Acetylsalicylic Acid Reduces Vegetation Bacterial Density, Hematogenous Bacterial Dissemination, and Frequency of Embolic Events in Experimental Staphylococcus aureus Endocarditis Through Antiplatelet and Antibacterial Effects

Leon Iri Kupferwasser, MD; Michael R. Yeaman, PhD; Shelley M. Shapiro, MD, PhD; Cynthia C. Nast, MD; Paul M. Sullam, MD; Scott G. Filler, MD; Arnold S. Bayer, MD

Background—Platelets are integral to cardiac vegetations that evolve in infectious endocarditis. It has been postulated that the antiplatelet aggregation effect of aspirin (ASA) might diminish vegetation evolution and embolic rates.

Methods and Results—Rabbits with Staphylococcus aureus endocarditis were given either no ASA (controls) or ASA at 4, 8, or 12 mg·kg⁻¹·d⁻¹ IV for 3 days beginning 1 day after infection. Vegetation weights and serial echocardiographic vegetation size, vegetation and kidney bacterial densities, and extent of renal embolization were evaluated. In addition, the effect of ASA on early S aureus adherence to sterile vegetations was assessed. In vitro, bacterial adherence to platelets, fibrin matrices, or fibrin-platelet matrices was quantified with either platelets exposed to ASA or S aureus preexposed to salicylic acid (SAL). ASA at 8 mg·kg⁻¹·d⁻¹ (but not at 4 or 12 mg·kg⁻¹·d⁻¹) was associated with substantial decreases in vegetation weight (P<0.05), echocardiographic vegetation growth (P<0.001), vegetation (P<0.05) and renal bacterial densities and renal embolic lesions (P<0.05) versus controls. Diminished aggregation resulted when platelets were preexposed to ASA or when S aureus was preexposed to SAL (P<0.05). S aureus adherence to sterile vegetations (P<0.05) or to platelets in suspension (P<0.05), fibrin matrices (P<0.05), or fibrin-platelet matrices (P<0.05) was significantly reduced when bacteria were preexposed to SAL.

Conclusions—ASA reduces several principal indicators of severity and metastatic events in experimental S aureus endocarditis. These benefits involve ASA effects on both the platelet and the microbe. (Circulation. 1999;99:2791-2797.)

Key Words: aspirin ▪ embolism ▪ endocarditis ▪ platelets ▪ fibrin

Despite advances in antibiotic therapies and cardiac surgery, infective endocarditis (IE) remains a severe illness, with high mortality and complication rates.¹ Embolic events in IE occur in 20% to 40% of patients, with no apparent decrease over the last decade.²³ Platelets are an integral component of the vegetation.⁴ Because of its antiplatelet aggregation effect, it has been postulated that acetylsalicylic acid (ASA) might diminish vegetation evolution and embolic rates in IE.⁵ However, clinical data elucidating the potential role of ASA in IE are scarce.

The current investigation was designed to determine whether ASA produces beneficial effects on the severity of experimental IE. The specific effects of ASA and its metabolite salicylic acid (SAL) on both the platelet and the infecting organism were determined in the context of important events in IE pathogenesis, including bacteria-induced platelet aggregation and bacterial adherence to platelets and fibrin.⁶⁷ We used Staphylococcus aureus, a prototypical endovascular pathogen, to induce IE. To reflect the clinical situation, ASA was administered after induction of IE. In vitro adherence was determined with the use of a variety of S aureus strains to assess the comparative interactions of distinct strains.

Methods

General Methods

Table 1 describes the S aureus strains used in this study. All strains have been well characterized and are known to induce experimental...

