CONCISE REPORT

Salicylate Blockade of Granulocyte Adherence and the Inflammatory Response to Experimental Peritonitis

By Philip J. Spagnuolo and Jerrold J. Ellner

Aspirin profoundly inhibited the in vitro augmentation of human and mouse granulocyte adherence to nylon fiber induced by the bacterial products Escherichia coli endotoxin and Staphylococcus aureus culture filtrate. Granulocytes obtained from normal volunteers during the 48 hr following ingestion of aspirin did not respond normally to endotoxin stimulation. Furthermore, pretreatment of mice with sodium salicylate prior to intraperitoneal infection with Streptococcus pneumoniae impaired granulocyte exudation and resulted in uncontrolled bacteremia and greater lethality of infection.

The in vitro assay of granulocyte adherence to nylon fiber (GA) correlates with adherence to vascular endothelium in vitro and has facilitated definition of this early parameter of granulocyte function. This report describes the enhancement of human and mouse GA by products of Gram-positive and Gram-negative bacteria and the abrogation of these effects by salicylate both in vitro and in vivo. These studies suggested possible deleterious effects of this drug on granulocyte-dependent confinement of acute bacterial infections that were confirmed in a model of experimental peritonitis.

MATERIALS AND METHODS

GA

GA was assessed as previously described. Thirty milligrams of nylon fiber were packed into standard 5.75-inch Pasteur pipettes to a column length of 15 mm. Substances that stimulate or inhibit GA were incubated at 37°C on a mechanical rotator with heparinized (15 units heparin/ml blood) whole human blood granulocytes for 1 hr before the cells were applied to triplicate nylon columns. Aliquots of white cells were counted before and after column passage with a Coulter counter, and the corresponding percentage GA was determined by comparing the number of granulocytes in the effluent sample to that in the original sample.

Peritonitis Model

Type 19 Streptococcus pneumoniae (kindly provided by Dr. Robert Austrian, University of Pennsylvania School of Medicine, Philadelphia, Pa.) were cultured in brain-heart infusion broth in a 5% CO₂ incubator. Pharmacologic agents diluted to 0.5 ml in sterile pyrogen-free saline or saline control were injected into the tail veins of 16-20-g female Swiss albino mice (Charles River Laboratories, Wilmington, Mass.) 30 min before intraperitoneal instillation of dilutions of an 18-hr broth culture of pneumococci. At 2 and 4 hr after infection, blood for culture was obtained from the retroorbital venous sinus via the retroorbital venous sinus.
plexus with sterile heparinized 5-μl capillary pipettes. Following sacrifice of the mice by cervical dislocation, peritoneal exudate cells were obtained by lavage of the peritoneal space with 5 ml of sterile heparinized (10 units/ml) Hanks balanced salt solution (HBSS) (KC Biological, Lanexa, Kan.). Cells were counted with a Coulter counter, and differential counts were determined using tetrachrome-stained smears of cytocentrifuge preparations of exudate cells. Peritoneal fluid cultures were quantitated by streaking serial 10-fold dilutions of peritoneal lavage fluid onto sheep blood agar plates with a calibrated 0.001-ml platinum wire loop. The LD₉₀ of treated and untreated mice were calculated by a method reported previously. Peritoneal granulocytes also were obtained 18 hr after induction of sterile peritonitis by intraperitoneal injection of 3% proteose-peptone (Difco Laboratories, Detroit, Mich.). Peritoneal cells harvested at that time contained 80%-95% granulocytes.

**Bacterial Products and Salicylate Preparations**

*Escherichia coli* endotoxin 026:B6 (ET) (Difco) was dissolved in HBSS at various concentrations. *Staphylococcus aureus* (American Type Culture Collection No. 25923) was grown overnight in medium 199 (GIBCO, Grand Island, N.Y.). Culture filtrates (CF) were prepared by centrifuging 24-hr cultures at 17,000 g and filtering the supernatant with 0.2-μ filters. Both *E. coli* ET and CF of *S. aureus* were stored at −70°C until used.

