Precociously Induced Resistance to Tyzzer's Disease of Mice

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ABSTRACT. By pretreating mice with sublethal dose of mouse liver homogenate infected with Tyzzer's organism, resistance was precociously induced to fatal challenge inoculation made days 1 to 4 posttreatment. Serum antibody became detectable on day 4 postchallenge in both pretreated and challenged mice and serum interferon remained undetectable. Such resistance could be induced by pretreating with formol vaccine or lysate of the bacteria, lipopolysaccharide or BCG but not with Freund complete adjuvant, poly-inosinic: poly-cytidylic acid or inactivated mouse hepatitis virus.—**KEY WORDS:** immunopotentiator, mouse, Tyzzer's disease.

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Tyzzer's disease in mice is known to be enhanced by macrophage blocking [18], splenectomy [18], glucocorticoids [7] or cyclophosphamide [19], suggesting the importance of macrophage activities in early phase of the infection [18]. 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 [2, 6, 20].

In mouse hepatitis virus (MHV) infection, in which the target cells are also the hepatocytes, macrophage-related resistance can be induced against challenge with high-virulent virus shortly after pretreating with low-virulent virus [24].

This paper deals with precocious induction of resistance to challenge infection by either specific or non-specific pretreatment probably due to macrophage activation in Tyzzer's disease [16, 22].

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, U.S.A.), *Escherichia coli* 055:B5 lipopolysaccharide (LPS, Difco, U.S.A.) and lyophilized viable BCG vaccine (Japan BCG, Tokyo) were used as immunopotentiators [1, 16, 22], and poly-inosinic:poly-cytidylic acid (poly[I]:poly[C], Sigma Chemical Co., U.S.A.) were used as an interferon (IFN) inducer [8].

Tyzzer's organisms and formol 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 10⁶ MSK strain [4] of Tyzzer's organisms. For bacterial pretreating, 10² to 10⁵ organisms in 0.2 ml were given i.v. 1 to 4 days before challenge inoculation. As control, 1:2,000 homogenate of normal mouse liver was given i.v.

For preparation of bacterial lysate a 1:200 homogenate of the same infected mouse

liver was made in distilled water and allowed to stand for 60 min for bacteriolysis [5]. 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 μ m-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 [3], formalin was added to the infected liver homogenate at a final concentration of 1%. After incubation at 4°C for 3 days, the formalized 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 formol 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.

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 [9] 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 [4]: About 0.01 ml of mouse liver homogenate infected with MSK strain of Tyzzer's organisms was placed on a 76×26 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-conjugated anti-mouse IgG goat serum (Cappel, U.S.A.). The titer of anti-MSK IgG antibody was expressed by the highest dilution of test sera

showing specific fluorescence.

IFN assay [11]: 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 [18]. 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. 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 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

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

Inoculation (i.v.)		No. of mice	Time-to-death in days p.i.						
Days -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 hydrocortisone acetate s.c.

b) No. of cases showing indicated time-to-death.

Table 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) Observed on day 7 postchallenge.

b) No. of cases showing indicated severity.

Table 3. Effect of various dose of bacterial pretreatment

Pretreatment ^{a)} (i.v., on day -2)	No. of mice	Score						DI ^{b)}
		0	1	2	3	4	5	
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.

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 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 chal-

lenged with 1.7×10^6 organisms. As shown in Table 3, with any pretreatment dose examined 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). However, the DI of PBS-pretreated group was 1.7 and showed no significant difference as compared with that of control.

To evaluate time period required for the

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

Pretreatment (i.v.) ^{a)}	No. of mice	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 or after i.v. challenge with 1.4×10^6 organisms.

b) and c) See Table 3.

*, **, *** Significantly different at $p < 0.05$, $p < 0.02$ and $p < 0.01$, respectively, as compared with nonpretreated control.

