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Platelet responses and anaphylaxis-like shock induced in mice by intravenous injection of whole cells of oral streptococci

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Intravenous injection of lyophilized whole cells of various oral streptococcal strains into muramyldipeptide (MDP)-primed C3H/HeN mice induces rapid anaphylactoid shock. Here we examined the mechanism underlying this shock. In non-primed mice, *Streptococcus intermedius* K-213K (SiK213) and *Streptococcus constellatus* T21 (ScT21) produced little or no sign of shock. In MDP-primed mice, SiK213 caused lethal shock, while ScT21 only had a weak effect. SiK213 induced decreases in blood platelets and 5-hydroxytryptamine (5HT) preceding the shock, while the effects of ScT21 were weak. The SiK213-induced 5HT decrease and shock were reduced by a complement-C5 inhibitor. These results suggest that (i) streptococcal bacterial cells can induce rapid platelet responses, (ii) complement-dependent degradation of platelets may be involved in streptococcus-induced shock, (iii) the streptococcus-induced platelet degradation or degranulation may occur largely in the systemic circulation, and (iv) platelets may play a role not only in infectious diseases caused by gram-negative bacteria, but also in diseases caused by gram-positive bacteria.

Key words: complement; 5-hydroxytryptamine (serotonin); platelets; shock; streptococci

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The *Streptococcus anginosus* (previously *Streptococcus milleri*) group consists of *S. anginosus*, *Streptococcus constellatus*, and *Streptococcus intermedius* (4). They are common inhabitants of the human mouth, and are implicated in oral and systemic suppurative infections. They are also an important cause of endocarditis (5). Interestingly, Isobe (9) found that lyophilized whole cells of many strains (106 out of 109 strains) of streptococci (including those obtained from the human mouth) induce a rapid anaphylaxis-like shock when injected intravenously into MDP-primed C3H/HeN mice. However, the mechanism has not been identified.

Intravenous injection into mice of certain types of lipopolysaccharide (lipopolysaccharide, a component of gram-negative bacterial cell wall) induces a rapid and profound decline in platelets in the blood, their extensive accumulation in the lung and liver, and their degranulation or degradation, leading to rapid anaphylaxis-like shock (2, 13, 14). These platelet responses to lipopolysaccharide are mediated by the complement system (14, 21). In the above studies, the *in vivo* translocation of platelets was quantitatively estimated by measuring 5-hydroxytryptamine (5HT, or serotonin).

In mice pretreated with synthetic peptidoglycan fragments, such as *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP), an intravenous injection of an lipopolysaccharide from some sources results in a high incidence of rapid anaphylaxis-like shock (15, 16). It is intriguing that just such rapid shock also occurs in MDP-primed C3H/HeJ mice, a well-known strain that is resistant to ''endotoxin shock'' (16). Recently, the rapid anaphylaxis-like shock induced by lipopolysaccharide in MDP-primed mice was also shown to be mediated by the complement-dependent degradation of platelets (10).

In view of the above, we decided to examine the effects induced by intravenous injection of lyophilized whole cells of streptococci on platelets in MDP-primed and non-primed mice. We did this by measuring the blood platelet count and the levels of 5HT in blood, lung, and liver. In this study, we used two strains of bacteria, *S. intermedius* K-213K (SiK213) and *S. constellatus*

T21 (ScT21). In terms of their ability to induce rapid shock, the former is potent and the latter much weaker (9). We then compared the effects induced by these bacteria with those of an lipopolysaccharide from *Prevotella intermedia*, a gram-negative oral bacterium. This lipopolysaccharide can induce the above-mentioned platelet response and rapid shock in C3H/HeN mice, and these effects are both augmented by MDP (2).

Material and methods

Mice

C3H/HeN mice (male, 6–7 weeks old) were obtained from SLC Japan (Shizuoka, Japan). All experiments complied with the Guidelines for Care and Use of Laboratory Animals in Tohoku University.

