

## Biphasic, Organ-Specific, and Strain-Specific Accumulation of Platelets Induced in Mice by a Lipopolysaccharide from *Escherichia coli* and Its Possible Involvement in Shock

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Platelets contain a large amount of 5-hydroxytryptamine (5HT, serotonin). Intravenous injection into BALB/c mice of a Boivin's preparation of lipopolysaccharide (LPS) from *Escherichia coli* induced rapid 5HT accumulation in the lung (within 5 min) and slow 5HT accumulation in the liver (2 to 5 h later). The rapid response required high doses of LPS (more than 0.1 mg/kg). On the basis of 5HT measurements, 70% or more of the platelets which disappeared from the blood appeared to have accumulated rapidly in the lung, and a large number of platelets were found there by electron microscopy. A shock, which was manifested by crawling, convulsion, or prostration, followed shortly after the rapid accumulation of 5HT in the lung. On the other hand, the slow accumulation of 5HT in the liver could be induced by much lower doses of LPS (1 µg/kg or less), even when given by intraperitoneal injection. This 5HT accumulation appears to be a reflection of platelet accumulation in the liver (Y. Endo and M. Nakamura, Br. J. Pharmacol. 105:613–619, 1992). The combination of a low dose of LPS with D-galactosamine amplified the hepatic accumulation of 5HT, and the mice developed a severe hepatic congestion resulting in death. The rapid response was not induced at all in C3H/HeN mice. These results and comparison with other LPS preparations indicate that some component(s) of LPS from *E. coli* induces a biphasic, organ-specific and strain-specific accumulation of platelets, and it is proposed that this effect is involved in the development of shock.

5-Hydroxytryptamine (5HT, serotonin) is located largely in enterochromaffin cells of the gastrointestinal tract, most of the remainder being present in platelets and in the brain (11). The blood (i.e., platelets) of the mouse contain a large amount of 5HT (1, 7). By measuring 5HT, therefore, it is possible to assess the translocation of platelets from blood to tissues.

The usefulness of this method as a means of investigating the *in vivo* behavior of platelets has been shown by our previous finding that the platelets of mice slowly translocate to the liver in response to small intraperitoneal doses of a lipopolysaccharide (LPS) from *Escherichia coli* (7, 8). The translocation or accumulation of platelets in the liver, as estimated by measuring 5HT, begins after 1 to 2 h and reaches its maximum within 5 to 6 h of the injection of the LPS.

On the other hand, as reviewed by Morrison and Ulevitch (12), it has been shown that intravenous injection of a large dose of an LPS induces a biphasic disappearance of <sup>51</sup>Cr-labeled platelets from the blood of rabbits and dogs: there is an initial, rapid disappearance within a few minutes and then a slow disappearance lasting for several hours. Electron microscopic analysis has demonstrated that the rapid platelet drop accompanies a local platelet aggregation in pulmonary and hepatic capillaries and that there is extensive platelet destruction (12). However, such a local aggregation has not been assessed quantitatively.

Given the background described above, we tested quantitatively the effect of an LPS preparation derived from *E. coli* on platelet behavior *in vivo* by measuring 5HT. In addition, because intravenous injection of a large dose of an LPS induces a rapidly occurring shock (14), and intraperitoneal injection of a small dose of an LPS combined with D-galactosamine (GalN)

induces a delayed shock (10), we compared the effects of these two types of LPS-induced shock on platelets.

### MATERIALS AND METHODS

**Animals and materials.** BALB/c and C3H/HeN mice (male, 6 weeks old) raised in the Mouse Center of our university were used. All experiments complied with the *Guideline for Care and Use of Laboratory Animals in Tohoku University*. LPS prepared from *E. coli* O55:B5 by Boivin's method (trichloroacetic acid extraction) was purchased from Difco Laboratories (Detroit, Mich.). LPS prepared from the same bacterium by Westphal's method (phenol-water extraction, protein content less than 3%) (17), detoxified LPS (delipidized and chromatographically purified) prepared from the same bacterium by the method of Ding et al. (2), and D-galactosamine (GalN) were all purchased from Sigma Chemical Co. (St. Louis, Mo.). These LPS samples were dissolved in sterile saline and intravenously or intraperitoneally injected (0.1 ml/10 g of body weight). GalN was dissolved in sterile saline, and its pH was adjusted to 7 with NaOH solution. The mixture of the GalN solution and LPS solution was injected intraperitoneally as described in Results.

