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Role of the Vegetation in Experimental *Streptococcus viridans* Endocarditis

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This study examines the role of the vegetation in catheter-induced experimental endocarditis in predisposing to bacterial colonization of cardiac valves and in influencing the course of the disease and response to penicillin therapy. Platelet-fibrin vegetations developed at areas of valvular trauma and were colonized when *Streptococcus viridans* were injected intravenously. Pretreatment with warfarin prevented vegetation formation, but animals still developed endocarditis at the same rate after injection of 10^6 *S. viridans*. The course of the disease in anticoagulated animals was more explosive, as determined by a more rapid rise in fever and level of bacteremia. Mean survival was shorter in anticoagulated rabbits (7 versus 12.7 days). Large vegetations containing 10^9 *S. viridans*/g were found in control animals, whereas anticoagulated rabbits developed only microscopic deposits. Large vegetations required a longer duration of penicillin therapy to sterilize than the infected valves of the anticoagulated group (7 versus 3 days). Therefore, a preformed platelet-fibrin deposit is not a prerequisite for bacterial colonization of cardiac valves. After infection, the vegetation is an important factor in determining the subacute course of disease and resistance to penicillin therapy.

The clinical characteristics of bacterial endocarditis are related to the formation and persistence of a fibrin-platelet-bacterial complex, the vegetation. It has been suggested that a pre-existing sterile vegetation, nonbacterial thrombotic endocarditis (NBTE), is a necessary prerequisite for bacterial colonization of cardiac valves (10). After infection, the vegetation is responsible for many of the clinical features of bacterial endocarditis, such as constant bacteremia and peripheral embolization. The vegetation may also protect bacteria from host defenses and may contribute to the unique resistance of bacterial endocarditis to antimicrobial agents (8).

A variety of stresses to the endocardium have been shown to cause NBTE (2). Placement of polyethylene catheters across the aortic valves of rabbits is known to stimulate formation of NBTE. This procedure has been shown to render cardiac valves susceptible to bacterial colonization after an intravenous injection of bacteria (3). Anticoagulation with warfarin will prevent both NBTE formation and reactive vegetation formation resulting from bacterial colonization of the valve. The purpose of this study was to compare the susceptibility to infection, clinical course, and response to antibiotic therapy of bacterial endocarditis in normal rabbits with vegetations with anticoagulated animals in which vegetation formation had been prevented.

MATERIALS AND METHODS

Production of bacterial endocarditis. Endocarditis was produced in 2-kg white New Zealand rabbits, utilizing the method previously reported by Perlman and Freedman (11). After anesthetization with 60 mg of sodium pentabarbitol intravenously, the right carotid artery was exposed and ligated cephalad, and a sterile polyethylene cannula (Intramedic; Clay-Adams PE-90) was inserted through a small incision in the arterial wall. The catheter was advanced toward the heart until pulsation and resistance indicated that it had reached the apex of the left ventricle. The catheter was then clamped off and tied to the artery with 00 silk thread, and the skin incision was closed over it. Strict asepsis was not observed; however, local wound infections were not noted. Four to 5 days after catheterization, an intravenous injection of 10^6 *Streptococcus viridans*, diluted from an overnight culture in broth, was given. Criteria used to indicate the existence of bacterial endocarditis were positive blood cultures and fever greater than 39.6 C (rectally). These parameters have been shown to accurately predict the existence of bacterial endocarditis in previous studies (12).

Test organism. A strain of *S. viridans* isolated

from a patient with bacterial endocarditis was used throughout this study. The minimal inhibitory or bactericidal concentration of penicillin for this strain was 0.06 μg/ml.

Administration of warfarin. One group of rabbits received an intramuscular injection of 4 mg of sodium warfarin 2 to 5 days prior to catheterization. Anticoagulation was maintained by administration of between 1 and 2 mg/day. For each rabbit the size of the maintenance dose was determined after consideration of the most recent prothrombin time. Efforts were made to keep prothrombin times greater than one and one-half times normal but less than three times normal levels. Plasma prothrombin determinations were carried out twice weekly.

Administration of antibiotics. Response to penicillin was examined in groups of control and anticoagulated rabbits. Procaine penicillin in a dose of 150,000 U/kg was administered intramuscularly twice daily. Serum levels were determined by the agar well diffusion technique and were in general equivalent to those considered therapeutic in humans. Penicillin levels 3 h after administration of the drug were 3.2 μg/ml and at 12 h 1.3 μg/ml. With this dosage of penicillin, serum diluted 1:8 or more was bactericidal when tested against an inoculum of 10^4 bacteria/ml in a tube dilution technique in broth. Therapy was initiated only after fever and positive blood cultures indicated that endocarditis had been established, usually within 2 to 3 days after injection of bacteria.

