Effect of Aspirin on Thrombogenesis and on Production of Experimental Aortic Valvular *Streptococcus viridans* Endocarditis in Rabbits

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Because thrombus formation at the site of endothelial injury has been thought to be a critical step in the pathogenesis of bacterial endocarditis, the effect of aspirin on experimental valvular thrombosis and bacterial endocarditis in rabbits was evaluated. Aortic valvular injury and thrombosis were induced in aspirin-treated and control rabbits with intracardiac catheters. A subsequent inoculation of *Streptococcus viridans* resulted in the development of infective endocarditis. Rabbits were sacrificed as early as 6 hr, and the effectiveness of aspirin was determined by the weight of the sterile vegetations and the quantitation of bacteria in the thrombotic vegetation. Aspirin, in levels in excess of 50 mg/dl did not attenuate the evolution of infective endocarditis, since the formation of sterile thrombotic vegetation and bacterial endocarditis in aspirin-treated rabbits was similar to those in controls.

THE USEFULNESS OF ASPIRIN for the prevention of thrombosis has been extensively investigated, and several studies have provided conflicting results.[1-5] Aspirin can increase the bleeding time in normal individuals[6] and alter platelet function in vitro, probably by interfering with prostaglandin synthesis in platelets.[7] The action of aspirin in vivo is not clear, but Weiss et al.[5] suggest that aspirin can decrease thrombus formation by inhibiting platelet-platelet cohesion, but not by affecting platelet-subendothelial adhesion.

We report the results of our investigation of the ability of aspirin to prevent experimental endocarditis in rabbits. Thrombosis is thought to be an important factor in the pathogenesis of experimental bacterial endocarditis.[8,9] Our study was designed to determine if aspirin would prevent thrombus formation on aortic valves damaged by a catheter which is inserted into the lumen of the carotid artery and passes across the aortic valve into the left ventricle. This method has been used to demonstrate that coumadin has an antithrombogenic action in rabbits.[10] Our study has assessed the effects of aspirin on the early phases of the genesis of thrombosis by electron microscopy and by quantitating the weight of sterile thrombi, as well as on the susceptibility to infection of the sterile thrombi by *Streptococcus viridans*.

MATERIALS AND METHODS

Microorganism. A strain of *S. viridans* isolated from the blood of a patient with endocarditis was used in all experiments. Stock cultures were maintained by storing aliquots of an

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18-hr culture in Todd-Hewitt broth (Difco Lab., Detroit, Michigan) at −20°C. For each experiment the stock culture was thawed, incubated in 10 ml of Todd-Hewitt broth overnight at 37°C, and centrifuged at 3000 rpm for 30 min. The bacterial sediment was washed three times in 10 ml of sterile saline and resuspended in saline. The suspension was serially diluted in saline to prepare appropriate inocula for injection.

Production of endocarditis. Experimental *S. viridans* endocarditis was produced in aspirin-treated and untreated rabbits by a previously described method.[11] Briefly, white female rabbits weighing 2 to 3 kg (West Jersey Biological Farm, Wenonah, N.J.) were anesthetized with 60 mg of sodium pentobarbital intravenously. Polyethylene tubing (Intramedic PE 90/S36, Clay Adams, Parsippany, N.J.) was passed through the right carotid artery into the left ventricle and secured. Twenty-four hours later, when thrombi had formed over the injured aorta and aortic valve,[9] a 1-ml inoculum containing 10^4–10^8 colony-forming units (CFU) of *S. viridans* was injected into a marginal ear vein. Two hours, 1 day, or 3 days later the animal was killed by injection of sodium pentobarbital intravenously. Rabbits dying spontaneously were discarded. The vegetations were excised, weighed, and homogenized in sterile saline with a Teflon tissue grinder.

Bacterial enumeration. Serial tenfold dilutions of the inocula or homogenized tissue suspension were made in sterile saline. The number of viable CFU of *S. viridans* per milliliter of blood or inocula or per gram of vegetation was determined by plating 1.0 and 0.1 ml of blood, inocula, or homogenized vegetation into trypticase soy agar blood plates and plating 0.1 ml of each serial dilution. The blood agar plates were incubated at 37°C for 24 hr.