**Determination of 5HT.** Blood samples (4 or 5 drops) were collected into preweighed test tubes containing 3 ml of 0.4 M HClO<sub>4</sub>-0.1% cysteine-HCl-2 mM Na<sub>2</sub>EDTA. After being weighed, the tube was cooled in an ice bath. The tissues were rapidly removed and kept in a jar with dry ice until needed. 5HT in the blood was determined on the day the blood was collected, because 5HT is unstable in the blood. 5HT in the tissues and blood was measured as previously described (7).

**Electron microscopy.** Immediately after decapitation, tissues were rapidly removed and electron microscopic analysis carried out as described previously (7).

**Statistical analysis.** The statistical significance of differences was analyzed by using the Dunnett multiple-comparisons test, and *P* values of less than 0.05 were considered to be significant.

### RESULTS

**Time course of changes in 5HT.** Intravenous injection of LPS into BALB/c mice induced a rapid change in 5HT levels which followed a characteristic time course in each tissue (Fig. 1).

**(i) Blood.** There was a rapid fall in the 5HT level, and the maximum decline had occurred within 5 min of the injection of LPS. Thereafter, the 5HT level recovered to an extent that was larger when the dose of LPS was smaller (data not shown).

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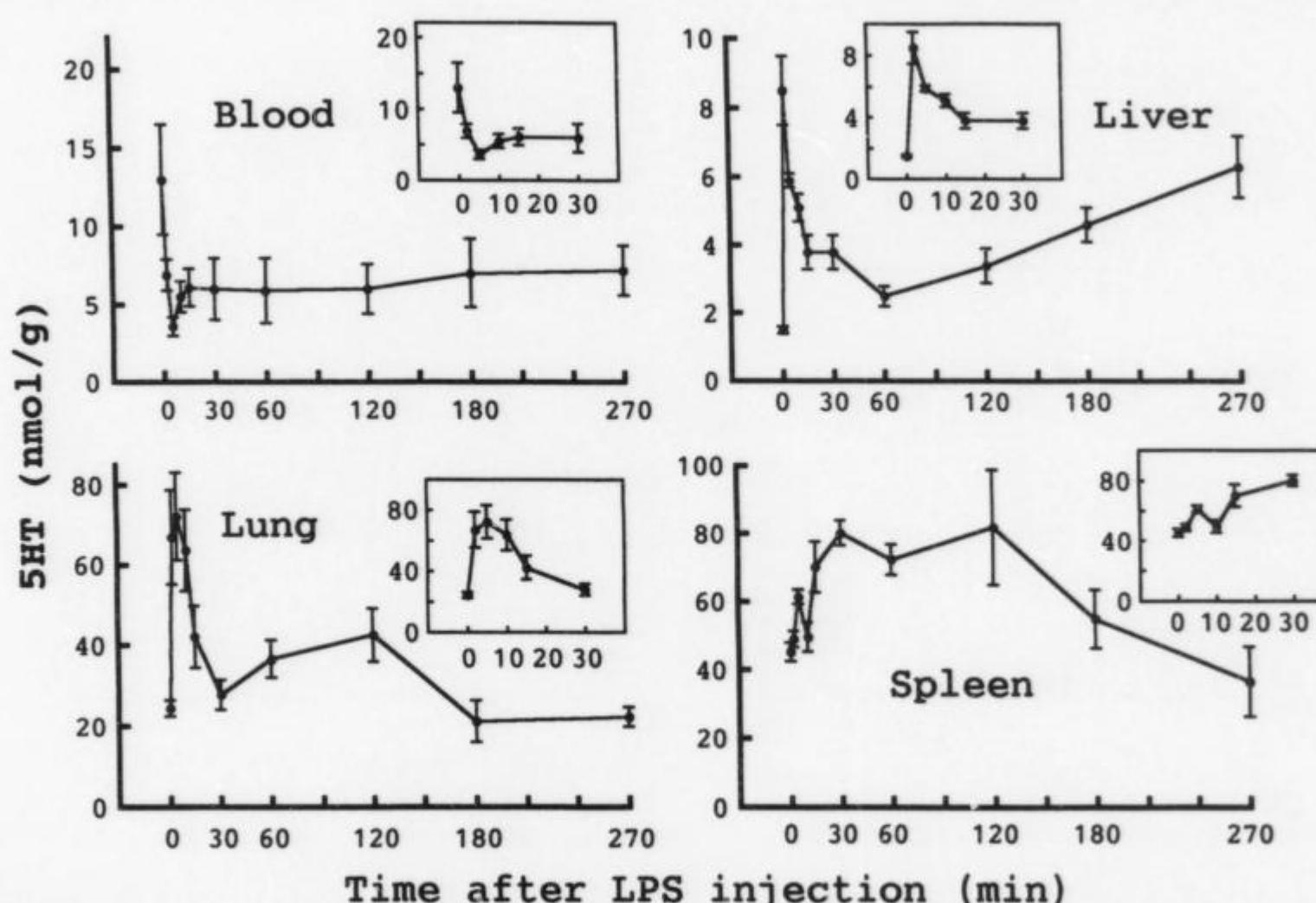