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TABLE 1. Bacterial Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Reference</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISP479C</td>
<td>8</td>
<td>Spontaneous plasmid-cured derivative of parental strain ISP479</td>
</tr>
<tr>
<td>ISP479R</td>
<td>8</td>
<td>Isogenic variant of ISP479 constructed by transposon mutagenesis</td>
</tr>
<tr>
<td>RN6390</td>
<td>9</td>
<td>Wild-type prototypic strain</td>
</tr>
<tr>
<td>6850</td>
<td>10</td>
<td>Clinically isolated wild-type strain</td>
</tr>
<tr>
<td>8325-4</td>
<td>11</td>
<td>Wild-type derivative of ATCC8325 cured of prophages</td>
</tr>
</tbody>
</table>

IE. Bacteria were grown overnight in brain-heart infusion (BHI) broth (Difco), washed, resuspended in PBS, adjusted to an OD

Microbiological Parameters
At the time the animals were euthanized, valvular vegetations from each animal were removed, pooled, weighed, homogenized in 0.5 mL of sterile normal saline, and quantitatively cultured. The total quantity of bacteria within vegetations was determined as log

Measurement of PGI
Prostacyclin (PGI

Kidney Histopathology
To assess and quantify the influence of ASA treatment on the incidence and extent of hematogenous embolization, renal histopathology was performed. Kidneys were removed, fixed in 10% neutral buffered formalin, and longitudinally sectioned. Grossly apparent lesions were identified, and representative sections from each kidney were submitted for histological assessment. Tissues were processed routinely, stained with periodic acid–Schiff, and examined in a blinded manner for areas of renal infarction and microabscess formation. Individual areas of infarction were outlined, measured, and added to generate an overall infarction area. The total area of kidney infarction was then calculated as a percentage of the entire longitudinal section area. A scoring system was applied that divided these percentages into 5 relative grades to compare the amount of kidney infarction between animal groups (see below).

Echocardiography
To serially assess the influence of ASA on vegetation growth, transthoracic echocardiography was performed daily in all animals starting 24 hours after catheterization (preinfection) until the time the animals were euthanized. A 7.5-MHz transducer linked to an ultrasound unit (Hewlett Packard, Sonos Intravascular) was used. The transducer was placed in the third or fourth left intercostal space to achieve a parasternal long-axis view, which was defined as the region of interest. The presence and maximal diameter of vegetations at the aortic or mitral valve were evaluated. Total maximal vegetation diameter was calculated as the sum of all single maximal vegetation diameters. Vegetations were only included for analysis when their presence could be confirmed during autopsy and when their attachment to the valvular surface was visible. Catheter-associated vegetations were not included.

In Vivo Adherence of S. aureus to Vegetations and Bacteremia Clearance
The growth of the infected vegetation in IE correlates with ongoing vegetation reseeding by microbes invading the bloodstream from extracardiac sites of infection. Therefore, microbial adhesion to the vegetation plays a major role in this regard. In vivo studies were performed to evaluate whether ASA would affect S. aureus adhesion to sterile vegetations. To differentiate ASA effects on platelets from ASA effects on the microorganism, 3 groups of rabbits were challenged intravenously with S. aureus ISP479C 48 hours after catheterization: (1) controls (no ASA); (2) ASA (8 mg/kg) administered intravenously 5 hours before bacterial challenge to obtain complete platelet inhibition after establishment of a sterile vegetation but before microbial challenge; or (3) no ASA given, but animals were challenged with S. aureus cultured overnight in medium containing SAL (50 µg/mL). An inoculum of 5 x 10³ CFU was chosen. In previous in vivo adherence studies, this inoculum was found to provide detectable bacterial levels within vegetations at an early time point after intravenous bacterial challenge. At 30 minutes after intravenous bacterial challenge, animals were euthanized and vegetations removed and quantitatively cultured. In vivo adherence was expressed as mean CFU±SD per vegetation. Quantitative blood cultures were obtained at 1 minute and 30 minutes after intravenous challenge to ensure that any adherence differences between groups were not due to differences in clearance of bacteremia.
measuring its stable metabolite, 6-keto-PGF_1α by radioimmunoassay as previously described. 18

*S. aureus*–Induced Platelet Aggregation

To evaluate whether ASA inhibits bacteria-induced platelet aggregation in vitro, *S. aureus*–induced platelet aggregometry was performed as previously described. 19 Aggregation was quantified by measurement of the maximum positive change in light transmission (extent of aggregation), the interval between the addition of bacteria to the platelet suspension and the onset of aggregation (lag time), and the duration required for complete aggregation (total aggregation time).

