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Lack of Effect of Salicylate on Pyrogen Release from Human Blood Leucocytes *in Vitro*

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Although salicylates are widely used as antipyretic agents, the mechanism by which they lower body temperature is controversial. It has been suggested that these compounds act at peripheral sites of inflammation, by suppressing release of endogenous pyrogen from leucocytes exposed to an inflammatory stimulus (1), or by suppressing synthesis and release of fever-inducing prostaglandins (2, 3). Alternatively, considerable evidence in both man (4) and rabbits (5, 6) indicates that salicylates act centrally to lower fever by blocking the action of endogenous pyrogen on certain temperature-sensitive hypothalamic neurons. For example, stable fevers induced by infusions of leucocyte (endogenous) pyrogen in humans persisted for 50 min or so after the infusion was discontinued (4). However, when sodium salicylate was injected intravenously during the continuous pyrogen infusion, temperature fell promptly. This rapid antipyretic effect of salicylate is best explained by a central action rather than by altered production or release of pyrogen from the host's leucocytes.

This postulated central action clearly does not exclude the possibility that these agents have peripheral antipyretic effects, as reported (1, 6). It seemed appropriate, therefore, to re-examine the action of salicylate on release of pyrogenic agents from leucocytes, with a model which approximates conditions of *in vivo* inflammation. The studies to be reported here indicate that salicylate does not suppress the release of pyrogen from human blood leucocytes following exposure to endotoxin or a phagocytic stimulus *in vitro*. By contrast, as expected, salicylate administered to rabbits suppressed fever to intravenous injection of endogenous pyrogen.

MATERIAL AND METHODS

All glassware and buffers were made sterile and pyrogen-free by heating at 160°C for 2 hr or by autoclaving for 1.5 hr. Na salicylate U.S.P. (Mallinckrodt) was dissolved in sterile pyrogen-free saline to 10 mg/ml, filtered through a Swinnex

millipore filter, and stored at 4°C. For infusion experiments, 100 mg/ml Na salicylate solution was prepared. One-gram amounts were injected intravenously into rabbits to test for possible endotoxin contamination. Human blood leucocytes were prepared by methods described previously (7). Briefly, heparinized venous blood was drawn from normal volunteers, separated by dextran sedimentation, and the leucocytes suspended in modified Krebs-Ringer phosphate buffer pH 7.4 with 10 units heparin/ml. Incubation flasks contained 5–7.5 ml of 15% autologous serum-KRP buffer with 1×10^7 leucocytes per ml, glucose 150 mg% and penicillin 6000 units. Salicylate 40 mg% was incubated with leucocytes for one hour before addition of bacteria. Heat-killed *Staphylococcus albus,* prepared as described previously (7) was used at a ratio of 5–40 bacteria per leucocyte. In experiments with endotoxin, a 1:10 dilution in saline of typhoid vaccine (Monovalent Reference Std NRV-LS #1, Walter Reed, Washington, D.C.) was filtered just before use through a Swinnex millipore filter, and 0.3–0.5 ml/ 3×10^7 leucocytes was used. Salicylate was added to leucocytes 1 hr after the endotoxin. Flasks were incubated with shaking on a Dubnoff incubator at 37°C for several hours, and then placed in a stationary incubator at 37°C overnight. In initial experiments with bacteria, phagocytosis was confirmed by examination of coverslip smears two hours after addition of staphylococci.

After incubation, flask contents were centrifuged at 2000*g* for 30 min, an aliquot of the supernatant was cultured in thioglycollate broth to confirm sterility, and the remainder was injected at once or stored at 4°C. for 1–5 days. Pyrogen release was determined by assay in rabbits as described previously (8). ΔT represents the maximum height of fever above pre-injection baseline temperature, occurring within 75 min after injection. In some experiments, rabbit temperatures were continuously monitored on a Rustrak recorder. In all experiments, the same rabbits received aliquots of supernatants from control and experimental preparations. Results of pyrogen assay of supernatants, reported in Tables 1 and 2, were tested for significant differences by means of *t*-test for paired samples, using responses of the same rabbit to a control and experimental supernatant as one pair. Supernatant from leucocytes incubated without salicylate or activators was determined to give an average temperature rise of less than 0.2°C. in all instances.

Rabbit endogenous pyrogen (EP) was prepared as follows. One-hundred and twenty ml. heparinized rabbit blood was centrifuged at 600*g*, the cells were resuspended in 25 ml plasma, and heparinized KRP buffer containing 150 mg% glucose and 6000 units of penicillin was added to a final volume of 150 ml. Heat-killed staphylococci (1.5×10^{10}) were added, and the mixture was shaken gently at 37°C overnight. After centrifugation at 2000*g* for 30 min, the supernatant containing EP was removed, an aliquot cultured and assayed for pyrogenicity, and the remainder stored at 4°C.