Table 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
1.3×10^5	2.6×10^6	0 ^{a)} (25>) ^{b)}	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.

induction of precocious resistance to challenge, 4 groups of 9 to 10 mice each were pretreated i.v. with 8.1 to 9.9×10^4 organisms and 1 to 4 days later, they were challenged with 1.4×10^6 organisms. Another group of 10 mice was given 7.9×10^4 organisms 1 day after challenge. As shown in Table 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 value of non-pretreated control ($P < 0.05$), while postchallenge treating (DI=1.1) was not effective.

An experiment was done to see relationship between severity of liver lesions,

serum antibody titers or IFN values in pretreated and challenged mice. Two groups of 18 mice each received i.v. inoculation with 1.3×10^5 organisms and 2 days later, one of them and non-pretreated control mice were inoculated i.v. with 2.6×10^6 organisms. Three mice of each group were killed each day after inoculation and examined for liver lesions. Sera were collected from all cases and tested for anti-MSK IgG antibody titers and IFN.

As presented in Table 5, necrotized lesions in the liver appeared already on the next day of challenge inoculation and slowly developed with time in pretreated mice with or without challenge. They were undetect-

Table 6. Effect of formol vaccine pretreatment

Pretreatment ^{a)} (on day -2)	No. of mice	Score						DI ^{b)}
		0	1	2	3	4	5	
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 1.5×10⁶ organisms.

b) and c) See Table 3.

* Significantly different at p<0.01 as compared with nonpretreated control.

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

Pretreatment ^{a)} (0.2 ml, i.v., on day -2)	No. of mice	Score						DI ^{b)}
		0	1	2	3	4	5	
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 2.0×10⁶ organisms.

b) and c) See Table 3.

* Significantly different at p<0.01 as compared with nonpretreated control.

able on day 7 postchallenge in some cases. Anti-MSK IgG antibody was detected on day 4 postchallenge in these pretreated and challenged mice. In contrast, non-pretreated and challenged mice were shown to have liver lesions only on day 2 or later postchallenge, while 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, while IFN remained undetectable in cases of all groups tested.

The following experiment was done to see whether pretreatment with formol vaccine was effective. Three groups of 8 to 11 mice received s.c. or i.v. injection of 8.2×10⁵, 8.2×10⁴ and 8.2×10³ formalin-killed organisms, respectively. Another group of

10 mice was inoculated i.v. with 2.4×10⁴ live organisms. Two days later all these mice and those of non-treated controls received i.v. challenge with 1.5×10⁶ organisms. As presented in Table 6, those pretreated with formol vaccine as well as live organisms showed a significantly smaller 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 1.5×10⁵ MSK organisms, (2) supernatant after centrifugation at 10,000 rpm for 20 min from the same homogenate, or (3)

Table 8. Effect of pretreatment of infected liver lysate

Pretreatment ^{a)} (0.2 ml, i.v., on day -2)	No. of mice	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.

* Significantly different at $p < 0.01$ as compared with other groups.

Table 9. Effect of LPS and BCG pretreatment

Pretreatment ^{a)} (on day -2)	No. of mice	Score						DI ^{b)}
		0	1	2	3	4	5	
LPS, 20 μ g, s.c.	10	7 ^{c)}		3				0.6*
BCG, 16 μ g, s.c.	10	2	6	2				1.0**
BCG, 16 μ g, i.p.	11	2	9					0.8*
MSK, 1.1×10^5 , i.v.	8	4	4					0.5*
—	10		4	5	1			1.7

a) Before i.v. challenge with 2.6×10^6 organisms.

b) and c) See Table 3.

*, ** Significantly different at < 0.01 and $p < 0.05$, respectively, as compared with nonpretreated control.

normal mouse liver homogenate (1:2,000). Two days later all these groups of mice and another non-pretreated control group received i.v. challenge of 2.0×10^6 MSK organisms. As shown in Table 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 infected liver 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 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 μ g live BCG vaccine while another group s.c. injection of 20 μ g LPS. One of other 2 groups of 8 or 10 mice were injected i.v. with 1.1×10^5 MSK organisms while the other remained non-treated. Two days later, all these mice were exposed to i.v. challenge of 2.6×10^6 organisms. As shown in Table 9, LPS- and BCG-treated mice as well as those pretreated with viable organisms showed significantly smaller DI as compared with that of non-pretreated control ($P < 0.05$).