The effect of ASA on bacteria-induced platelet aggregation was assessed in 2 parallel ways: (1) *S. aureus*–induced aggregation of platelets, either control or preexposed to ASA, and (2) *S. aureus*–induced aggregation of control platelets with the organism grown in either plain or SAL-containing medium.

### Direct Platelet–*S. aureus* Binding

The influence of ASA on the direct binding of *S. aureus* to platelets in suspension was quantified by flow cytometry as described previously. 19 We calculated the percentage of bacteria bound to platelets by dividing the number of dually labeled particles (representing bacteria bound to platelets) by the number of particles labeled with Hoechst 33342 (ie, total bacteria), and multiplying by 100. The effects of ASA on bacteria-platelet binding were analyzed in parallel either with platelets exposed to ASA or with *S. aureus* strains precultured in the presence of SAL.

### *S. aureus* Adherence to Fibrin-Platelet and Fibrin Matrices In Vitro

To simulate binding of *S. aureus* to vegetations, the adherence of *S. aureus* was evaluated to either a fibrin-platelet matrix or a fibrin matrix as previously described. 6 Adherence was expressed as the number of adherent organisms in percent of the original inoculum. As in the platelet binding and aggregation studies described above, the effects of ASA on adherence were determined either by exposure of the platelets to ASA or preincubation of *S. aureus* in the presence of SAL.

### Statistics

Mean±SD values were calculated for continuous variables. Differences between groups were analyzed by the appropriate nonparametric tests. A 2-way repeated-measures analysis was performed to test for time-related and group-related differences in the echocardiographic data (SAS). A P value <0.05 was considered significant.

### Results

#### In Vitro Susceptibilities and SAL Serum Levels

At the bacterial inoculum used in the ASA treatment study, all *S. aureus* strains tested had identical MICs to ASA and SAL (7.5 and 8 mg/mL, respectively), which indicates that these compounds have no significant growth-inhibitory effect against *S. aureus* at serum-achievable levels. SAL serum levels in healthy rabbits were nondetectable 12 hours after ASA administration at all doses. In contrast, the decline of SAL levels in animals with IE given an 8 mg/kg dose was substantially less rapid than in uninfected animals, with SAL serum levels ranging between 47 and 53 μg/mL over a 12-hour period and 26 to 30 μg/mL 24 hours after ASA administration. Because renal seeding is a regular feature of experimental *S. aureus* IE (see below), the decreased clearance of SAL in animals with IE is likely due to functional renal impairment in the presence of this systemic infection.

### ASA Treatment Study

#### Endocardial Lesions

Treatment with ASA at 4 mg · kg⁻¹ · d⁻¹ yielded a trend toward lower vegetation weights and vegetation bacterial densities than in the control group, although statistical significance was not reached (Table 2). These parameters were significantly different when the 8 mg · kg⁻¹ · d⁻¹ dose regimen was compared with controls. In contrast, the 12 mg · kg⁻¹ · d⁻¹ ASA dose regimen showed no substantial decreases in any of these parameters compared with controls.

Differences in vegetation growth as determined echocardiographically are illustrated in Figures 1 and 2. In control animals, mean vegetation size increased 4-fold between 24 and 72 hours after infection. The 4 and 8 mg · kg⁻¹ · d⁻¹ ASA dose regimens essentially eliminated increases in vegetation size over this time period. In control rabbits, a substantial and rapid drop in vegetation size was observed between 72 and 96 hours after infection, which suggests vegetation fragmentation and embolization in the turbulent blood stream. The 12