Clinical and bacteriological parameters. Anticoagulated and control rabbits with endocarditis were followed with daily temperatures and quantitative blood cultures in pour plates. Mortality was 100% in rabbits not receiving penicillin. Groups of anticoagulated and control rabbits were treated with penicillin as described above. These animals were sacrificed after variable periods of treatment. Vegetations and/or aortic valves were aseptically removed, and bacteria were enumerated in the manner described below. No attempt was made to inactivate the penicillin; however, animals were sacrificed at a time after the final antibiotic dose when serum levels were below the minimal inhibitory concentration for the organism.

All rabbits were autopsied, using aseptic techniques, after death or sacrifice. The aortic valve cusps and vegetations were removed, weighed, and homogenized in a tissue grinder. Pour plates of the serially diluted homogenate were then made in Trypticase (BBL) soy agar containing 2% sheep erythrocytes. After incubation at 37 C for 24 h, colonies were counted, and titers were recorded as colony-forming units per gram of tissue (wet weight). When no macroscopic vegetation was present, the aortic valve and annulus were excised and titered in the manner described above. Since individual specimens usually weighed only 0.01 to 0.1 g, sterile specimens were recorded as $<10^2$ bacteria/g ($<\log 2$). At autopsy, portions of the valves and vegetations when present were excised, fixed in 10% formalin, and sectioned for histological examination after staining of sections with a tissue gram stain, a fibrin stain, and hematoxylin and eosin.

RESULTS

Several rabbits were catheterized and sacrificed 5 days post-operatively. As expected from the work of previous investigators (5), all had small (1 to 2 mm in diameter), sterile vegetations (NBTE) present on the arterial side of the aortic valve cusps. Microscopic examination showed NBTE to be a compact network of fibrin strands tightly packed with platelets (Fig. 1A). In six rabbits adequately anticoagulated before and after catheterization, no NBTE was visible at autopsy. Microscopic examination of each of these traumatized valves confirmed the absence of NBTE, although the endothelium of the valves was found to be disrupted (Fig. 1B).

Bacterial endocarditis was produced with equal regularity in controls and rabbits receiving warfarin anticoagulation. After a single injection of 10^6 *S. viridans*, 40 of 58 (69%) control rabbits developed endocarditis as manifested by positive blood cultures and fever greater than 39.6 C, whereas infection developed in 17 of 23 (74%) anticoagulated rabbits ($P = 0.66$).

The course of bacterial endocarditis in rabbits receiving warfarin varied from that of control rabbits. Temperatures increased more rapidly after infection in anticoagulated rabbits than in controls (Fig. 2A). These differences in temperature were statistically significant ($P = 0.001$) for days 2 to 5 after infection. The development of bacteremia followed a similar trend (Fig. 2B). The mean number of bacteria per 0.5 ml of blood was greater in anticoagulated rabbits than in controls on every day except the first day of infection.

In anticoagulated rabbits not receiving antibiotic therapy, mean survival was reduced when compared with survival of untreated control animals. Rabbits on the warfarin regimen had a mean survival of 7 days, whereas mean survival for control rabbits was 12.7 days, a figure which agrees quite well with the findings of previous investigators (5).

The cause of death in control and anticoagulated animals could not be determined using the limited autopsy procedures employed in these studies. In control rabbits, postmortem examination revealed large friable vegetations which partially occluded the aortic outflow tracts. Upon histological examination, these vegetations were found to have large numbers of bacteria dispersed throughout an amorphous framework consisting of fibrin and platelets (see Fig. 3A). Other postmortem findings of possible consequence in control rabbits were evidence of embolization to a variety of organs (spleen,

