Suppression and Enhancement of the Freund's Incomplete Adjuvant-Induced Writhing Reaction by Sodium Ascorbate in Mice

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We noticed that an intraperitoneal injection of Freund's incomplete adjuvant (FIA) into mice could stimulate the induction of a writhing reaction. The FIA emulsion-induced writhing reaction was found to be remarkably inhibited by preadministration of oral indomethacin, a non-steroidal anti-inflammatory and analgesic drug. The induction of the writhing reaction was also inhibited by intravenous preadministration of sodium ascorbate (SAs) in saline. In the experiments where SAs was added to FIA, it was demonstrated that SAs had dual activity of suppression and enhancement. At lower concentrations SAs functioned as a suppressor of the writhing reaction, while at concentrations higher than about 1 mg/50 μ l/mouse it acted as an enhancer of the reaction. Furthermore, this writhing reaction induced by FIA+SAs emulsion was also inhibited by preadministration of SAs itself as well as indomethacin. These results suggested that the mechanism of the writhing reaction induced by FIA was concerned with the production of prostaglandins (PGs), and SAs might be involved in regulation of the writhing reaction.

In this paper, we propose a mouse writhing model induced by FIA or FIA+SAs emulsion as a novel pain model useful for assessment of analgesic and anti-inflammatory agents.

Key words writhing reaction; Freund's incomplete adjuvant; sodium ascorbate; prostaglandin endoperoxide synthase

Writhing reaction is a characteristic syndrome where animals exhibit painful and repeated stretching movements against such substances as acetic acid or kaolin. The mouse writhing assay system has been widely used as a simple screening for analgesic or anti-inflammatory agents.^{1–3} For the mechanism of the kaolin-induced writhing reaction, Fujiyoshi *et al.*³ reported the involvement of released bradykinin through the activation of factor XII.

In the immunological and immunochemical fields, Freund's incomplete adjuvant (FIA), which is composed of mineral oil–surfactant (85:15), has been commonly used as an enhancing agent for the immune response. In our preliminary experiments, it was found that an intraperitoneal injection of FIA could induce in mouse a remarkable writhing reaction.

In this paper, we propose a novel mouse writhing model induced by FIA, which could be regarded as a prostaglandin (PG) endoperoxide synthase associated phenomenon.

Ascorbate, along with its antioxidant activity which scavenges active oxygen species and free radicals, is known not only to have anti-inflammatory, anti-allergic, and immunostimulatory properties,⁴ but also to be related to the regulation of prostaglandin (PGs) formation.⁵ Thus, we examined the effects of ascorbate on the FIA-induced writhing reaction, and several interesting results were found in the present paper.

MATERIALS AND METHODS

Animals Male ddY mice (SPF, 5 weeks old) were purchased from Japan SLC Co. (Hamamatsu). These animals were maintained under a constant temperature (24 $\pm$ 2 $^{\circ}$ C) and were fed commercial laboratory diet (CE-2, Clea Japan, Tokyo) and tap water *ad libitum*.

Reagents The following reagents were used in the present study: FIA (Difco Laboratories, Detroit, MI); sodium ascorbate (SAs), indomethacin, and sodium carboxymethyl-

cellulose (CMC-Na) (Nacalai Tesque, Inc., Osaka, Japan); sodium erythorbate (SEr) and sodium chloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Reagent solution was prepared with ultra pure water (resistivity of distilled water $\geq$ 18.2 M Ω ·cm) to avoid contamination with slight amounts of metal ions, and solutions of SAs or SEr were freshly prepared before use.

FIA-Induced Writhing Reaction Mice were intraperitoneally injected with FIA in the form of either solution or emulsion with equal volume of saline. In most experiments, the amount of injection was set to 50 μ l/mouse unless otherwise indicated. As a FIA-writhing inducer, "FIA emulsion" means a fixed preparation in the present work where 25 μ l of both FIA and saline were emulsified for injection. The number of induced writhings was counted for 30 min in each 5 min period after injection of FIA.

FIA+SAs (or FIA+SEr)-Induced Writhing Reaction In several experiments, instead of FIA emulsion, emulsions were prepared by FIA and an equal volume of either SAs or SEr. The amount of intraperitoneal administration was 50 μ l/mouse unless otherwise indicated. For the FIA+SAs-induced writhing reaction, "FIA+SAs emulsion" especially means another constant preparation in this text that the dose of SAs was set to 10 mg/50 μ l/mouse. Similarly, the number of induced writhings was counted as above.