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**TABLE 2. ASA Treatment Study: Microbiological Data**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Vegetation Weight, mg</th>
<th>Vegetation Bacterial Counts</th>
<th>Kidney Bacterial Counts,</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11</td>
<td>74±41</td>
<td>7.11±1.24</td>
<td>8.36±1.03</td>
<td></td>
</tr>
<tr>
<td>ASA 4 mg · kg⁻¹ · d⁻¹</td>
<td>8</td>
<td>36±30</td>
<td>4.97±1.45</td>
<td>6.59±1.77</td>
<td>0.05</td>
</tr>
<tr>
<td>ASA 8 mg · kg⁻¹ · d⁻¹</td>
<td>11</td>
<td>19±8*</td>
<td>4.35±1.26*</td>
<td>6.13±1.37*</td>
<td></td>
</tr>
<tr>
<td>ASA 12 mg · kg⁻¹ · d⁻¹</td>
<td>10</td>
<td>55±22</td>
<td>6.17±1.59</td>
<td>7.48±1.69</td>
<td>0.05</td>
</tr>
</tbody>
</table>

*P<0.05 vs control.
mg · kg⁻¹ · d⁻¹ ASA dose regimen did not affect vegetation size as compared with controls.

Kidney Lesions
Compared with controls, there were substantial decreases in bacterial densities within kidney lesions in the 4 and 8 mg · kg⁻¹ · d⁻¹ ASA with a single vegetation (⁎) (total size = 0.21 cm). Bar = 1 cm. LV indicates left ventricle; LA, left atrium.

In Vivo S aureus Adherence to Vegetations and Bacteremia Clearance
S aureus precultured in the presence of SAL exhibited a significantly reduced ability to bind to sterile vegetations. In contrast, a lower impact on vegetation adherence was observed when animals were pretreated with ASA before challenge with unmodified S aureus (Table 3). No differences in quantitative blood cultures were seen at 1 or 30 minutes after IV challenge, which confirms that the observed differences in vegetation adherence data described above were not due to differences in bacteremia clearance rates.

6-Keto-PGF₁α Plasma Levels
Figure 5 illustrates the 6-keto-PGF₁α plasma levels in rabbits with established IE relative to different ASA dose regimens.

In the 12 mg/kg ASA dose regimen, PGI₂ synthesis was inhibited to a substantially greater extent and for a longer duration than with the 4 and 8 mg/kg ASA dose regimens.

S aureus–Induced Platelet Aggregation
In control studies, complete inhibition of collagen-induced platelet aggregation was observed in rabbit platelets after intravenous ASA therapy at 8 and 12 mg/kg. At 4 mg/kg, ASA-induced inhibition of platelet aggregation was incomplete, with 57% maximal light transmission.

A significant decrease in the extent of S aureus–induced platelet aggregation was seen when PRP was obtained from ASA-pretreated rabbits compared with control PRP (Table 4). A significant prolongation in lag time and total aggregation time was found when aggregation of control PRP was induced by S aureus precultured in the presence of SAL. Aggregation data for S aureus ISP 479C were virtually identical to those seen with strains ISP479R, RN6390, 6850, and 8325-4 (data not shown).

S aureus–Platelet Binding
S aureus RN6390 cells cultured in the presence of SAL exhibited significantly less binding to control platelets than S aureus cells cultured in the absence of SAL (20.4±24% and 71±21%, respectively; P=0.004; Figure 6). In contrast, no differences in S aureus–platelet binding occurred when platelets from ASA-pretreated or control rabbits were mixed with control S aureus cells (65±12% or 66±9% of the bacteria were bound to platelets, respectively). An identical effect of ASA and SAL on adhesion of S aureus to platelets was seen with ISP479C (data not shown).

Fibrin-Platelet Matrix
Binding of S aureus strain ISP479C to fibrin-platelet and fibrin matrices was significantly reduced when bacteria were precultured in SAL (Table 5). Similar significant reductions of fibrin-platelet and fibrin matrix binding by S aureus strains ISP479R, RN6390, 6850, and 8325-4 were observed after preexposure to SAL (data not shown).
Discussion

Our data demonstrate significant decreases in vegetation growth (echocardiographically) and mass (weights), as well as bacterial densities, in animals with established IE given ASA therapy compared with untreated controls. Our findings are consistent with previous data in a similar model of S aureus IE. However, results of our treatment study identified an additional major beneficial effect of ASA by showing a significant reduction in the incidence of embolization. Thus, both the bacterial densities within the renal lesion and the degree of embolic renal infarction were substantially reduced by ASA therapy. Potential mechanisms by which ASA renders its antiembolic effects include smaller vegetations, yielding embolic fragments that are less likely to lodge within the vascular system, or less-friable vegetations, which infrequently fragment to cause embolization.