Streptococci and lipopolysaccharides

S. intermedius K-213K (SiK213) (with strong shock-inducing activity) and *S. constellatus* T21 (ScT21) (with weak shock-inducing activity) were cultured anaerobically (80% N_2 – 10% CO_2 – 10% H_2) in brain-heart infusion broth (BBL Microbiology Systems, Becton Dickinson, Cockeysville, MD) at 37°C for 18 h. After this culture, bacterial cells were harvested by centrifugation (10,000 × *g*, for 10 min), washed twice with distilled water, and lyophilized. Lipopolysaccharide from *P. intermedia* ATCC 25611 was prepared by the phenol-chloroform-petroleum-ether method, as described previously (8). The protein contamination is 5.2 μg/mg in the dried preparation (14). All experiments were carried out at 26–28°C. An anti-complement agent, K76 monocarboxylic acid (7), was provided by Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan). This agent was dissolved in saline with the addition of enough NaOH solution to bring the pH to about 7.5. MDP was provided by Daiichi Pharmaceutical Co. (Tokyo, Japan). Test samples were dispersed in sterile saline with a vortex mixer, and injected intravenously (0.1 ml/10 g body weight).

Platelet count

Two or three drops of blood from each decapitated mouse (unanesthetized) were collected directly into a pre-weighed test tube containing 1.0 ml of 4 mM EDTA in 0.01 M phosphate-buffered saline (pH 7.0). The tube plus blood was weighed, and the volume of blood estimated from the weight of the blood. The number of platelets was then ascertained using a cell counter (Sysmex SF-3000; Toa Medical Electronics Co. Ltd., Kobe, Japan).

Measurement of 5HT in the blood and tissues

After collecting the blood required for measuring the platelet count, we collected the next one or two drops of blood from the same mouse into another pre-weighed test tube containing 3 ml of 0.4 M $HClO_4$, 0.1% *N*-acetyl cysteine-HCl, and 2 mM EDTA-2Na. After the tubes had been weighed, they were cooled in an ice-bath until needed. The lung and liver were rapidly removed and kept in a jar with dry ice until needed. The determination of the 5HT level in the blood was carried out soon after the blood was collected, while the 5HT levels in the liver and lung were determined within 3 days of collection. After 5HT had been separated by column chromatography, it was measured fluorometrically as previously described (1). The values for 5HT levels reported here are somewhat lower than those shown in our previous reports. This is due to the fact that in the present study we used a newly purchased standard 5HT, while in previous studies we used one purchased at least 10 years ago.

Scoring of rapid shock

The incidence of the rapid shock and the score given for its severity were recorded as described previously (14). The scoring of the severity of the shock was as follows: 0 (no signs of shock), 1 (staggering), 2 (crawling and prostration), 3 (prostration and weak convulsions), 4 (prostration and strong convulsions), and 5 (death).

Statistical analysis

Experimental values for 5HT levels and platelet count are given as mean ± standard deviation (SD). The statistical significance of differences was assessed using a Student's unpaired *t*-test to compare two groups or ANOVA to compare data obtained at various times after injection of SiK213, ScT21, or lipopolysaccharide. *P*-values of less than 0.05 indicated a significant difference.

Results

Abilities of SiK213 and ScT21 to induce rapid anaphylaxis-like shock

First, we confirmed the findings made by Isobe (9). At 10 mg/kg, ScT21 produced no clear signs of shock in MDP-primed or non-primed mice (Table 1), while SiK213 produced only weak signs of shock in non-primed mice, but lethal shock in MDP-primed mice. After injection of SiK213 in MDP-primed mice, signs of anaphylaxis-like shock began to occur after 5–7 min, with death occurring after 15–60 min.

Effects of SiK213 on platelet count and 5HT in blood, lung, and liver

The effects of SiK213, a potent inducer of rapid shock, on the blood platelet count and the 5HT levels in the blood, lung, and liver in MDP-primed and non-primed mice are shown in Fig. 1. MDP itself produced no significant changes in the platelet count in the blood or in the 5HT levels in blood and lung, although the 5HT level in the liver sometimes tended to increase slightly. As shown in Fig. 1, the blood 5HT and platelet responses to SiK213 were markedly augmented by prior treatment with MDP. It should be noted that within 5 min of the injection of SiK213 in MDP-primed mice, the platelet count fell by about 60% of its control level, while the 5HT level in the blood fell by more than 80% of its control level. The shock score was 1–2 at 5 min and 4–5 at 10 min in these mice. In non-primed mice, the effects on both platelet count and the blood 5HT level were smaller, particularly in the first 5 min.