Inhibition of Writhing Reaction Indomethacin, which is a typical non-steroidal anti-inflammatory and analgesic drug, suspended in a 0.5% CMC-Na solution, was orally given 1 h prior to an intraperitoneal injection of the writhing inducer. Control group was orally administered 0.5% CMC-Na alone. SAs in saline was administered intravenously 60 or 10 min before the injection of the writhing inducer, and control groups were intravenously administered saline alone under the same conditions as the experimental groups. The number of induced writhings was counted for 10 min or 30 min in each 5 min period after the injection of the writhing

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inducer.

Statistical Analysis Statistical analysis was performed by analysis of variance, followed by Fisher's PLSD test. Asterisks indicate significant differences from the control group (***, $p < 0.001$; **, $p < 0.005$; *, $p < 0.05$).

RESULTS AND DISCUSSION

We found that a writhing reaction was induced by an intraperitoneal injection of FIA solution (25 μ l) or FIA emulsion (FIA 25 μ l+saline 25 μ l). The time course of the writhing response induced by FIA solution and FIA emulsion is shown in Fig. 1a. To investigate the inductive ability of the FIA more precisely, writhing responses were assessed for 30 min after intraperitoneally administration at 4 different doses (25, 50, 100, and 300 μ l) of FIA solutions or FIA+

Fig. 1. FIA Solution or FIA Emulsion-Induced Writhing Reaction

(a) Mice were injected intraperitoneally with FIA (25 μ l)+saline (25 μ l) emulsion ($\square$) or FIA solution (25 μ l) ($\blacksquare$). The number of writhings was measured by counting for 30 min in each 5 min period. Each point and bar represent the mean $\pm$ S.E. for 8 mice. (b) Dose-dependences of the solution and emulsion were examined. Mice were injected intraperitoneally with 25, 50, 100, or 300 μ l/mouse of FIA+saline (1:1) emulsion ($\square$) and FIA solution ($\blacksquare$). The number of writhings was measured by counting for 30 min. The writhing number of FIA+saline (1:1) emulsion is indicated by the corresponding FIA-dose. Each point and bar represent the mean $\pm$ S.E. for 8 mice. Statistical analyses were performed by analysis of variance, followed by Fisher's PLSD test. Asterisk indicates significant differences from the injection of the FIA solution (*, $p < 0.05$).

Fig. 2. Inhibition of FIA Emulsion-Induced Writhing Reaction by Indomethacin

Indomethacin suspended in 0.5% sodium carboxymethylcellulose (CMC-Na) (0.1, 1, 5, or 10 mg/kg) was administered orally to mice 1 h before the intraperitoneal injection of FIA+saline emulsion (50 μ l/mouse). The number of writhings was measured by counting for 30 min. Control group was orally administered 0.5% CMC-Na alone. The data were expressed as the percentage of the average of the control group. Each column and bar represent the mean $\pm$ S.E. for 8-9 mice. Statistical analyses were as in Fig. 1b. Asterisks indicate significant differences from the control group (***, $p < 0.001$).

saline (1:1) emulsions to mice (Fig. 1b). The results revealed that the inducing ability of the FIA emulsion surpassed the FIA solution at all indicated doses of FIA. The writhing-inducing ability was most intense at 25 μ l dose of FIA in emulsion (*i.e.*, inducer of the FIA emulsion-induced writhing reaction).

The primary candidate for the mediators of the present writhing reaction was PGs, because PGs mediate several biological activities such as inflammation, pain, and fever. The formation of PGs from polyunsaturated fatty acids such as arachidonic acid (AA) can be summed up as $AA \rightarrow PGG_2 \rightarrow PGH_2 \rightarrow \dots \rightarrow PGs$. The first steps indicated within the parentheses are catalyzed by PG endoperoxide synthase. PG endoperoxide synthase functions both in the initial reaction of oxidation of AA (cyclooxygenase (Cox) activity), and at the conversion of PGG_2 to PGH_2 (PG hydroperoxidase activity).

In this study, indomethacin was employed as an anti-inflammatory and analgesic drug having inhibitory action on Cox activity. Indomethacin suspended in a 0.5% CMC-Na solution was orally given 1 h prior to an intraperitoneal injection of the FIA emulsion. As shown in Fig. 2, indomethacin significantly inhibited the FIA emulsion-induced writhing reaction in a dose-dependent manner, and the reaction was almost completely suppressed by a 10 mg/kg dose. It was suggested that the FIA emulsion induced-writhing reaction may be caused by PGs catalyzed by Cox.