In vivo, ASA effects on IE were dose dependent, with lesser or no influence in animals given ASA at 4 or 12 versus 8 mg·kg⁻¹·d⁻¹, associated with an incomplete blockade of platelet aggregation at the 4 versus 8 mg/kg dose as shown by collagen-induced aggregometry. In parallel with our data, a previous study²⁰ reported an attenuation of principal IE parameters at lower (5 and 10 mg·kg⁻¹·d⁻¹ PO) compared

TABLE 3. In Vivo Adherence and Clearance of Bacteremia

<table>
<thead>
<tr>
<th></th>
<th>Blood Culture (Mean log₁₀ CFU/ml±SD) After</th>
<th>Adherence, Mean CFU/Vegetation±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n 1 min 30 min</td>
<td></td>
</tr>
<tr>
<td>Group I</td>
<td>7 4.2±3.3 1.5±1.1</td>
<td>110±54</td>
</tr>
<tr>
<td>Group II</td>
<td>7 4.1±3.6 1.6±1.3</td>
<td>75±32</td>
</tr>
<tr>
<td>Group III</td>
<td>7 4.4±4.1 1.2±0.6</td>
<td>53±15*</td>
</tr>
</tbody>
</table>

*P<0.05 vs controls.

*Group I indicates controls; group II, animals pretreated with 8 mg/kg ASA before challenge; and group III, nonpretreated animals challenged with S aureus preexposed to SAL.

Figure 4. Representative sections of kidneys from rabbits with S aureus endocarditis. A and C, Rabbits without ASA treatment. There is considerable infarction of cortical (C) and medullary (M) areas. Note the adjacent viable area (V). B and D, Rabbits treated with ASA at 8 mg·kg⁻¹·d⁻¹. There are no areas of infarction or necrosis in the cortex or medulla. C and D, Hematoxylin and eosin stain; magnification ×150.

Figure 5. 6-Keto-PGF₁α plasma levels after an intravenous bolus administration of 4, 8, or 12 mg/kg ASA in each of 2 rabbits with established IE.
with higher (20 and 50 mg \cdot kg^{-1} \cdot d^{-1}) ASA doses. As shown in the present study, the mechanisms underlying this apparent paradoxical effect of ASA in this model correlate with a dose-dependent suppression of PG12 synthesis by ASA. Thus, we observed substantial inhibition of PG12 synthesis with the 12 mg \cdot kg^{-1} \cdot d^{-1} regimen, whereas the 4 and 8 mg \cdot kg^{-1} \cdot d^{-1} regimens had little effect. PG12 inhibits platelet adhesion and thrombus formation on the subendothelium. Therefore, it is reasonable to suggest that inhibition of PG12 synthesis at higher ASA dose regimens results in a proaggregation state of platelets at the site of endothelial damage and vegetation formation, fostering evolution rather than mitigation of the IE process.

![Flow-cytometric analysis of S aureus ISP479C adherence to platelets.](image)

**Figure 6.** Flow-cytometric analysis of *S aureus* ISP479C adherence to platelets. I, FITC-labeled platelets appear as a focused population of cells. II, *S aureus* cells labeled with Hoechst 33342 dye appear as a distinct population. III, Dually labeled particles indicate that adherence has occurred. *S aureus* grown overnight in SAL (B) exhibited reduced adherence compared with controls (A). CMFDA indicates chloromethylfluorescein diacetate (molecular probes).