In the lung, 5HT was slightly, but significantly, increased by SiK213 in the MDP-primed mice, but not in the non-primed mice. Conversely, 5HT increased

Table 1. Abilities of 10 mg/kg of SiK213 and ScT21 to induce anaphylaxis-like shock

		Shock		
Treatment		Incidence	Score*	Mortality**
Saline	ScT21	0/6	0 ~ 1	0/6
MDP	ScT21	4/6	1 ~ 2	0/6
Saline	SiK213	6/6	1 ~ 2	0/6
MDP	SiK213	6/6	4	6/6

Saline or MDP (5 mg/kg) was injected intravenously, and 4 h later a suspension of SiK213 or ScT21 (each 10 mg/kg) was injected intravenously.
*Maximum shock scores observed 8–15 min after the injection SiK213 or ScT21.
**Within 60 min of the injection of SiK213 or ScT21.

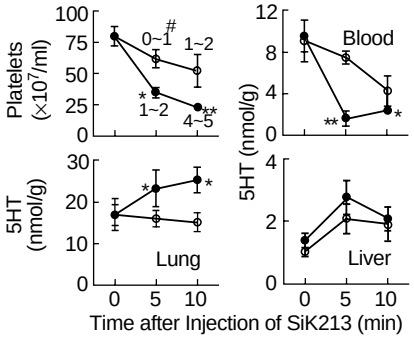

Fig. 1. Effects of SiK213 on platelet count in blood and on 5HT levels in blood, lung, and liver. Saline (○) or MDP (5 mg/kg) (●) was injected intravenously, and 4 h later a suspension of SiK213 (10 mg/kg) was injected intravenously. Mice were killed at the times indicated after the injection of SiK213. The values shown for time 0 are from mice not given SiK213. Each value is the mean (SD) from four mice. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. the corresponding saline-injected group. #Scores allocated to shock signs at the times indicated.

in the liver in both groups of mice, and although the increase tended to be larger in the primed mice, there was no significant intergroup difference.

Effects of ScT21 on platelet count and 5HT in blood, lung, and liver

MDP also augmented the 5HT and platelet responses to ScT21, a weak inducer of rapid shock. However, in MDP-primed mice ScT21 produced weaker decreases in blood platelet count and blood 5HT than SiK213 (compare Fig. 2 with Fig. 1). It should be noted that the decreases in the platelet count and 5HT level in the blood were nearly parallel to each other in the primed mice given ScT21. The ScT21-induced increases in the levels of 5HT in the lung and liver were also larger, or tended to be larger, in the MDP-primed mice, and the levels reached were similar to those seen with SiK213 (compare Fig. 2 with Fig. 1).

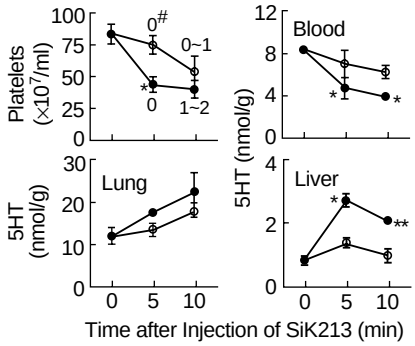

Fig. 2. Effects of ScT21 on platelet count in blood and on 5HT levels in blood, lung, and liver. Saline (○) or MDP (5 mg/kg) (●) was injected intravenously, and 4 h later a suspension of ScT21 (10 mg/kg) was injected intravenously. Mice were killed at the times indicated after the injection of ScT21. The values shown for time 0 are from mice not given ScT21. Each value is the mean (SD) from four mice. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. the corresponding saline-injected group. #Scores allocated to shock signs at the times indicated.

Effects of *P. intermedia* lipopolysaccharide on 5HT levels in the blood and lung

We previously reported that in both MDP-primed and non-primed C3H/HeN mice, *P. intermedia* lipopolysaccharide (1 mg/kg) induced marked changes in 5HT within 5 min of its intravenous injection: a decrease in the blood and a concomitant increase in the lung and liver (2, 10). The 5HT increments in the lung corresponded to 80% or more of the 5HT lost from the blood (2), indicating that the platelets lost from the blood mostly accumulate in the lung in response to that lipopolysaccharide. Such a large accumulation of 5HT in the lung was confirmed in the present study, too (Fig. 3). It should be noted that the increase in 5HT in the lung in mice given *P. intermedia* lipopolysaccharide was much greater than that in mice given MDP+SiK213 (Fig. 1), although the shock scores in mice given *P. intermedia* lipopolysaccharide were much smaller than those in mice given MDP+Sik213.