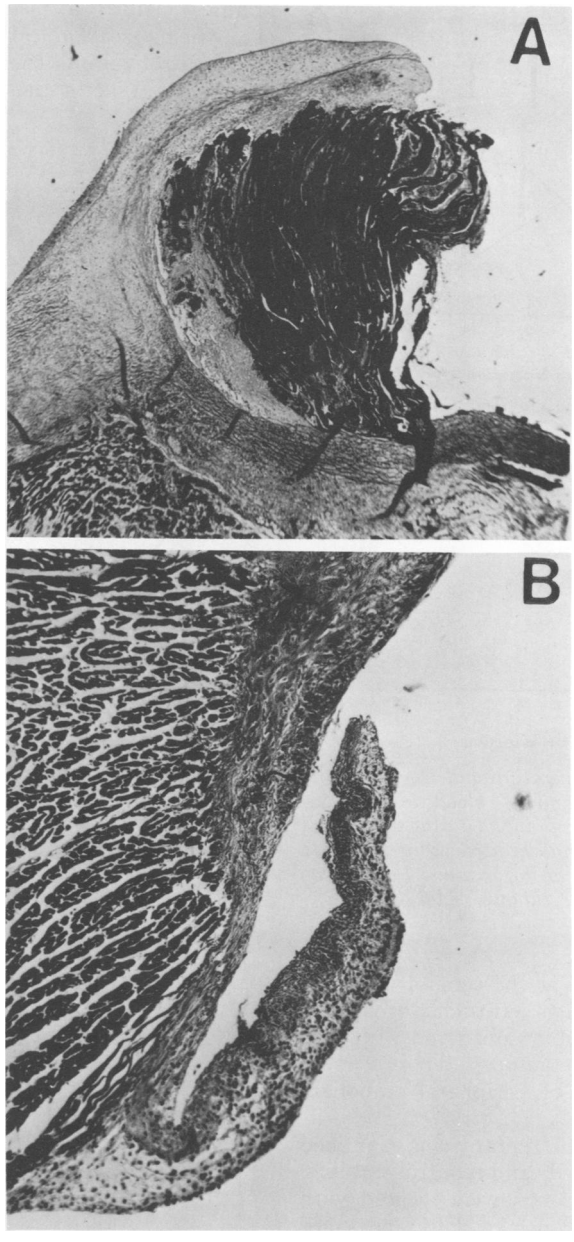

FIG. 1. *Microscopic sections of rabbit aortic valves 5 days after catheterization (fibrin stain). (A) Nonbacterial thrombotic endocarditis in a nonanticoagulated rabbit (original magnification, × 36). (B) Traumatized valve cusp without NBTE in an anticoagulated rabbit (original magnification, × 116).*

kidney, intestine), pleural and pericardial effusions, ascites, cardiac hypertrophy, and dilatation. In rabbits receiving adequate warfarin anticoagulation, vegetation formation was prevented. If, however, the prothrombin time was allowed to drop below 1.5 × normal levels at any time after catheterization, small valvular vegetations were formed. Small vegetations were occasionally seen as well on aortic walls or within the ventricles of insufficiently anticoagulated rabbits. Upon microscopic examination of aortic valves which had no visible vegetations present, large numbers of bacteria were seen tightly adhering to the inner surface of valve cusps (see Fig. 3B). Often evidence of hemorrhage into body cavities was also seen in

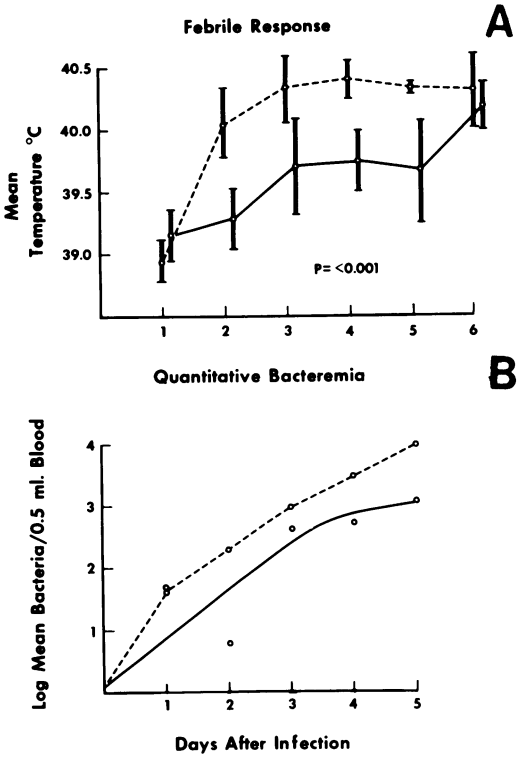

FIG. 2. *(A) Mean temperature and (B) log of the mean bacteria per 0.5 ml of blood in five anticoagulated and six control rabbits during first 5 days after development of endocarditis. Bars represent standard error of the mean logarithms. Dotted line represents anticoagulated rabbits; solid line represents control rabbits.*

anticoagulated rabbits at the time of postmortem examination. Ascites, effusions, or cardiac enlargement were not observed in anticoagulated rabbits at autopsy. There was no macroscopic evidence of peripheral embolization.