Aspirin administration. Aspirin treatment was begun 1 hr prior to the introduction of the intracardiac catheter. One group of rabbits received a single 600-mg dose of aspirin (St. Joseph's Aspirin for Children, Plough, Memphis, Tenn.) that was suspended in 10 ml of water and was administered intragastrically via an infant feeding tube (No. 8 French).

To maintain continuously high serum salicylate levels, a second group of animals received multiple doses of 600-mg suspensions which were also begun 1 hr prior to the catheterization and which were repeated every 6 hr to insure that newly formed platelets were exposed to aspirin until the animal was sacrificed, 24 hr after intravenous injection of streptococci. These repeated doses were administered via a permanent gastrostomy tube placed aseptically through the anterior abdominal wall 24 hr prior to the intracardiac catheterization. Serum salicylate levels were determined by the method of Trinder[12] at various times after aspirin administration. Control groups of rabbits received only 10-ml doses of water. In order to monitor the pharmacologic activity of the intragastrically administered aspirin, platelet aggregation to collagen was measured before and at periods from 1 hr to 3 days after aspirin administration.[13]

Microscopic examination of vegetations. The thrombi which formed in aspirin-treated and control rabbits were compared by light and electron microscopic examination by previously reported methods.[14] Rabbits treated with single and multiple doses of aspirin and control rabbits were killed by injecting pentobarbital intravenously 6 and 24 hr after the intracardiac catheterization. The thrombi were removed together with the underlying aorta and valve leaflets; were fixed for 12 hr in 2.5% glutaraldehyde in 0.1 *M*, pH 7.4 phosphate buffer; were postfixed for 3 hr in 1% osmium tetroxide in the same buffer; and were embedded in Epon 812. Thick sections (1 μm) were stained with toluidine blue and examined by light microscopy, while thin sections were stained with lead and examined with a Philips EM300 electron microscope.

RESULTS

In order to ensure that newly formed platelets were exposed to aspirin, salicylate levels were determined throughout the time of the experiment. A single

Table 1. Weight of Sterile Aortic Valve Vegetation After 24 hr of Intracardiac Catheterization

Rabbits (*N*)	Vegetation Weight (Mean ± SE)
Control (9)	30.4 ± 3.4 mg
Aspirin-treated (11)	50.9 ± 15.4 mg

N, number of rabbits.