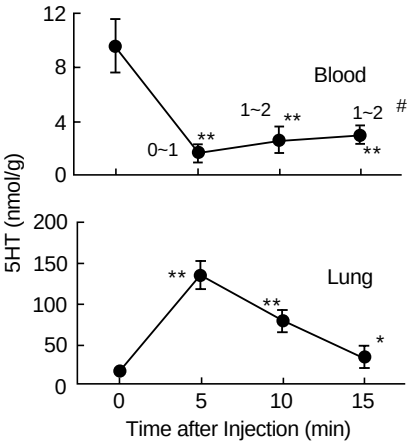

Fig. 3. Changes in 5HT levels in blood and lung induced by *P. intermedia* lipopolysaccharide. *P. intermedia* lipopolysaccharide (1 mg/kg) was injected intravenously into non-primed mice, and they were killed at the indicated times. The values shown for time 0 are from mice not given lipopolysaccharide. Each value is the mean (SD) from four mice. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. time 0. #Scores allocated to shock signs at the times indicated.

Effects of a complement-C5 inhibitor, K76, on the changes in 5HT levels and rapid shock induced by SiK213

In our previous study, a complement inhibitor, K76, was effective at reducing the score allocated to the rapid shock induced by lipopolysaccharide, evidently by preventing platelet destruction (14, 21). Consequently, in the next experiment we examined the effects of this inhibitor on the Sik213-induced rapid shock and 5HT changes in blood, lung, and liver. As shown in Fig. 4, K76 itself had no detectable effects on 5HT levels. However, this agent reduced the severity of shock (from score 4

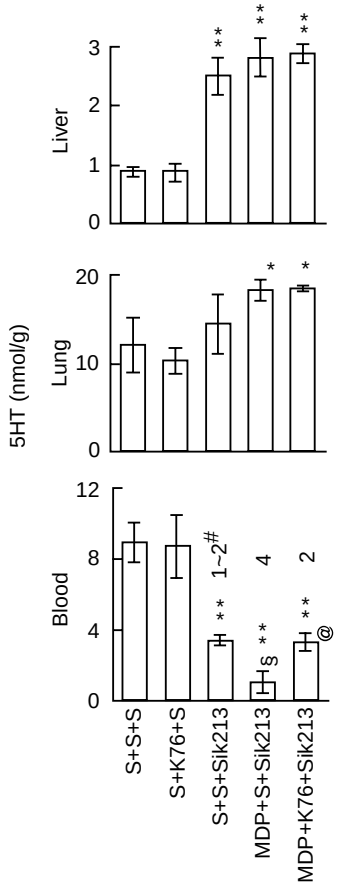

Fig. 4. Effects of K76, an inhibitor of complement C5, on the signs of shock and 5HT changes induced by SiK213. MDP (5 mg/kg) was injected intravenously, and 3 h later K-76 (100 mg/kg) was injected intraperitoneally. At 1 h after the injection of K76, a suspension of SiK213 (10 mg/kg) was injected intravenously. Mice were killed 10 min after the injection of SiK213. As a control for each injection, saline (S) was injected into other groups. Each value is the mean (SD) from four mice. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. S + S + S; $^{\S}P < 0.01$ vs. S + S + Sik213; $^{@}P < 0.01$ vs. MDP + S + Sik213; #Scores allocated to shock signs just before decapitation.

to 2) and significantly reduced the decrease in blood 5HT induced by SiK213. In fact, the 5HT level in the blood of the group given MDP + K76 + SiK213 was similar to that of the group given saline + saline + SiK213. In contrast, K76 produced no detectable effect on the 5HT elevations induced in the lung and liver by SiK213.

Discussion

We confirmed the Isobe's finding (9) that intravenous injection into MDP-primed C3H/HeN mice of a lyophilized preparation of the gram-positive bacterium SiK213 (but not of ScT21) induces lethal rapid shock. In the present study, we found that SiK213 induces concomitant and profound decreases in the platelet count and 5HT level in the blood, and that these responses precede the development of rapid shock. In non-primed mice, these responses were small. By comparison with SiK213, ScT21 produced similar, but smaller, effects on platelet and 5HT levels in the blood. In contrast to the marked effects of *P. intermedia* lipopolysaccharide on the 5HT levels in the blood and lung, the effect of SiK213 on the lung 5HT level was small, although it, too, caused a marked decrease in blood 5HT. Both the shock-inducing and 5HT-decreasing activities of SiK213 were markedly and significantly reduced by K76, an inhibitor of complement C5. These findings are discussed below.