Response to antibiotic therapy was examined in both anticoagulated and control rabbits. Rabbits from both groups were treated with 300,000 U of penicillin per kg daily and sacrificed at specific intervals. Despite differences in absolute numbers, in both control and anticoagulated groups all animals not receiving therapy were found to have concentrations of greater than 10^9 organisms/g of tissue on valves (and in vegetations in the case of control animals).

Recoverable organisms had been eliminated in three out of four adequately anticoagulated rabbits without visible vegetations present at autopsy and receiving 4 or fewer days of penicillin therapy. At sacrifice the valves of these four animals yielded a mean log of 2.28 ± .55 organisms/g (see Table 1).

In control rabbits there was a regular, stepwise diminution in the numbers of organisms

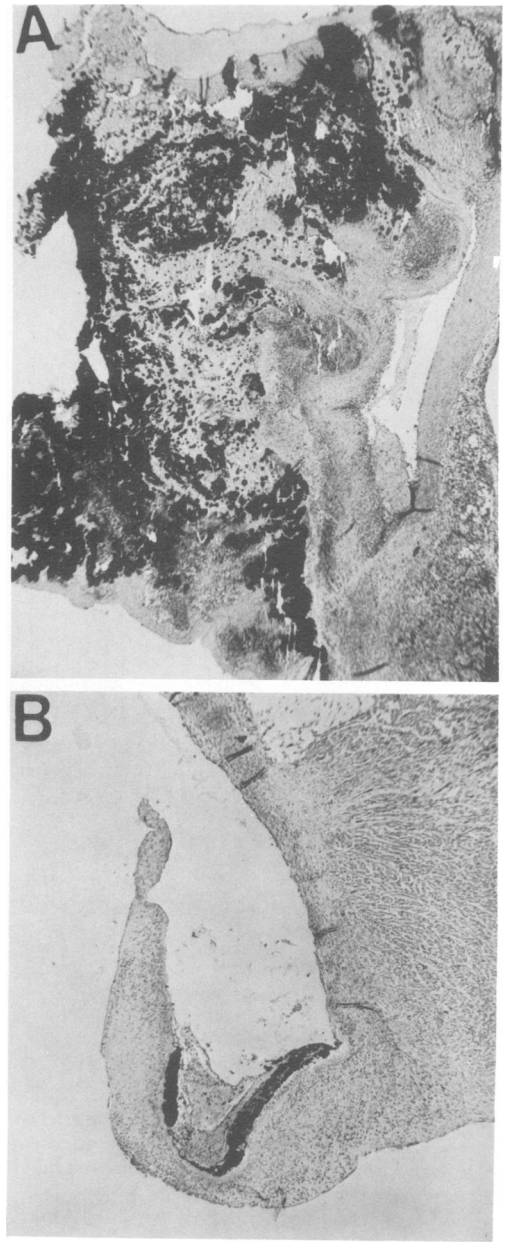

FIG. 3. *Microscopic sections of infected aortic valves from control and anticoagulated rabbits (dark staining areas represent bacterial colonies-tissue gram stain). (A) Vegetation from control animal (original magnification, × 36). (B) Bacterial colonies on inner surface of aortic valve cusp of anticoagulated animal (original magnification, × 42).*

TABLE 1. *Rate of killing of S. viridans in control (with vegetations) and anticoagulated rabbits (without vegetations) treated with penicillin*

Days of therapy	Log viable bacteria/g of tissue	
	Rabbits with vegetations	Rabbits without vegetations
0	9.5	9.3
1	8.8	<2
2	7.2	3.1
3	5.4	<2
4	4.4	<2

recoverable from vegetations with penicillin therapy. The mean log of viable organisms recovered from four animals with vegetations present and treated for 1 to 4 days was 6.45 ± 1.95/g of vegetation (see Table 1). The difference between numbers of organisms recovered from treated animals with and without vegetations present if highly significant ($P = 0.003$).

DISCUSSION

These studies were stimulated by observations made by Valone and Freedman, Durack and ourselves (Clin. Res. **22:**33a, 1974) that anticoagulation with either heparin or warfarin prevented vegetation formation but not the development of endocarditis in the rabbit model (personal communications). This phenomenon enabled us to examine and compare the disease in animals with and without vegetations, contrasting the susceptibility to infection, clinical course, and response to antibiotic therapy.