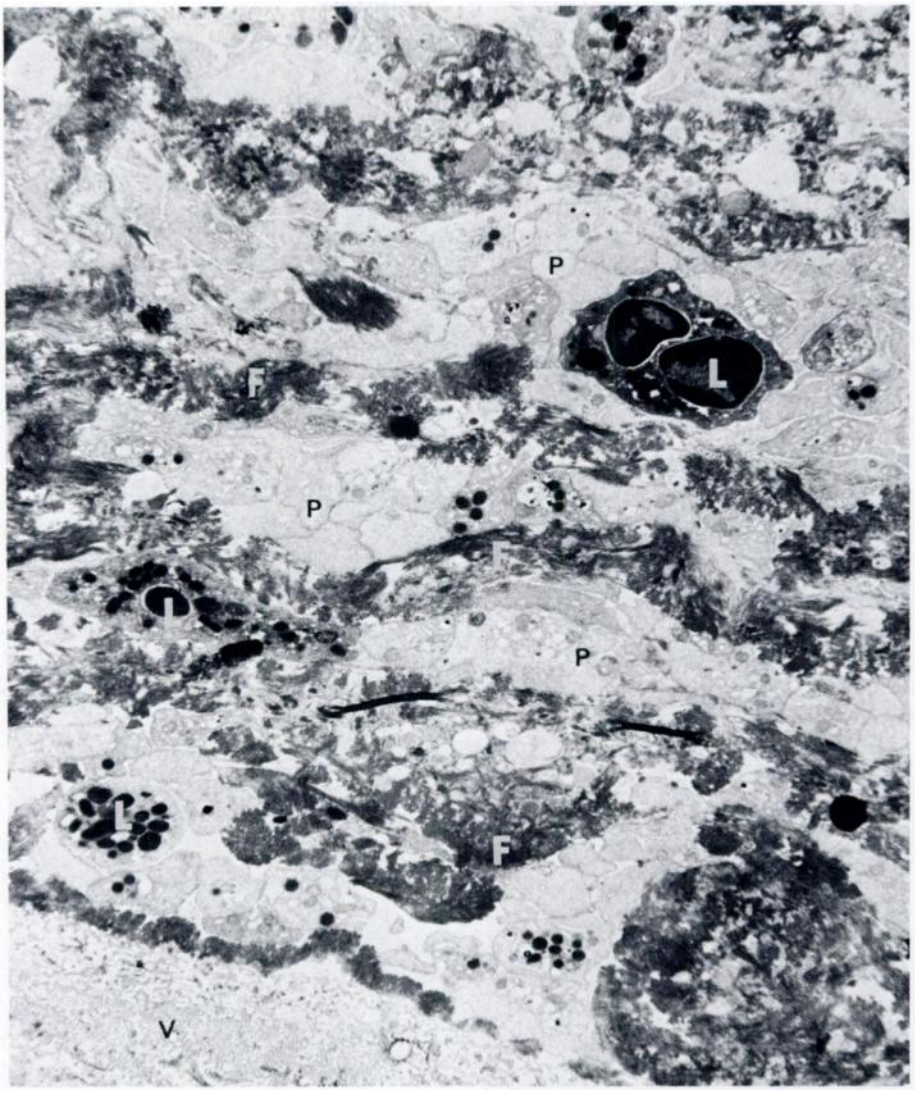

Fig. 1. Electron microscopy of thrombus from aspirin-treated rabbit. This thrombus and the underlying aortic valve leaflet (V) had been removed 6 hr after intracardiac catheterization from a rabbit receiving a single dose of aspirin. No difference was noted between the fine structure of this thrombus and those from untreated animals. Most of the aggregated platelets (P) were extensively degranulated. The endothelium covering the valve had been removed by the catheter. F, fibrin; L, polymorphonuclear leukocytes.

600-mg dose of aspirin in seven rabbits produced mean salicylate levels of 28 mg/dl at 2 hr and 20 mg/dl at 5 hr. Repetition of this dose every 6 hr in two rabbits produced levels of 72 and 76 mg/dl 2 hr after the last of five doses, and in four rabbits produced a mean level of 51 mg/dl 6 hr after the last of eight doses. Evidence of the effect of aspirin on platelet function was the marked attenuation of platelet aggregation in response to collagen (average concentration of 2 μg/ml) in vitro of platelets from aspirin-treated rabbits in

comparison to platelets from untreated rabbits. Approximately half of each of the groups of rabbits that received multiple 600-mg doses of aspirin died spontaneously within 48 hr. Autopsy failed to disclose the cause of death, which was presumably due to the excessively high serum concentrations of salicylate found in some animals.

Aspirin treatment had no apparent effect on intracardiac thrombus formation or subsequent development of *S. viridans* endocarditis. The mean weight of sterile aortic valve thrombi after 24 hr of intracardiac catheterization was not significantly different ($p > 0.05$) between 11 rabbits receiving repeated aspirin doses (mean weight ± SE, 50.9 ± 15.4 mg) and 9 control rabbits (mean weight ± SE, 30.4 ± 3.4 mg) (Table 1). The sterile thrombi which formed in both the treated and control rabbits appeared similar on light and electron microscopy after 6 and 24 hr of intracardiac catheterization. Thrombi from aspirin-treated rabbits contained well-aggregated platelets that were extensively degranulated (Fig. 1).