Since free 5HT in the blood is rapidly cleared from the circulation (18, 19) – indeed, its half-life in mice is $<<10$ s (20) – the observed levels of 5HT in the blood reflect the amount of 5HT retained within platelets (12). Thus, the ratio of the observed 5HT level to the platelet count in the blood is an indicator of the degree of platelet degradation. In the present study, ScT21 produced nearly parallel decreases in platelet count and 5HT level in the blood (Fig. 2). However, in MDP-primed mice, SiK213 induced a profound decrease in 5HT in the blood 5 min after its injection, although the decrease in the platelet count at this time was less marked (Fig. 1). These results suggest that SiK213 induced a significant release of 5HT from platelets (i.e. a degradation or degranulation of platelets) during their circulation.

We have shown that 5HT levels in the lung and liver also reflect the amount of 5HT retained within platelets, which are known to be translocated into these tissues (12). As shown in Fig. 3, *P. intermedia* lipopolysaccharide induced a large elevation of 5HT in the lung, indicating that the platelets lost from the blood largely accumulated in the lung in response to the lipopolysaccharide. Indeed, electron micrographs have demonstrated a marked platelet accumulation in the lung, and have provided evidence that the accumulated platelets are destroyed there (2). However, as shown in Figs 1 and 3, in spite of a very large reduction in blood 5HT following SiK213 injection into MDP-primed mice, the 5HT increase in the lung was much smaller than that induced by *P. intermedia* lipopolysaccharide. In no tissues or organs other than the lung and liver was 5HT increased after SiK213 injection (data not shown). These results lead us to suppose that in MDP-primed mice given SiK213, platelets accumulated in the lung and/or liver after their degranulation (or release of 5HT) in the circulation. In MDP-primed mice, ScT21 produced a significant increase in 5HT in the liver (Fig. 2), suggesting that such a degradation (or release of 5HT) is small in mice given ScT21.

We previously showed that K76, an inhibitor of complement C5, can prevent not only lipopolysaccharide-induced platelet destruction, but also the development of rapid shock (10, 14, 21). This agent also significantly reduced the SiK213-induced decline in 5HT in the blood and reduced the severity of the subsequent shock, suggesting that complement system may be involved in the SiK213-induced degranulation of platelets.

Various microbial materials are known to sensitize animals to the actions of lipopolysaccharide (3). We have already shown that the lipopolysaccharide-induced platelet response is augmented by MDP (2). In the present study, we found that the gram-positive bacteria-induced platelet response was also augmented by MDP, although the mechanism underlying this effect of MDP remains unknown. Takada et al. (17) showed that some bacterial and synthetic muramyl peptides potently primed mice with respect to lethal anaphylactoid shock, and they suggested that these materials may be produced from bacteria *in vivo*.

Infusion of *Streptococcus sanguis* into rabbits has been shown to induce a marked accumulation of platelets in the lung (6). In the present study, however, oral streptococci did not produce such a marked pulmonary accumulation of 5HT in mice. Although we cannot explain this difference at present, these results indicate that various bacteria (both gram-negative and -positive bacteria) induce *in vivo* platelet aggregation, degranulation, or degradation. As emphasized by Herzberg & Meyer (6), it is likely that through dental surgical procedures and daily oral hygiene procedures, there is a far more frequent exposure over a lifetime to dental microorganisms than to any other atherosclerosis-associated microbes. In addition, the augmentation of the platelet response by MDP suggests that such an augmentation of the inflammatory reactions induced by bacterial products (and by bacteria themselves) may occur in the host during the course of an infection. It is possible that such an augmentation may occur locally within the microcirculation close to where bacteria proliferate, leading to a release of inflammatory mediators, such as thromboxane A2 and 5HT (or both 5HT and histamine in the case of humans) (11). However, it should be noted that our data do not provide direct information concerning the quantity of bacteria that might pose a real risk for human subjects, although they do suggest that oral bacteria have the potential to stimulate platelet responses.

In conclusion, the present results suggest the following:

- streptococcal bacterial cells can induce rapid platelet responses;
- complement-dependent degradation of platelets may be involved in streptococcus-induced rapid shock;
- the streptococcus-induced platelet degradation or degranulation may occur largely in the systemic circulation (unlike that induced by lipopolysaccharide);
- platelets may play a role not only in infectious diseases caused by gram-negative bacteria, but also in those caused by gram-positive bacteria.

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