No differences in the susceptibility of the two groups of animals to bacterial colonization after trauma to cardiac valves were detected. Animals receiving warfarin in doses preventing formation of NBTE at the site of trauma (Fig. 1B) developed infections on the valve at the same frequency (74%) as animals with NBTE (69%) after a single intravenous injection of 10^6 *S. viridans*. Angrist has popularized the concept that an aggregation of platelets and fibrin on an area of endocardial pathology is a critical prerequisite to bacterial colonization (2). This study, although not disproving this hypothesis, does suggest that bacterial adherence and growth may occur at areas of endocardial trauma without preformed vegetations. It is possible, however, that microscopic platelet aggregations may have been present at the time of initial infection and not detected at the time of pathological examination. These experiments do not rule out the possibility that smaller inocula may have caused endocarditis in a greater number of rabbits with NBTE than anticoagulated animals, since a strict infectious dose response curve was not determined. Prior studies have demonstrated that the incidence of endocarditis after a single intravenous injection increases as the size of the inoculum is increased until 100% of animals become infected (infectious dose, 100) (Valone and Freedman, personal communication). With *S. viridans*, injections of 10^7 to 10^8 organisms produce endocarditis in all rabbits with catheters in the left heart (5). Therefore, since the same proportion of rabbits with and without pre-existing NBTE developed endocarditis at an infectious dose below 100, the presence of NBTE does not appear to significantly increase susceptibility of the traumatized valve to infection.

The presence of the vegetation did appear to influence the clinical and bacteriological manifestations of the disease once infection was established. Differences clearly exist between infected animals in which vegetation formation had been prevented and those in which vegetations were allowed to develop. In those without vegetations, the disease was more explosive and rapidly progressive, i.e., high fever developed earlier in the course and was paralleled by higher levels of bacteremia. Similarly, anticoagulated rabbits survived only half as long as the non-warfarinized animals. Several possibilities exist to explain these phenomena. Warfarin administration may result in an altered rate of phagocytic activity in the reticuloendothelial system when maintained for long periods (1, 6). We, however, found no differences in the clearance of bacteria between anticoagulated and control rabbits in our short-term model. The more accelerated course followed by anticoagulated rabbits could also be a direct consequence of the prevention of vegetation formation. In non-warfarinized animals vegetations were composed of tightly packed fibrin, platelets, and large numbers of bacterial colonies. The entire structure is largely covered by a complex fibrin mesh (3). The absolute numbers of bacteria present approached maximal population densities of 10^9 to 10^{10} bacteria/g. At these population densities bacteria assume a resting state (4). Thus, the bacteria may be reproducing relatively slowly within the fibrin matrix and have limited access to the surrounding blood stream. These factors may in part explain the rather low grade but constant bacteremia characteristic of this disease. On the other hand, in animals in which vegetation formation had been inhibited by anticoagulation the entire mass of actively multiplying organisms is on the surface of the valve, with ready access to the passing blood. This could result in the more explosive disease.

The large friable vegetations also contribute

to the unique resistance of this disease to penicillin therapy. Seven to 8 days of therapy were required to completely eradicate the *S. viridans* from the large vegetations of non-warfarinized rabbits. The group of rabbits in which anticoagulation had prevented vegetation formation responded more rapidly, i.e., valves were sterile with 3 days of therapy. This latter rate is similar to the rate at which penicillin kills large numbers of *S. viridans* actively growing in broth (12). It seems unlikely that this difference in response can be explained by inadequate penetration of penicillin into the vegetation, since both vegetations and clots have been found to be highly permeable to the drug (8, 13). It seems more likely that differences in killing may be attributed to the large percentage of metabolically inactive organisms deep within the vegetation. Durack and Beeson measured metabolic activity in vegetations using tritium-labeled L-alanine and found a general reduction in uptake, especially over still viable colonies deep within the tissue (4). Hobby et al. (7) and McDermott (9) have demonstrated that bacteria in the resting state with reduced metabolic activity are less susceptible to the bactericidal activity of penicillin than rapidly dividing organisms. Thus, the decreased metabolic activity of bacteria deep within the vegetation may also contribute to the resistance of this disease to antimicrobial therapy.

These studies have, therefore, demonstrated that the infected fibrin vegetation of bacterial endocarditis may be responsible for the subacute characteristics of this disease and its relative resistance to penicillin therapy. A preformed vegetation, however, does not seem to be a critical prerequisite to initial bacterial colonization of a traumatized aortic valve.

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