Aspirin (acetylsalicylic acid, ASA) (Sigma Chemical, St. Louis, Mo.) and sodium salicylate were dissolved in HBSS at a concentration of 3 mg/ml and further diluted for use. For the mouse peritonitis model, sodium salicylate was diluted in sterile pyrogen-free normal saline, pH 6.5 (Travenol Laboratories, Deerfield, Ill.). For the in vivo study, 1.5 g of ASA were given to normal volunteers, and whole blood was obtained by venipuncture for determination of GA at various intervals following ingestion.

**RESULTS**

Incubation of normal whole blood with *E. coli* ET resulted in significant augmentation of GA within 5 min; maximal response occurred at an ET concentration of 5 μg/ml, and significant increases were observed with ET concentrations of 0.05 μg/ml. A 1% (v/v) dilution of *S. aureus* 24-hr CF also augmented GA; maximal increments occurred with 10% CF. Aspirin preincubated with whole blood and ET at 5.0 μg/ml or 10% CF for 1 hr reduced the stimulation of GA by both ET (Fig. 1A) and CF (Fig. 1B). Higher concentrations of bacterial products did not overcome inhibition by ASA. Sodium-bicarbonate-buffered ASA (pH 7.4) and sodium salicylate also interfered with augmentation of GA. The highest concentrations of ASA used in these experiments had no effect on baseline GA or on granulocyte viability assessed with trypan blue dye.

Salicylate blockade of GA did not occur via interference with ET activation of plasma, since it was still observed when ET and plasma were preincubated for 30 min at 37°C prior to the addition of ASA and granulocytes. Moreover, the granulocytes in whole blood incubated with ASA at 1000 μg/ml for 60 min at 37°C and washed three times in HBSS remained unresponsive to ASA, thus suggesting a direct effect of salicylates on cells.

Endotoxin-augmented GA also was studied following ingestion of 1.5 g of ASA by normal volunteers (Fig. 2). This dose should allow a peak salicylate level of less than 150 μg/ml, and by 24-48 hr all of such an oral dose should be excreted. Nonetheless, maximal depression of ET-augmented GA was noted 24 hr following ASA, and the blockade persisted at 48 hr. In contrast, baseline unstimulated GA (not shown) was transiently depressed, but it returned to normal by 6 hr following ingestion in each case.

The finding that in vitro and in vivo exposure of human granulocytes to ASA inhibited augmentation of GA by bacterial products suggested that salicylates
Fig. 1. Aspirin inhibition of augmented GA. Heparinized (15 units/ml) whole blood was incubated with either ET at 5 μg/ml (A) or 10% CF (B) alone (hatched bar) or with various concentrations of ASA (open bars) for 1 hr at 37°C. Baseline GA was 23 ± 4% in 10 healthy controls. Augmented GA was calculated by subtracting percentage unstimulated GA from percentage ET- or CF-stimulated GA. Inhibition of augmented GA at all concentrations of ASA differed significantly from those of augmented GA without ASA (p < 0.05. Student's t test). The data represent means ± SEM of four experiments.

might interfere with the granulocyte inflammatory response to bacterial infection in vivo. To explore this possibility, we used a mouse model of bacterial peritonitis. In preliminary experiments, ET at 5.0 μg/ml was shown to augment GA of mouse peritoneal granulocytes obtained after induction of sterile peritonitis. Aspirin at 1000 μg/ml (pH 7.4) blocked this increment in adherence, as was observed with human granulocytes. Type 19 pneumococcus, a strain of limited virulence, was used to induce bacterial peritonitis; 10⁵ organisms provoked an acute peritoneal exudate of granulocytes that could be quantitated. Five milligrams of sodium salicylate (chosen because of poor aqueous solubility of ASA), one-third the LD₅₀ of this drug, resulted in a 50% reduction in total peritoneal exudate cells and a 67% reduction in peritoneal exudate granulocytes recovered 2 hr following infection, as

Fig. 2. Effects of oral ingestion of ASA on augmented GA in four normal volunteers. Impairments of ET-augmented GA at 2, 4, 5, 24, and 48 hr differed from preingestion (time 0) augmented values (p < 0.05, Student's t test). Each data point represents the mean ± SEM of determinations in the 4 subjects.
SALICYLATE BLOCKS GRANULOCYTE ADHERENCE

Fig. 3. Effect of salicylate pretreatment on peritoneal exudate formation in experimental pneumococcal peritonitis. Total peritoneal exudate cells (hatched bars) and total granulocytes (open bars) in salicylate-treated mice (SAL) differed from those in saline controls (C) at 2 hr (p < 0.01, Student's t test). Data are expressed as means ± SEM of a representative experiment that included 12 animals at each time point.