On the other hand, the formation step from AA to PGH_2 may be affected by ascorbate, which functions not only as a biological cofactor but also as an antioxidant, because this step proceeds by both oxidase reaction and a reaction requiring an oxygen radical scavenger, *e.g.*, tryptophan.^{6,7)} If ascorbate was concerned with the formation of PGH_2 , the FIA-induced writhing reaction might be affected also by SAs.

The next experiment was conducted to investigate the influence of SAs on the FIA emulsion-induced writhing reaction. As shown in Fig. 3, the FIA emulsion-induced writhing reaction was suppressed significantly by the intravenous injection of SAs (0.1 mg/50 μ l/mouse) 10 min before the in-

Fig. 3. Inhibition of FIA Emulsion-Induced Writhing Reaction by Pre-administration of SAs

Mice were injected intravenously with SAs in saline (10 or 0.1 mg/50 µl/mouse) 60 or 10 min before intraperitoneal injection of FIA emulsion (50 µl/mouse). The number of writhings was measured by counting for 30 min. The data were expressed as the percentage of the average of the control group. Each column and bar represent the mean $\pm$ S.E. for 12 mice. Statistical analyses were as in Fig. 1b. Asterisk indicates significant differences from the control groups which were intravenously administered saline alone at the indicated time before the induction of the writhing reaction (*, $p < 0.05$).

Fig. 4. Effect of Additional SAs in FIA Emulsion-Induced Writhing Reaction

Effect of SAs dose on time courses of the writhing reaction for 30 min in each 5 min is indicated. Mice were injected intraperitoneally with FIA emulsion containing 10 (□) and 0.1 (△) mg/50 µl/mouse of SAs. For comparison, mice were injected intraperitoneally with FIA emulsion (■) (50 µl/mouse) and saline solution of SAs (▲) (10 mg/50 µl/mouse) alone. Each point and bar represent the mean $\pm$ S.E. for 8–9 mice.

duction of the reaction.

To ascertain the effect of SAs on the writhing reaction, the influence of the addition of SAs to the FIA emulsion was then investigated. Figure 4 illustrates the dose- and time-dependence of the writhing responses, when mice were injected intraperitoneally with two different doses (10 and 0.1 mg/50 µl/mouse) of FIA+SAs (1:1) emulsion. The potency of the writhing reaction was enhanced by addition of 10 mg of SAs compared with FIA emulsion-induced writhing reaction, while the reaction was not induced by the intraperitoneal in-

Fig. 5. Dose Dependence of FIA+SAs Emulsion-Induced Writhing Reaction

Effect of SAs dose on the writhing reaction for 10 min is indicated. Mice were injected intraperitoneally with the FIA emulsion of indicated SAs dose. Each point and bar represent the mean $\pm$ S.E. for 8–9 mice. See text for further details.

jection of a saline solution of SAs (10 mg) alone. A peak enhancement of this reaction caused by additional SAs appeared remarkably within 10 min after the injection of FIA+SAs (1:1, 10 mg/50 µl/mouse) emulsion (i.e., FIA+SAs-induced writhing reaction). Contrary to addition of 10 mg of SAs, the induction of the writhing reaction was clearly suppressed by addition of 0.1 mg of SAs within 10 min. The influence of additional SAs on the writhing response was clearly observed within 10 min after the injection of the inducer, so inductive effects versus doses of SAs on the writhing reaction was investigated for 10 min. As indicated in Fig. 5, the writhing reaction was linearly suppressed with an increasing dose of SAs, and when 0.1 mg of SAs was added to the FIA emulsion, the maximum suppression was observed. On the other hand, the writhing response linearly increased with doses more than 0.1 mg of SAs. It is an interesting phenomenon that the writhing response and additional dose of SAs were both negatively and positively correlated in a dose dependent manner. In the experiments where SAs was added to FIA, these results suggested that SAs at lower concentrations functioned as a suppressor of the writhing, while at concentrations higher than about 1 mg/50 µl/mouse as an enhancer of the writhing reaction. These data presumably suggested that SAs can display dual effects on PGH₂ generation, suppressive action to the Cox activity and enhanced action to the PG hydroperoxidase activity.

Harada *et al.* reported that a phenolic anti-inflammatory and analgesic agent, 2-aminomethyl-4-*tert*-butyl-6-iodophenol hydrochloride (MK-447), acts as a cofactor in the PG endoperoxide (PGH₂) formation, and exerts the dual action of stimulation and inhibition on the reaction, depending on the concentration of hemoglobin and tryptophan.⁸⁾ The dual effects of SAs on the writhing reaction imply activity similar to MK-447; however, our results *in vivo* differ from those of MK-447 in the dose-response pattern.