**TABLE 4.** *S aureus*–Induced Platelet Aggregation (ISP479C)

<table>
<thead>
<tr>
<th>Adherence %</th>
<th>Lag Time, min</th>
<th>Total Aggregation Time, min</th>
<th>Maximum Light Transmission, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>26.1 ± 3.9</td>
<td>35.8 ± 4.3</td>
</tr>
<tr>
<td>Plasma exposed to ASA</td>
<td>28.9 ± 3.8</td>
<td>36.5 ± 4.1</td>
<td>59.7 ± 4.9*</td>
</tr>
<tr>
<td><em>S aureus</em> precultured in SAL</td>
<td>39.4 ± 5.6*</td>
<td>50.2 ± 5.2*</td>
<td>84.3 ± 4.6</td>
</tr>
</tbody>
</table>

*P<0.05 vs control.

**TABLE 5.** In Vitro Adherence of *S aureus* (ISP479C)

<table>
<thead>
<tr>
<th>Adherence %</th>
<th>Fibrin-Platelet Matrix Adherence, % of Inoculum</th>
<th>Fibrin Matrix Adherence, % of Inoculum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.7 ± 0.4</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>PRP/PFP from ASA pretreated rabbits; <em>S aureus</em> grown in conventional medium</td>
<td>2.6 ± 0.5</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>PRP/PFP from nonpretreated rabbits; <em>S aureus</em> cultured in SAL</td>
<td>1.5 ± 0.3*</td>
<td>0.7 ± 0.3*</td>
</tr>
</tbody>
</table>

*P<0.05 vs control.

Several studies have shown that the capacity of selected IE pathogens to evoke platelet aggregation in vitro parallels their propensity to induce experimental IE. Because of the in vivo effects of ASA on IE, its influence on *S aureus*–induced platelet aggregation was also examined. The use of either platelets isolated from animals after receiving ASA or *S aureus* precultured in the presence of SAL resulted in an inhibition of platelet aggregation. *S aureus*–induced rabbit platelet aggregation is a biphasic process dependent on the capacity of the organism to attach to the platelet surface and subsequently to bind to and cross-link adjacent platelets. Thus, the inhibition of bacteria-induced platelet aggregation by ASA involves both antimicrobial and antiplatelet mechanisms, each of which influences distinct mechanisms in the process of aggregation.

Collagen-mediated platelet aggregation is completely blocked by ASA in vitro. This has key relevance to IE, because damage of valvular endothelium exposes the collagen-rich subendothelial stroma. Thus, ASA inhibition of collagen-mediated platelet aggregation would be predicted to reduce the size of the evolving vegetative lesion on damaged heart valves. Because circulating bacteria can adhere directly to platelets on damaged endocardium, this ASA effect might reduce the net number of functional binding sites for circulating microbes. Therefore, we evaluated whether ASA/SAL also interferes with adhesion of the microbe to the vegetation. Moreover, to discriminate between effects of ASA on the platelet from those on the organism, we used parallel strategies in which either the platelet or the organism was exposed separately to ASA or its metabolite, SAL, respectively, before study. In animals with indwelling transaortic valve catheters and sterile vegetations, administration of ASA before *S aureus* challenge reduced early colonization of the valve by 27%, whereas pretreatment of the organism with SAL reduced valvular adhesion to an even greater extent (51%). Thus, for the first time, the antistaphylococcal effects of SAL were shown in vivo, revealing an attenuation of a key component in the pathogenesis of IE. Additionally, pretreatment of several *S aureus* strains with SAL substantially reduced their ability to bind to an artificial vegetation (fibrin-platelet matrix) as well as to its components (platelets and platelet-free fibrin matrix) by ≈50% each. Collectively, these in vivo and in vitro adherence studies clearly implicate 2 distinct effects of ASA and its metabolite SAL on mitigating attachment of *S aureus* to the...
vegetative lesion and its component constituents: effects on the platelet and on the organism.

Conclusions
Data from the present study provide compelling evidence of the salutary and dose-dependent influence of ASA and SAL in mitigating several distinct aspects of established S aureus IE, including vegetation growth, vegetation microbial proliferation, and renal embolization. Moreover, our studies demonstrate that the beneficial effects of ASA in this model are due to inhibition of platelet aggregation as well as of the capabilities of the infecting organism to adhere to key components of the vegetative lesion. These findings have significant implications for the use of ASA as adjunctive therapy in the treatment of S aureus IE.

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References
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