Na salicylate for intraperitoneal injection was diluted to 20 mg/ml in KRP buffer, and injected slowly through a blunt infusion needle to provide 250 mg/kg, the dose used by others (1).

RESULTS

The results of experiments designed to investigate the effect of salicylate on pyrogen release from human blood cells *in vitro* are shown in Tables 1 and 2. Pre-incubation of leucocytes with a high concentration of sodium salicylate (40 mg%) did not alter the subsequent release of pyrogen after stimulation by phagocytosis

TABLE 1

EFFECT OF Na SALICYLATE ON PYROGEN RELEASE *in vitro* FROM LEUCOCYTES STIMULATED BY PHAGOCYTOSIS

No. recipients	Average cells per dose	ΔT(°C) + Salicylate	ΔT(°C) − Salicylate	Bacteria per WBC
6	1.3×10^7 (1.2–1.4)[b]	.54 ± .07[a]	.62 ± .06	40:1
8	2.5×10^7 (2.1–3.2)	.75 ± .07	.86 ± .05	5:1
6	5×10^6	.48 ± .08	.44 ± .07	5:1

[a] SEM.
[b] Range.

TABLE 2

EFFECT OF Na SALICYLATE ON PYROGEN RELEASE *in vitro* FROM LEUCOCYTES STIMULATED BY ENDOTOXIN

No. recipients	Average cells per dose	ΔT(°C) + Salicylate	ΔT(°C) − Salicylate
8	2.3×10^7 ($2.2–2.4 \times 10^7$)[a]	.57 ± .08[b]	.45 ± .07

[a] Range.
[b] SEM.

(Table 1) (all p values $>.05$). Varying the numbers of leucocytes or the ratios of bacteria to leucocytes did not change the results. Addition of salicylate to leucocytes stimulated by endotoxin also did not suppress pyrogen release (Table 2). In fact, in the presence of salicylate, this small dose of endotoxin caused somewhat greater release of pyrogen $p = <.02$).

A more sensitive, quantitative assay for pyrogen was also carried out in several of these experiments by injecting both a standard pyrogenic dose of each supernatant and one half of this dose. Amounts were adjusted so that the average height of fever for one aliquot of supernatant was about 0.6°C and was proportionately decreased by injection of half that amount (for example, see Fig. 1). It could then be assumed that the dose–response curve was in its most sensitive range.[1] As shown in Fig. 1, using this technique, again no difference could be detected in the amounts of pyrogen present in supernatants derived from the same number of leucocytes incubated with or without salicylate and bacteria. Similar results were obtained in experiments with endotoxin. These findings, then, do not provide evidence for a direct action of sodium salicylate on endogenous pyrogen release by human blood leucocytes *in vitro*.

In order to confirm the central antipyretic action of salicylate, the following experiment was done. Five rabbits received 1- and 0.5-ml aliquots of a preparation of rabbit EP (see METHODS); the average temperature responses are shown in

[1] At low concentrations of human leucocyte pyrogen, the height of fever in rabbits is proportional to the amount of supernatant injected (unpublished observations). With higher concentrations of pyrogen, a logarithmic dose–response curve results (9, 10) and small changes in the amount of pyrogen cannot be detected.

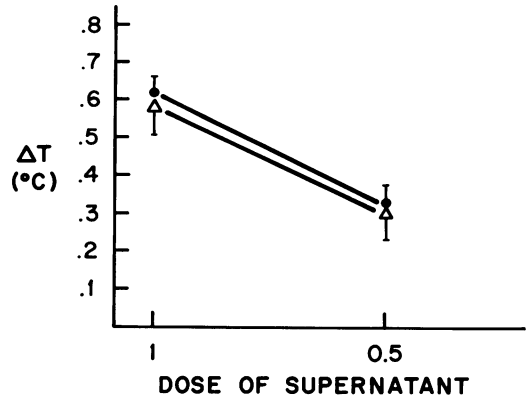

FIG. 1. Assay of endogenous pyrogen release from normal or salicylate-treated leucocytes. Average maximum height of fever ± SEM in nine rabbits after injection of supernatants from 18-hr incubations of human blood leucocytes with heat-killed staphylococci (·—·) or leucocytes pre-incubated for 1 hr with 40 mg% Na salicylate plus heat-killed staphylococci (Δ—Δ). One dose in different experiments was derived from 9.5–14.0 × 10^6 leucocytes (see text for details).