Two hours after injection of streptococci intravenously, the weights of vegetation and the number of the streptococci (CFU per gram of vegetation) were not significantly different ($p > 0.05$ by t test for unpaired observations) between rabbits treated with a single dose of aspirin and control rabbits (Table 2). The numbers of infected rabbits and weights and streptococcal titers of vegetation were similar at 3 days after inoculation of the bacteria (Table 3). The dose of streptococci required to infect 50% of rabbits, as estimated by the Reed-Muench method,[15] was $10^{5.2}$ CFU in both 14 rabbits treated with multiple doses of aspirin and 12 control rabbits which had been challenged with 10^4–10^7 streptococci and killed 24 hr later.

DISCUSSION

This study indicates that aspirin does not retard thrombosis on a traumatized aortic valve in rabbits. It demonstrates that the extent of sterile thrombus formation and the subsequent evolution of infective endocarditis are identical in control and aspirin-treated animals. The failure of aspirin to inhibit the development of infective endocarditis also provides indirect evidence that aspirin does not inhibit thrombosis under these experimental conditions, based on

Table 2. Infective Endocarditis 2 hr Following $\log_{10}$ 8.6 CFU *S. viridans* Injected Intravenously

	Rabbit	Vegetation		Blood
		Weight (mg)	$\log_{10}$ CFU/g	($\log_{10}$ CFU/ml)
Aspirin	1	33	5.3	<1.0
	2	56	3.5	<1.0
	3	130	4.4	<1.0
	4	45	5.0	<1.0
Mean ± SE		66.0 ± 18.9	4.6 ± 0.3	
No aspirin	5	31	6.9	<1.0
	6	33	6.4	<1.0
	7	8	4.8	<1.0
	8	37	4.9	<1.0
Mean ± SE		27.3 ± 5.7	5.8 ± 0.5	

Rabbits either given a single 600-mg dose of aspirin intragastrically 1 hr prior to intracardiac catheterization, or untreated.

Table 3. Infective Endocarditis 3 Days Following 10^4–10^8 CFU *S. viridans* Injected Either Into Rabbits Given a Single 600-mg Dose of Aspirin Intragastrically 1 hr Prior to Intracardiac Catheterization or Into Untreated Rabbits

Aspirin				No Aspirin			
Inoculum	Vegetation		Blood	Inoculum	Vegetation		Blood
(log_{10}CFU)	Weight	log_{10}CFU/g	log_{10}CFU/ml	(log_{10}CFU)	Weight	log_{10}CFU/g	log_{10}CFU/ml
8.0	42	7.1	<1.0	8.0	67	8.5	2.0
	56	8.0	<1.0		26	3.4	<1.0
	70	3.6	<1.0				
6.0	65	8.9	<1.0	6.0	81	9.0	2.0
	48	8.5	<1.0		60	8.5	2.0
	150	8.1	3.3		78	8.5	2.0
4.0	43	4.4	<1.0	4.0	17	4.1	<1.0
	28	6.5	<1.0		22	<2.0*	<1.0

Weight given in milligrams.
*Sterile.

evidence that thrombus formation is required for subsequent development of endocarditis. Durack and Beeson[9] have provided data that experimental bacterial endocarditis will develop at the site of the catheter-induced valvular thrombosis. However, Hook and Sande[10] question the primary role of thrombus formation in the pathogenesis of experimental endocarditis because of the absence of histologic evidence of thrombosis at the sites of bacterial adherence and growth in warfarin-treated rabbits. Their study does not invalidate the contention of Durack and Beeson of the importance of thrombosis for the development of infective endocarditis. In Hook and Sande's study, rabbits were sacrificed 5 days following infection. This time point was most likely too late in the evolution of the endocarditis to detect platelet interactions or thrombus formation at the time of initial infection. Indeed, in a study of the evolution of a thrombus in a large artery, platelets at 7–24 hr were found to have undergone disintegration with replacement by fibrin.[16]

In our study, aspirin administration did not attenuate the induction of valvular thrombosis and the evolution of infective endocarditis. The rabbits were sacrificed and the valves examined for weight of vegetation within 6 hr, which would be sufficiently early to detect differences in vegetation size in aspirin-treated rabbits. It should be pointed out that our study demonstrated that platelet-subendothelial interaction was not altered in vivo in aspirin-treated rabbits and that platelets appeared to be degranulated. Weiss et al.,[5] using an ex vivo aortic vessel model, have also commented on this point, suggesting that aspirin did not inhibit platelet-subendothelial adhesion or platelet degranulation. However, Weiss et al.[5] concluded that aspirin inhibited platelet-platelet cohesion and thus thrombus formation.