FIG. 1. Time course of changes in 5HT induced by LPS. Boivin's preparation of *E. coli* LPS (0.5 mg/kg; 12.5 µg/mouse) was intravenously injected into mice, and the blood and tissues were collected or removed at the time indicated. Each value is the mean  $\pm$  standard deviation from four or five mice.

(ii) **Lung.** As the 5HT in the blood fell, there was a corresponding rapid elevation in lung 5HT. At its peak (about 70 nmol/g), the increase in lung 5HT was the highest recorded among the tissues tested. The level of 5HT decreased to its control level within 30 min, then rose slightly but reproducibly again, and finally returned to a steady level 3 h later. At present, we are unable to explain this second small rise.

(iii) **Liver.** As in the lung, there was a rapid rise and fall in 5HT. After this rapid response, there was a slowly developing increase. The 5HT level reached a plateau 3 to 5 h after LPS injection and returned to its normal level within 1 to 2 days.

(iv) **Spleen.** Rather than a rapid elevation of the type seen in the lung and liver, there was a more delayed and more prolonged elevation. The timing of this increase seemed to correspond to the timing of the rapid fall in the lung and liver.

(v) **Kidney and intestine (whole thickness).** No significant variations in 5HT were observed (data not shown).

**Dose dependency of the rapid 5HT fluctuations.** The effects induced in BALB/c mice by various doses of LPS were examined at 8 min after injection (Fig. 2). The fall in the blood and the rise in the lung were reciprocally dose dependent. The rapid rise in the liver had reached its maximum with a dose of 1 to 2 mg/kg. There was no significant effect in the spleen at this time point.

**Electron microscopic analysis.** There was a large number of aggregated platelets in the capillaries of the lungs removed 2 and 8 min after injection of LPS (Fig. 3). At 8 min, when shock had been developed, there were many degranulated platelets along the capillary walls (Fig. 3B).

**The rapid shock induced by large doses of LPS.** The *E. coli* LPS induced a dose-dependent shock which started 6 to 15 min after intravenous injection; i.e., mice exhibited crawling, convulsion, or prostration (Table 1). Three of five mice died after receiving a dose of 1 mg/kg, death occurring within 1 h of the injection, but no deaths occurred at lower doses.

**Dose dependency of the slow 5HT accumulation in the liver.** Although a rapid accumulation of 5HT in the lung required a high dose of LPS (more than 0.1 mg/kg), as described above, the slow 5HT accumulation in the liver was maximal with as little as 1 µg/kg, if the dose was given intravenously (Table 2). A slow accumulation of 5HT was induced even by intraperitoneal injection of LPS, though maximal accumulation required at least 100 µg/kg by this route. Intraperitoneal injection of LPS, even at 1 mg/kg, did not induce a rapid 5HT accumulation in the lung, spleen, kidney, or intestine (data not shown).