Comparison with control mice (Fig. 3). Cultures of peritoneal fluid and blood in control mice at 2 and 4 hr showed few organisms (Table 1). In contrast, salicylate-treated mice had 1 log unit more pneumococci culturable in peritoneal fluid at 2 hr and 2 log units more organisms in blood at 2 and 4 hr. Furthermore, salicylate-treated mice showed a threefold reduction in the LD50 from 5 x 10^5 to 1.6 x 10^5 organisms (differ from saline-treated group, p < 0.01, Student's t test).

DISCUSSION

Previous studies have indicated that ASA either does not affect GA in vitro or only transiently depresses baseline GA in man after a single oral dose. Other investigations have demonstrated that sodium salicylate in vivo inhibits the adherence of granulocytes in sterile saline-induced rabbit peritoneal exudates. Our data indicate that in vitro exposure to ASA significantly blocks augmentation of human and mouse GA by bacterial products. Furthermore, oral administration of ASA to normal volunteers inhibits augmented GA in response to ET as long as 48 hr after ingestion, well beyond the normal clearance of ASA and its metabolites. The implications of these findings were evaluated in an experimental infection. Salicylates interfered with the in vitro augmentation of mouse peritoneal GA by ET and the in vivo acute granulocytic inflammatory response to pneumococcal peritonitis. Thus, pretreatment of mice with sodium salicylate significantly reduced granulo-

Table 1. Recovery of Pneumococci From Peritoneal Fluid and Blood Cultures in Experimental Pneumococcal Peritonitis

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Time After Infection With Type 19 Pneumococci</th>
<th>Quantitative Peritoneal Fluid Cultures (CFU/ml)</th>
<th>Quantitative Blood Cultures (CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>2 hr</td>
<td>80 ± 2*</td>
<td>7 ± 3</td>
</tr>
<tr>
<td>Salicylate</td>
<td>2 hr</td>
<td>860 ± 2†</td>
<td>3000 ± 3‡</td>
</tr>
<tr>
<td>Saline</td>
<td>4 hr</td>
<td>3 ± 3</td>
<td>0</td>
</tr>
<tr>
<td>Salicylate</td>
<td>4 hr</td>
<td>27 ± 9</td>
<td>2400 ± 3§</td>
</tr>
</tbody>
</table>

*Geometric mean ± SEM of a representative experiment with 12 animals per group.
†Diffs from saline control, p < 0.05. Student's t test.
‡Diffs from control, p < 0.01.
§Diffs from control, p < 0.001.
cyte migration into the exudate during the first 2 hr following bacterial challenge. The greater numbers of pneumococci in the blood at 2 and 4 hr suggested resultant impaired localization of infection, presumably accounting for the lowered LD50 inoculum of pneumococci in the salicylate-treated mice.

One interpretation of these observations is that salicylates reduced the influx of peritoneal granulocytes early in the course of infection and allowed pneumococci to multiply unchecked. The end result was inability to localize the infection, sepsis, and death. Although the current studies delineate effects of salicylate on GA, the greater lethality of pneumococcal infection in salicylate-treated mice also could reflect impairment of other granulocyte functions. However, aspirin in vitro did not inhibit lysosomal enzyme release or phagocytosis of zymosan by guinea pig granulocytes. Furthermore, in vivo ingestion of aspirin did not inhibit phagocytosis of Candida albicans by human granulocytes. Obvious difficulties are incurred in extrapolation of mouse data to humans; nonetheless, our data suggest that ASA may have an adverse influence on the outcome of infections.

REFERENCES

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