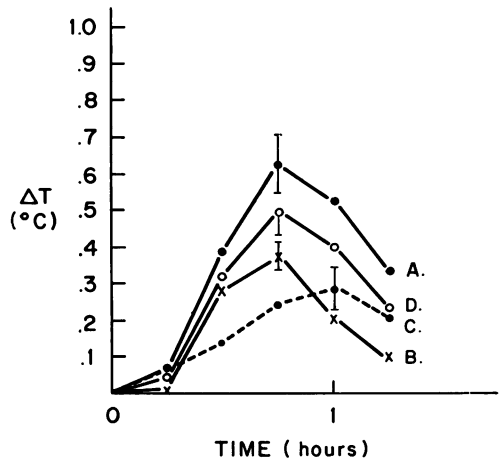

FIG. 2. Effect of salicylate on febrile responses in rabbits. Average fevers in a group of five rabbits after injection of a preparation of rabbit endogenous pyrogen (EP). A: 1 ml EP, B: 0.5 ml EP injected on Day 1; C: 1 ml EP injected on Day 5, 1–2 hr after intraperitoneal Na salicylate 250 mg/kg; D: 1 ml EP injected on Day 7. SEM of the peak responses is shown.

Fig. 2, curves A and B. Five days later, each rabbit received 250 mg/kg sodium salicylate by intraperitoneal infusion. Temperatures were then recorded, and 1 or 2 hr later, each rabbit was injected intravenously with a second 1-ml aliquot of the pyrogenic supernatant. As shown in Fig. 2, C, this injection produced only half as much fever as did the first (Curve A). To insure that the test solution had not lost its pyrogenicity, a third 1-ml aliquot was injected two days later. Although the stored material was slightly less pyrogenic than the sample tested initially (Fig. 2, Curves D and A), it clearly produced more fever than the preceding injection which followed salicylate treatment (Fig. 2, C). Salicylate thus interfered *in vivo* with the pyrogenic action of pre-formed endogenous pyrogen.

DISCUSSION

The experiments reported in Tables 1 and 2 indicate that incubation of human blood leucocytes *in vitro* with a high concentration of salicylate does not alter their capacity to release endogenous pyrogen after stimulation by endotoxin or phagocytosis. The experiments by others (1, 11) in which salicylate appeared to suppress leucocyte pyrogen production differed in several respects from ours. First, rabbit blood or exudate cells were used, rather than human blood leucocytes. Differences in the effects of activators and inhibitors on pyrogen production by cells of different species have been noted (8, 12), and both metabolic (13, 14) and pyrogen-releasing (15, 16) properties of exudate and blood cells differ. Second, blood cells in one study (1) were incubated with endotoxin in phosphate-buffered saline without serum or Ca^{2+}. The activation of leucocytes and the induction of pyrogen production may differ under these circumstances. In preliminary studies (not reported), pre-incubation of leucocytes with salicylate in a serum-buffer medium appeared to augment rather than inhibit the action of endotoxin on leucocyte pyrogen release. A similar, but smaller, effect occurred when salicylate was added after the endotoxin (see Table 2). Salicylate may increase the availability of endotoxin for leucocyte activation by interfering with binding sites for endotoxin on plasma proteins (17). Altered protein binding of other compounds by salicylate has been described (18).

Although we do not have a clear explanation for the differences between our results and those of others (1, 11), our experimental conditions more closely approximate *in vivo* clinical situations. Normal human leucocytes were maintained in a serum-buffer medium and exposed to salicylate both before and after activation by two inflammatory agents. The lack of effect of the drug under these conditions, then, seems significant.

In addition to the unimpaired capacity for pyrogen release shown in these studies, salicylate-treated leucocytes exhibit normal phagocytosis and release of lysosomal enzymes (19). The stability of isolated human leucocyte lysosomes is reportedly not enhanced by salicylate (19), in contrast to rat liver lysosomes (20). Data on anti-inflammatory effects of salicylates do not include evidence for altered leucocyte function (21).

Recently, it has been proposed that salicylate may act as an antipyretic by inhibiting the synthesis and release of certain pyrogenic prostaglandins at sites of inflammation or in the brain (2, 3). Release of pyrogenic prostaglandins from rabbit exudate leucocytes *in vitro* after phagocytosis has been reported (22), although the effect of salicylate on this process was not examined. Our studies did not detect any reduction in the release of pyrogenic substances from salicylate-treated leucocytes. Thus, they do not support the hypothesis that salicylate suppresses fever by inhibiting prostaglandin release from leucocytes at peripheral sites of inflammation.

Our experiments confirmed the findings of some investigators (5, 10), but not another (1), that after administration of parenteral salicylate, rabbits have less febrile response to injection of preformed endogenous pyrogen. This salicylate effect, although definite, is small, and its demonstration may require careful attention to accurate assay techniques. Also, when salicylate is given after the onset of experimental (6) or clinical fever, rather than before, as in this model, its antipyretic effect is pronounced.

Our findings, then, do not support the theory that the antipyretic action of salicylate results from peripheral effects on release of pyrogen from blood leucocytes. They do provide support for the concept that the action of salicylate on fever is primarily a central one.

Note added in proof: Other work indicating lack of effect of salicylate on progen release from cells of other species has recently been published (23–25).

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