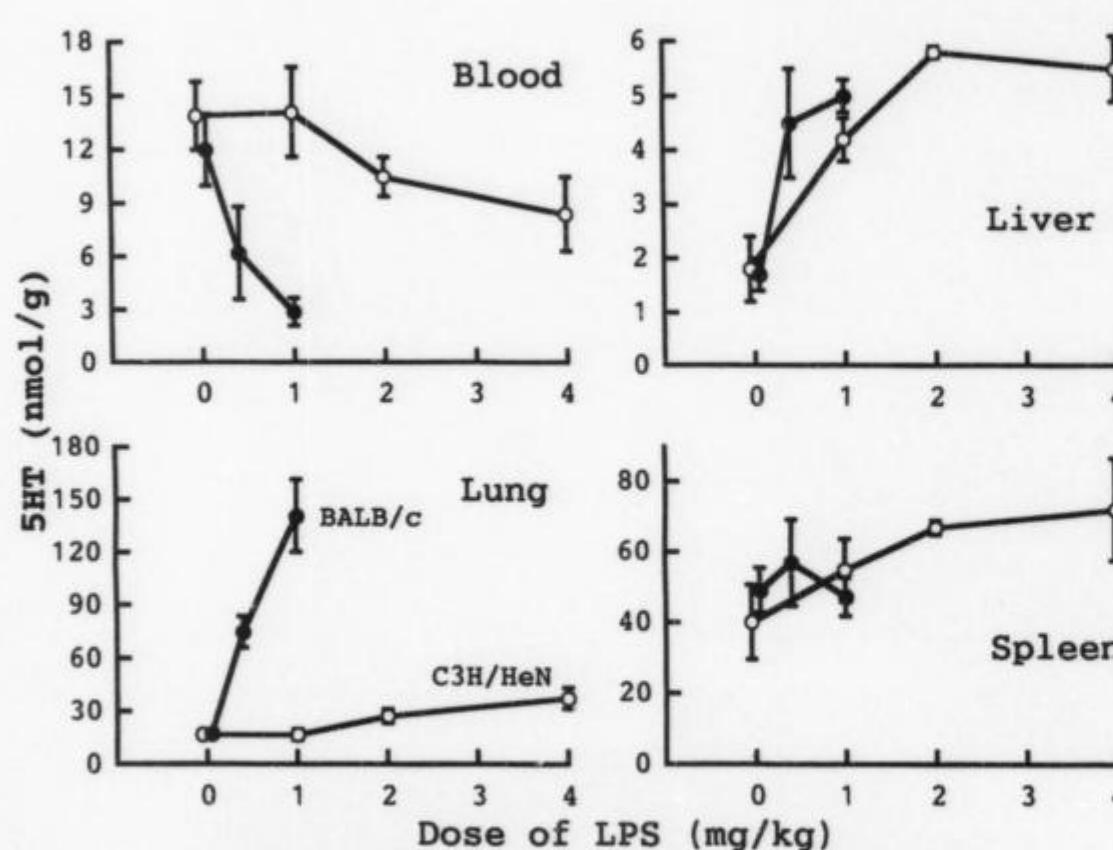


FIG. 2. Dose dependency of the rapid change in 5HT induced by LPS. Various doses of Boivin's preparation of *E. coli* LPS were intravenously injected into mice, and the blood and tissues were collected or removed 8 min later. Each value is the mean  $\pm$  standard deviation from five mice. Filled circles, BALB/c mice; open circles, C3H/HeN mice.

TABLE 1. Dose dependency of the shock induced rapidly after LPS injection

LPS dose (mg/kg)	Incidence of shock <sup>a</sup> (within 15 min)	Mortality (within 60 min)
0.25	0/4	0/4
0.5	6/6	0/6
1.0	5/5	3/5

<sup>a</sup> Assessed by the development of crawling, convulsion, or prostration.