To see effect of either an IFN inducer poly[I]:poly[C] or an immunostimulator FCA, 4 groups of 10 mice each were given: (1) 8 μ g 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 controls received i.v. challenge with 3.5×10^6 organisms. As shown in Table 10, except for those pretreated with 7.4×10^4 bacteria, no signifi-

Table 10. Effect of pretreatment of poly-inosinic-poly-cytidylic acid and Freund complete adjuvant

Pretreatment ^{a)} (on day -2)	No. of mice	Score						DI ^{b)}
		0	1	2	3	4	5	
Poly[I]: poly[C], 8 μ g, 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.

* Significantly different at $p < 0.01$ as compared with nonpretreated control.

Table 11. Effect of inactivated MHV pretreatment

Pretreatment ^{a)} (i.v., on day -2)	Challenge	No. of mice	Score						DI ^{b)}
			0	1	2	3	4	5	
Inactivated MHV	+	10			4 ^{c)}	3	3		2.9
	—	11	11						0.0*
MSK, 1.1×10^5	+	10	2	6	2				1.0*
—	+	10			2	5	2	1	3.2

a) Before i.v. challenge with 2.1×10^6 organisms.

b) and c) See Table 3.

* Significantly different at $p < 0.01$ as compared with nonpretreated control.

cant protective effect was observed. As presented in Table 11, any protective effect was not shown by pretreating with MHV, which is known to be an inducer of IFN [10] as well as a macrophage activator [24, 25].

DISCUSSION

The present study revealed that mice were protected from challenge infection which was made 2 or 3 days after primary 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 the previous report [19]. Pretreating with 10^2 to 10^4 bacteria was effective enough, though the higher dose of bacteria was more effective.

Similar precocious resistance was also obtained by formol 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 infected mouse liver homogenate was not effective. BCG vaccine [16], LPS [22] or muramyl dipeptide (MDP), a synthesized adjuvant [15, 21] 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 *Tetrahymena pyriformis* [13, 14] and *Lactobacillus casei* [17, 23] was also

reported in infections with toxoplasma and pseudomonas or listeria. 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 [24]. 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 [18], and possibility of indirect inhibition of bacterial growth within hepatocytes by macrophages during early phase of infection was proposed [18]. On the other hand, the severity of Tyzzer's disease was found to depend closely upon protein metabolism of host hepatocytes [12]. West *et al.* [27] suggested a close relationship between protein metabolism within hepatocytes and macrophage activities, since mouse hepatocytes showed decreased ^3H -leucine incorporation when cocultured with peritoneal cells from mice treated with either LPS, MDP or *Escherichia coli* killed by gentamycin. After pretreating with 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 [26], 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 undetectable in sera from pretreated mice which resisted to challenge infection. The pretreatment with IFN inducers, poly[I]:poly[C] [8] or also MHV [10] did not

induce any protective effect to challenge infection.

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要 約

マウス Tyzzer 病感染早期に発現する抵抗性：中山裕之・二井愛介・久和 茂¹⁾・寺西宗広・井上 智・藤原公策（東京大学農学部家畜病理学教室，¹⁾医科学研究所）——マウスに Tyzzer 菌接種後 1～4 日に大量菌攻撃に対する抵抗性がみられた。攻撃時に血清抗体は検出されず，攻撃後 4 日に検出された。インターフェロンは攻撃後 7 日までは検出されなかった。このような前処置効果はホルマリン死菌，溶菌上清，lipopolysaccharide，BCG によっても誘導されたが，Freund complete adjuvant，poly-inosinic:poly-cytidylic acid，不活化マウス肝炎ウイルス処置などでは誘導できなかった。