Sodium erythorbate (SEr), a stereoisomer of SAs, does not function as a biological cofactor but does function as an re-

Fig. 6. Comparison of the FIA+SAs and FIA+SEr Emulsion-Induced Writhing Reaction

The experimental method for the writhing reaction is the same as in Fig. 1. Mice were injected intraperitoneally with FIA emulsion containing 0.5 or 0.1 mg/50 μ l/mouse of SAs or SEr. For comparison, mice were injected intraperitoneally with FIA emulsion (50 μ l/mouse) and saline solution of SAs or SEr (0.5 mg/50 μ l/mouse) alone. Each column and bar represent the mean $\pm$ S.E. for 8 mice. Statistical analyses were as in Fig. 1b. Asterisks indicate significant differences from the injection of the FIA solution (***, $p < 0.001$; **, $p < 0.005$).

Fig. 7. Inhibition of FIA+SAs Emulsion-Induced Writhing Reaction by Indomethacin

Indomethacin of indicated dose was administered orally to mice 1 h before the intraperitoneal injection of FIA+SAs (10 mg/50 μ l/mouse) emulsion. The writhing number for 10 min period are shown. Control was orally administered 0.5% CMC-Na alone. Expression of each column and bar, and statistical analyses are the same as in Fig. 2. Asterisks indicate significant differences from the control group (***, $p < 0.001$; **, $p < 0.005$; *, $p < 0.05$). $n = 8-10$.

ducing agent on scavengers of oxygen radicals. The influence of additional SEr on the writhing reaction induced by FIA was also examined, because SAs is not an essential vitamin in mouse. Figure 6 shows the comparison of writhing induced by FIA+SEr or FIA+SAs (1:1) emulsion, or SEr or SAs in saline solution. Owing to the low solubility of SEr (16 g/100 ml, in water), the doses of SAs and SEr were examined at 0.5 mg/50 μ l/mouse for comparison at high concentration. The writhing reaction was also suppressed by addition of 0.1 mg/mouse of SEr to the FIA emulsion, and the reaction was not induced by the administration of the saline solution of SEr (0.5 mg/50 μ l/mouse) alone. These preliminary experiments revealed that the induced ability of writhing by SEr was almost the same as that of SAs. These results suggest that SAs functions on the writhing reaction induced by

Fig. 8. Influence of FIA+SAs Emulsion-Induced Writhing Reaction by Preadministration of SAs

Mice were injected intravenously with SAs in saline (10 or 0.1 mg/50 μ l/mouse) for 60 or 10 min before intraperitoneal injection of FIA+SAs (10 mg/50 μ l/mouse) emulsion. The number of writhings was measured by counting for 10 min. Expression of each column and bar, and statistical analyses are the same as in Fig. 3. Asterisks indicate significant differences from the control groups which were intravenously administered saline alone at the indicated time before the induction of the writhing reaction (***, $p < 0.001$; **, $p < 0.005$; *, $p < 0.05$). $n = 18-22$.

FIA as an antioxidant agent, (*i.e.*, inhibition of oxygenation reaction and the radical scavenger action), and this action also relates to the regulation of PGs formation.

Next, the inhibitory effects by the Cox inhibitor, indomethacin, on the enhanced writhing reaction by SAs were also investigated. Figure 7 illustrates that indomethacin inhibits the FIA+SAs-induced writhing reaction in a dose-dependent manner. This was consistent with the above results that FIA emulsion-induced writhing reaction was inhibited by indomethacin. It was suggested that the writhing reaction induced by both FIA and FIA+SAs emulsion could be due to pain which may be caused by the biosynthesis of PGs.

Furthermore, the influences of preadministered SAs on the enhanced writhing reaction by SAs were studied. It was shown that the FIA+SAs-induced writhing reaction was significantly suppressed by intravenous preadministration of SAs (0.1 mg/50 μ l/mouse) in saline, in two experimental conditions (Fig. 8).

A detailed study on the mechanism of the FIA emulsion-induced writhing reaction, on the action of SAs against the writhing reaction, and on the effect of Cox-2 selective inhibitors are in progress in our laboratory.

In conclusion, we found a novel mouse writhing model induced by FIA emulsion which could be related to the formation of PGs. Furthermore, addition of SAs to the FIA emulsion shows the dual action of suppression and enhancement in a dose-dependent manner, on the FIA-induced writhing reaction. In addition, these findings suggest that FIA and FIA+SAs emulsion-induced writhing reactions are useful for assessment of analgesic and anti-inflammatory agents as pain models involving the formation of the PGs.

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