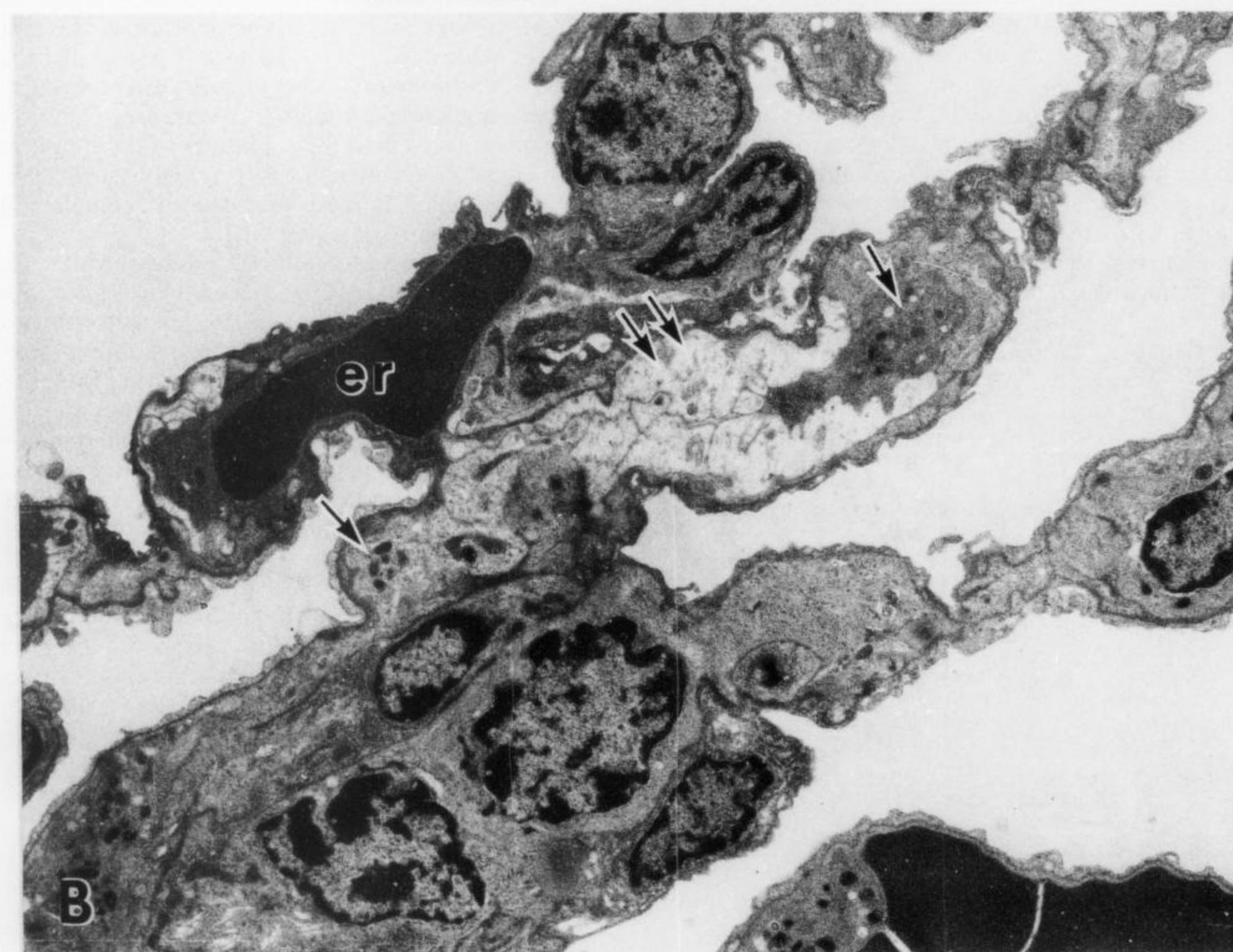
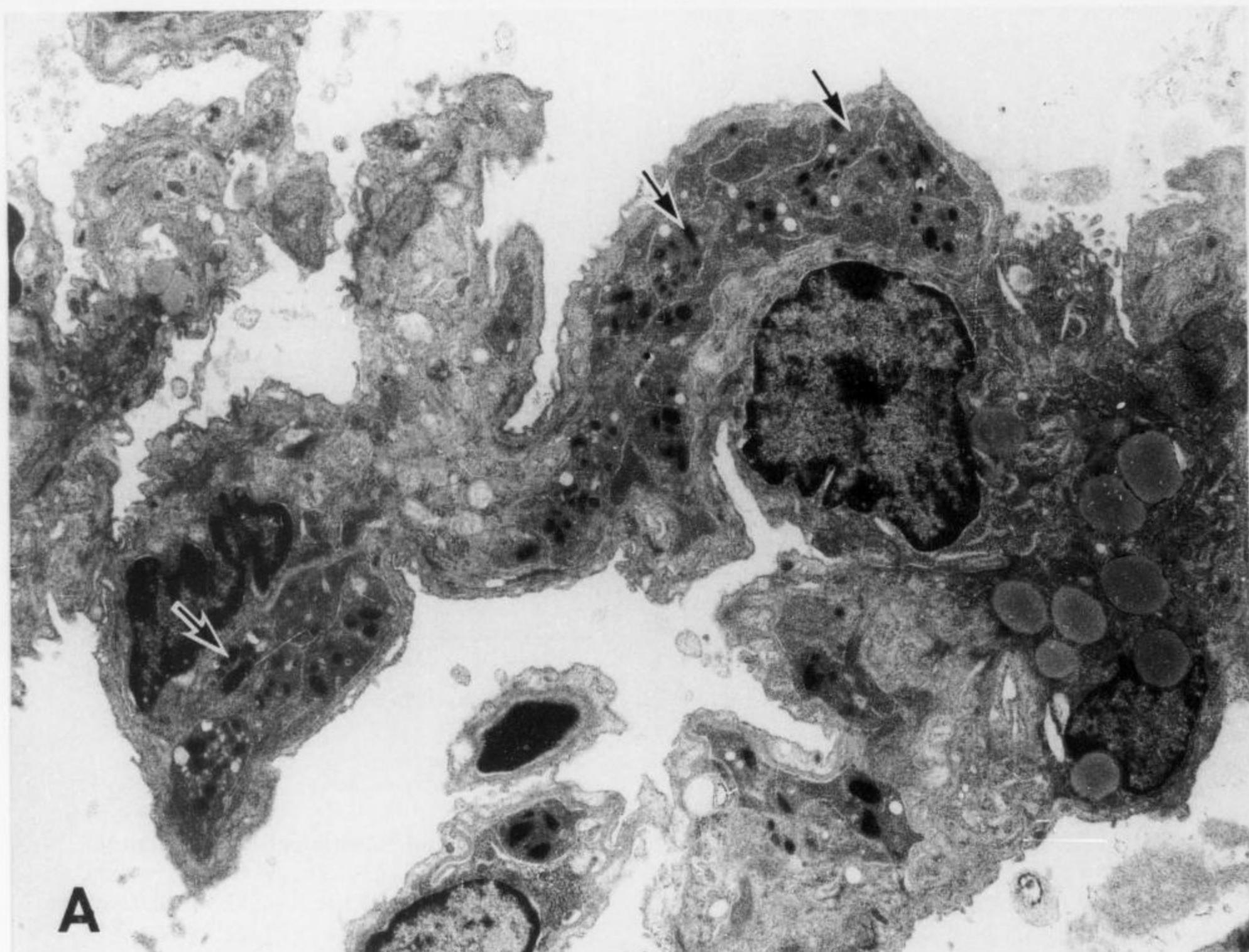