There are several possible reasons why aspirin was ineffectual under our experimental conditions. Although aspirin is known to alter platelet function for the life span of the cell, newly-formed platelets may not have been exposed to aspirin since aspirin is rapidly hydrolyzed in blood and tissues to salicylic acid, which is not an anti-platelet agent. This possibility is not likely because high

doses of aspirin had been administered every 6 hr. The intensity of the stimulus for thrombogenesis due to the continuous presence of an intracardiac catheter may have been sufficiently strong and persistent to overcome the aspirin-induced platelet defect. Another possibility is that local hemodynamic factors at the site of the aortic valve may have rendered aspirin ineffective in inhibiting thrombosis.

A similar failure of aspirin to inhibit thrombosis has been reported in the traumatized canine coronary and femoral arteries.[4] The present study provides evidence that high doses of aspirin do not have any effect on the evolution of thrombosis in a new experimental model.

REFERENCES

1. Boston Collaborative Drug Surveillance Program: Regular aspirin intake and acute myocardial infarction. Br Med J 1:440-443, 1974

2. Salzman EW, Harris WH, DeSanctis RW: Reduction in venous thromboembolism by agents affecting platelet function. N Engl J Med 284:1287-1292, 1971

3. Sullivan JM, Harken DE, Gorlin R: Pharmacologic control of thromboembolic complications of cardiac valve replacement. N Engl J Med 284:1391-1394, 1971

4. Moschos CB, Lahiri K, Peter A, et al: Effect of aspirin upon experimental coronary and non-coronary thrombosis and arrhythmias. Am Heart J 84:525-530, 1972

5. Weiss HJ, Thomas BT, Baumgartner HR: Impaired interaction (adhesion-aggregation) of platelets with the subendothelium in storage-pool disease and after aspirin ingestion. N Engl J Med 293:619-623, 1975

6. Evans G, Mustard GF, Parkham MA: Spontaneous bruising. Lancet 2:724-725, 1970

7. Smith JB, Willis AL: Aspirin selectively inhibits prostaglandin production in human platelets. Nature (New Biol) 231:235-237, 1971

8. Angrist AA, Oka M: Pathogenesis of bacterial endocarditis. JAMA 183:249-252, 1963

9. Durack DT, Beeson PB: Experimental bacterial endocarditis. I. Colonization of a sterile vegetation. Br J Exp Pathol 53:44-49, 1972

10. Hook EW III, Sande MA: Role of the vegetation in experimental *Streptococcus viridans* endocarditis. Infect Immun 10:1433-1438, 1974

11. Tanphaichitra D, Reis K, Levison ME: Susceptibility to *Streptococcus viridans* endocarditis in rabbits with intracardiac pacemaker electrodes or polyethylene tubing. J Lab Clin Med 84:726-730, 1974

12. Trinder P: Rapid determination of salicylate in biological fluids. Biochem J 57:301-303, 1954

13. Born GVR: Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature 192:927-929, 1962

14. Rubin W, Ross LL, Sleisenger MH, et al: The normal human gastric epithelia: A fine structural study. Lab Invest 19:598-626, 1968

15. Muench H, Reed LJ: A simple method of estimating fifty per cent endpoints. Am J Hyg 27:493-497, 1938

16. Jorgensen L, Rowsell HC, Hovig T, et al: Resolution and organization of platelet-rich mural thrombi in carotid arteries of swine. Am J Pathol 51:681-719, 1967