0	1	2	3	4	5	
Infected liver lysate	10	3 c)	6	1				0.8*
Normal liver lysate	10			2	5	3		3.1
Distilled water	11	1	1	6	2	1		2.1
----------	10			3	5	1	1	3.0

a) Before i.v. challenge with 3.1×10^6 organisms. b) and c) See Table 3-3.
* Significantly different at $P<0.01$ as compared with other groups.

Table 3-9. Effect of LPS and BCG pretreatment

Pretreatment [a] (on day -2)	No.of mice	Disease score 0	1	2	3	4	5	DI [b]
LPS, 20µg, s.c.	10	7 [c]		3				0.6*
BCG, 16µg, s.c.	10	2	6	2				1.0**
BCG, 16µg, i.p.	11	2	9					0.8*
MSK, 1.1 x 10^5, i.v.	8	4	4					0.5*
----------	10		4	5	1			1.7

a) Before i.v. challenge with 2.6 x 10^6 organisms. b) and c) See Table 3-3.
*, ** Significantly different at $P<0.01$ and $P<0.05$, respectively, as compared with nonpretreated control.

Table 3-10. Effect of pretreatment of Poly[I]:Poly[C] and FCA

Pretreatment [a] (on day -2)	No.of mice	Disease score						DI [b]
		0	1	2	3	4	5	
Poly[I]:Poly[C], 8 µg, i.p.	10		1 [c]	4	5			2.4
FCA, 0.25 ml, s.c.	10		1	3	4	1	1	2.8
PBS, 0.2 ml, i.p.	10		2	2	4	2		2.6
MSK, 7.6×10^4, i.v.	10	1	8	1				1.0*
----------	10			3	4	3		3.0

a) Before i.v. challenge with 3.5×10^6 organisms. b) and c) See Table 3-3.
* Significantly different at $P<0.01$ as compared with nonpretreated control.

Table 3-11. Effect of inactivated MHV pretreatment

Pretreatment [a] (i.v., on day -2)	Challenge	No.of mice	Disease score 0	1	2	3	4	5	DI [b]
Inactivated MHV	+	10			4 [c]	3	3		2.9
	-	11	11						0.0*
MSK, 1.1 x 10^5	+	10	2	6	2				1.0*
----------	+	10			2	5	2	1	3.2

a) Before i.v. challenge with 2.1 x 10^6 organisms. b) and c) See Table 3-3.
* Significantly different at $P<0.01$ as compared with nonpretreated control.

CONCLUSION

Experimental Tyzzer's disease of mice was enhanced by administration of CY, showing prominent atrophy of the thymus and spleen. CY-treated mice remained serum antibody-negative 7 days after inoculation when most of them died, whereas, without the CY treatment, antibody incresed in titers on day 4 or later attaining a maximum level on days 7 to 15 (Part 1).

The disease was also aggravated in splenectomized mice and those treated with carrageenan, India ink or silica. Splenectomy performed 3 days before inoculation as well as reticuloendothelial blocking on the following day were the most effective. The course of infection did not differ between athymic nude mice and euthymic counterparts. Significant enhancement was observed in nude mice when they were splenectomized 6 days before and treated with carrageenan 1 day after inoculation as seen in cortisone-treated euthymic mice (Part 2).

Resistance to fatal challenge inoculation was induced in mice 1 to 4 days after treating with sublethal dose of infected mouse liver homogenate, while serum antibody became detectable on day 4 or later postchallenge. Similar precocious resistance probably due to macrophage activation could be induced by formol vaccine, bacterial lysate and LPS or BCG. Serum interferon remained undetectable in the resistant mice, and interferon-inducers such

as poly[I]:poly[C] or inactivated mouse hepatitis virus was unable to induce the resistance (Part 3).

The present results suggested that the phagocytic system might have important role(s) during the early phase of Tyzzer's disease in mice without detectable serum antibody production.

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