FIG. 3. Electron micrographs of lung tissues from mice injected intravenously with Boivin's preparation of *E. coli* LPS (1 mg/kg; 25 µg/mouse). (A) Lung removed after 2 min (before shock). Inside the blood vessels, many platelets (arrows) which contain granules are aggregated. (B) Lung removed after 8 min (during shock). There are many platelets which had been degranulated (double arrow), some nondegranulated platelets (single arrows) and an erythrocyte (er).

TABLE 2. Dose dependency of the slow 5HT accumulation in the liver

LPS dose ( $\mu\text{g}/\text{kg}$ )	Hepatic 5HT accumulation at 5 h <sup>a</sup>	
	i.v. injection	i.p. injection
0	2.1 ± 0.3	1.8 ± 0.3
1	5.8 ± 0.8*	1.5 ± 0.1
10	5.2 ± 0.4*	3.2 ± 0.6**
100	5.3 ± 0.3*	5.4 ± 1.4*
1,000	ND <sup>b</sup>	6.3 ± 0.3*

<sup>a</sup> Mice were intravenously (i.v.) or intraperitoneally (i.p.) injected with various doses of LPS, and their livers were removed 5 h later. Each value is the mean ± standard deviation from four mice. \*,  $P < 0.01$ ; \*\*,  $P < 0.05$  versus dose 0.

<sup>b</sup> ND, not determined.

**Estimation of the magnitude of the platelet accumulation in lung and liver.** The amount of whole blood in normal mice has been shown to represent about 7% of the body weight (19). Therefore, from the data in Fig. 2 for BALB/c mice at an LPS dose of 1 mg/kg, and taking the mean body weight of the mice as 25 g and the mean weight of the lung as 0.10 g, the following values can be calculated: weight of whole blood =  $25 \times 0.07 = 1.75$  g; total 5HT in the blood before LPS =  $1.75 \times 12 = 21.0$  nmol; 5HT lost from the blood =  $(12 - 3) \times 1.75 = 15.8$  nmol; and 5HT accumulated in the lung =  $(140 - 15) \times 0.10 = 12.5$  nmol. Thus, 5HT accumulated in the lung represents 60% of the total 5HT in the blood and 79% of the 5HT lost from the blood. Similarly, the total 5HT accumulated in the liver (liver weight, about 1.4 g) amounts to 4.9 nmol, representing about 31% of the 5HT lost from the blood. These values must be considered as approximations, because  $79 + 31 = 110\%$ . In any case, these values clearly indicate that the platelets lost from the blood largely translocate to the lung.

**The shock induced slowly by GalN plus a low dose of LPS.** A combined intraperitoneal injection of GalN (800 mg/kg) and LPS (10  $\mu\text{g}/\text{kg}$ ) induced a lethal shock. The signs of shock appeared suddenly 9 to 11 h after the injection, and most of the mice had died within a further 1 h. There was a severe congestion in the liver of the dead mice. The effect of GalN on the 5HT level in the liver was examined at 8.5 h after injection, a time at which there was no sign of shock (Table 3). GalN itself did not increase liver 5HT. In the mice in which congestion had already occurred by combined administration of GalN and LPS, there was a marked accumulation of 5HT in the liver. However, in the mice in which congestion had not yet occurred, there was no such a marked 5HT elevation. There was no 5HT increase in the lung, spleen, kidney, or intestine in all of the groups of mice (data not shown).

**Comparison of different preparations of LPS from *E. coli*.** The LPS used in the above experiments was that prepared

TABLE 3. Potentiation by GalN of the slow 5HT accumulation in the liver

Treatment	Hepatic 5HT (nmol/g) <sup>a</sup>	Hepatic congestion
Saline	1.7 ± 0.4	No
GalN	1.7 ± 0.1	No
LPS	2.6 ± 0.3	No
LPS + GalN	2.9 ± 0.7**	No
LPS + GalN	8.5 ± 0.8*#	Yes

<sup>a</sup> Mice were intraperitoneally injected with saline (5 mice), GalN (800 mg/kg; 5 mice), LPS (10  $\mu\text{g}/\text{kg}$ ; 5 mice), or a mixture of GalN and LPS at the same doses (10 mice). Some 8.5 h later, a time at which there was no sign of shock, the mice were killed and their livers were removed. The livers in the LPS + GalN group were separated into two groups, depending on whether there was congestion (four mice) or no congestion (six mice). Each value is the mean ± standard deviation from four to six mice. \*,  $P < 0.01$ ; \*\*,  $P < 0.05$  versus the saline group. #,  $P < 0.01$  versus the no-congestion group in the LPS + GalN group.

TABLE 4. Comparison of effects of various LPS preparations from *E. coli* O55:B5

Prepn	Dose ( $\mu\text{g}/\text{kg}$ )	5HT accumulation (nmol/g) <sup>a</sup>	
		Lung (7 min)	Liver (5 h)
Saline		17.2 ± 2.1 (10 <sup>b</sup> )	1.5 ± 0.2 (10)
Delipidized LPS	0.05	ND <sup>c</sup>	2.4 ± 0.1** (5)
	0.5	24.3 ± 6.4 (5)	ND
	5.0	15.5 ± 5.2 (5)	ND
Westphal's LPS	0.05	ND	3.3 ± 0.8* (5)
	1.0	33.0 ± 7.9* (5)	4.0 ± 0.9* (5)
Boivin's LPS	0.05	20.0 ± 3.0 (5)	5.6 ± 0.6* (5)
	0.5	81.0 ± 10.2* (5)	4.5 ± 0.5* (5)
	1.0	140.0 ± 20.6* (4)	ND

<sup>a</sup> One of the preparations of LPS was injected intravenously, and the mice were killed 7 min or 5 h later. Each value is the mean ± standard deviation from 4 to 10 mice. \*,  $P < 0.01$ ; \*\*,  $P < 0.05$  versus the saline group.

<sup>b</sup> Number of mice.

<sup>c</sup> ND, not determined.

from *E. coli* O55:B5 by Boivin's method. Subsequently, we compared the activities of LPS prepared from the same bacterium by different methods (Table 4). The delipidized LPS was ineffective in inducing a rapid 5HT accumulation in the lung (at 7 min). Although this LPS did induce a slow 5HT accumulation in the liver (at 5 h), its effect was smaller than those of the other two preparations. The ability of Westphal's preparation to induce a rapid 5HT accumulation in the lung was much less than that of Boivin's preparation, but there was no such a marked difference in their ability to induce a slow 5HT accumulation in the liver.

**Comparison of the platelet response to LPS in BALB/c and C3H/HeN mice.** Takada et al. have shown that intravenous injection of some kinds of LPS produces a rapid shock in C3H/HeN mice but not in BALB/c mice (15). Therefore, we tested the effect of *E. coli* LPS on C3H/HeN mice. The rapid platelet response to Boivin's *E. coli* LPS was very poor or absent in C3H/HeN mice (Fig. 2). Moreover, in contrast to the result described by Takada et al., this LPS failed to induce the rapid shock in C3H/HeN mice, even at a dose as large as 8 mg/kg. However, no such a marked difference was seen in the slow platelet response (data not shown).

## DISCUSSION

On the basis of 5HT measurement, we were able to estimate quantitatively the magnitude of the translocation of platelets from blood to tissues. Our results indicate that intravenous injection of a large dose of LPS induces a translocation of platelets largely to the lung, although there was a small accumulation in the liver as well.

It is recognized that the 5HT in platelets is released during platelet aggregation. Indeed, the electron micrographs confirmed the presence of many degranulated platelets in the lung. Therefore, the rapid fall of the elevated level of 5HT may be a reflection of the release of 5HT from the aggregated platelets. From the delayed time course of the rise and fall of 5HT in the spleen (Fig. 1), it is also likely that some of the platelets that accumulated in the lung and liver may be removed and subsequently trapped in the spleen.

Shortly after the rapid rise and decline in 5HT in the lung and liver, a shock developed. The dose dependency of the shock and its severity both corresponded well to those of the rapid accumulation of platelets in the lung. 5HT is known to

cause both potent arteriolar vasodilation and coronary chemoreflex (Bezold-Jarisch reflex; inhibition of sympathetic outflow and activation of cardiac vagus) leading to profound hypotension and bradycardia (11). Indeed, Wiggins et al. (18) have reported evidence suggesting that the acute hypotension in rabbits that is induced by the intravenous injection of a large amount of dextran sulfate results from a chemoreflex evoked by the 5HT released from platelets accumulated in the lung. Therefore, our results strongly suggest that 5HT derived from platelets accumulated in the lung is involved in the development of the rapid shock, although it is possible that other components of platelets are also involved. Takada and Galanos (14) found that in mice pretreated with a muramyl dipeptide, some kinds of LPS produced an anaphylactoid shock within 10 to 20 min of the intravenous injection of large doses. Later, Takada et al. found that this anaphylactoid shock was suppressed by an antagonist of 5HT (15). Although they did not refer to platelets, their results well support the involvement of platelets in the development of the rapid shock.

The rapid platelet response to Boivin's *E. coli* LPS was particularly marked in the lung of BALB/c mice but extremely weak in C3H/HeN mice, in whom the rapid shock was not induced, either. These results clearly indicate that the action of LPS is due neither to a simple physicochemical property of the LPS nor to a physicochemical interaction between the LPS and platelets and depends on genetically controlled factors. BALB/c and C3H/HeN mice have different genetic backgrounds, i.e., *H-2<sup>d</sup>* and *H-2<sup>k</sup>* haplotypes, respectively. At present, however, there are no available data capable of explaining this phenomenon at the molecular level.

Injection of LPS also induces a slowly developing increase in 5HT in the liver. Our previous and present results indicate that this slow increase is specific to the liver and can be induced by much lower doses of LPS (3, 4). Similar 5HT increases are also induced by interleukin-1 and tumor necrosis factor (5, 9). Recently, we established that this liver-specific accumulation of 5HT is a reflection of the accumulation of platelets (7, 8). This slow platelet response is induced even by an intraperitoneal injection of LPS (Table 2), suggesting that it is not due to a direct action of LPS on the platelets but may be mediated by interleukin-1 and/or tumor necrosis factor.

GalN is an inhibitor of mRNA synthesis in the liver. GalN, when administrated to mice in combination with a low dose of either LPS or tumor necrosis factor, produces a shock and death with accompanying hepatic failure (6, 10, 16) and severe congestion in the liver (6). In contrast to the rapid shock described above, the shock induced by LPS plus GalN occurs at a later period (see Results). Although its detailed mechanism has not been clarified, an impaired production of some kinds of protein, which are formed via mRNA synthesis in the liver, has been suggested to be implicated in this phenomenon (7). On the other hand, Piguet et al. (13) showed that the depletion of platelets with antiplatelet antibody afforded significant protection against the mortality induced by LPS plus GalN. In the present study, it was shown that the accumulation of platelets in the liver precedes the shock, and there was a marked accumulation of 5HT or platelets in the liver with congestion. Therefore, the accumulation of platelets in the liver might be involved, at least in part, in the development of the shock in the presence of GalN, although the direct link of the platelet response to the development of the shock needs further investigation.

The ability of the Boivin's preparation to induce the rapid 5HT accumulation was much greater than that of the West-

phal's preparation, although there was no such marked difference in their abilities to induce the slow 5HT accumulation. Therefore, the active component(s) capable of inducing the rapid and slow platelet responses may be different from each other. However, the results for delipidized LPS suggest that the lipid structure of LPS may be important for inducing these responses.

Since platelets contain a variety of biologically active components, such as 5HT, histamine, catecholamines, ATP, hepatocyte growth factor, and transforming growth factor, our finding may open a new field of study on the role of platelets in immune responses. Finally, it is worth emphasizing the usefulness of measurements of 5HT as a tool for following the translocation of platelets in vivo.

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