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Effect of Warfarin on the Induction and Course of Experimental Endocarditis

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The effect of warfarin treatment on an experimental endocarditis was studied in rabbits. Warfarin had no effect on the induction of a *Streptococcus sanguis* infection in catheter-induced endocardial vegetations, and the course of this infection was also unaltered. However, warfarin treatment resulted in rapidly progressive bacteremia, probably due to impaired circulation in clearing organs such as the lungs, liver, and spleen. Warfarin also reduced the survival time of the infected rabbits, in which pulmonary edema and extensive lung hemorrhages may have been a contributory factor.

Despite extensive study, many factors in the pathogenesis of infectious endocarditis remain obscure. For instance, the relative predominance of certain bacteria causing this disease is unexplained (13). Presumably, a combination of host resistance factors and bacterial properties determines whether microbial invasion of the bloodstream will result in an infection of the endocardium (10, 14).

Recently, an experimental model was designed for the study of infectious endocarditis on the basis of the hypothesis that prior to infection, sterile vegetations consisting of fibrin-platelet thrombi are formed on an endocardial lesion. This "nonbacterial thrombotic endocarditis" (NBTE) is then secondarily infected by blood-borne bacteria (1, 5, 9).

The mechanisms involved in the infection of the endocardial vegetations are unknown, but a two-stage process seems to be involved. First, the bacteria adhere to the surface of the vegetation. In this stage a role is probably played by platelets, phagocytic cells, and specific antibodies (8, 14). Subsequently, these bacteria are covered by a layer of fibrin and, after 18 to 24 h, grow out to colonies, possibly because the fibrin makes the bacteria less accessible to normal host defense factors. Since radioactive labeling showed older colonies near the core and younger colonies near the surface of the vegetations (6), adherence of bacteria and outgrowth to colonies seem to be continuous processes in which fibrin formation could play an important role. Therefore, interference with fibrin formation by anticoagulant treatment could influence both the induction and course of an infection of the endocardium by reducing the formation either of a suitable surface for the adherence of bacteria or of the protective layer needed for the outgrowth of bacteria.

An effect of anticoagulant treatment on bacterial adherence would be reflected in an altered number of bacteria required for the induction of endocarditis, and an effect on the outgrowth in an altered numerical course of bacteria recovered from the vegetations. In this regard, a study done by Hook and Sande (12) is relevant. Before the introduction of a sterile catheter into the heart, which normally produces NBTE, they treated rabbits with the anticoagulant warfarin sodium. Although this treatment prevented the formation of sterile endocardial vegetations, a subsequent injection of 10^6 streptococci nevertheless resulted in an infection of the endocardium. The authors concluded that the prior formation of sterile vegetations is not an essential prerequisite for the induction of a bacterial infection of the endocardium. However, in this study only a single infecting dose was applied, and it was not determined whether the absence of vegetations influenced the number of bacteria necessary to induce an infection (7). It therefore remained uncertain whether preformed vegetations facilitate the induction of bacterial endocarditis by offering a suitable surface for the deposition of bacteria. It also remained possible that an effect of anticoagulant treatment on the adherence or outgrowth of bacteria could influence the induction or course of bacterial endocarditis, which would be reflected by either the number of bacteria necessary to induce an infection of the vegetations or the numerical course of the bacteria recovered from the vegetations. The present study was undertaken to investigate the effect of treatment with warfarin sodium on the induction and course of a *Streptococcus sanguis* endocarditis in rabbits with previously induced NBTE, while various infecting doses of streptococci were applied.

MATERIALS AND METHODS

Animals. The study was done in male chinchilla rabbits weighing 2 to 2.5 kg, raised in the Central Institute for the Breeding of Laboratory Animals, Bilthoven, The Netherlands.

NBTE. NBTE was induced according to Durack and Beeson (5). Briefly, a sterile saline-filled 17-gauge catheter was introduced via the left carotid artery into the left ventricle and was kept there for 2 days before the experiments were started.

Anticoagulant treatment. Warfarin sodium (Endo) was used as anticoagulant. A daily dose of 8 mg was given intramuscularly except for the first dose, which was given intraperitoneally. Within 24 h this treatment resulted in a reduction of the factors of the prothrombin complex from a control value of 130% to less than 5%, as measured by the Normotest method according to the manufacturer's instruction (Nyegaard, Oslo, Norway). No attempt was made to keep blood coagulability within certain limits.

Microorganism. The microorganism was *S. sanguis*, kindly made available by H. Engel, Bilthoven, The Netherlands. The minimal inhibitory concentration for penicillin was 0.125 μg/ml. The streptococci were maintained in a Tarozzi medium. Overnight cultures in Todd-Hewitt medium, usually giving 10^8 colony-forming units per ml, were washed twice with saline and resuspended in saline. Dilutions were made such that the volume to be injected was 1 ml. For infection of the vegetations, bacteria were injected into an ear vein.

Quantitative bacteriology. Blood cultures were taken from an ear vein or, after the animal was killed, from the inferior caval vein. For the measurement of blood clearance of bacteria, samples were taken via a catheter introduced into the left carotid artery. To determine bacterial numbers in the blood, 2 ml of blood was added to 1 ml of liquoid, and 1.0, 0.5, and 0.1 ml of this mixture was added to agar pour plates. When high bacterial numbers were expected, a 10-fold dilution was also made and 1-ml portions were plated. After 24 to 48 h of incubation at 37°C, plates with 6 to 500 colonies were counted, and the number of bacteria per milliliter was calculated from the mean of two consecutive dilutions.

To determine the degree of infection of the vegetations, the rabbits were killed by intravenous injection of 5 ml of pentobarbital sodium (Nembutal), and the heart was removed and opened under aseptic precautions. The vegetations were isolated, brought into a sterile plastic petri dish, weighed, and homogenized in a ground-glass potter containing 5 ml of glucose broth. Ten-fold serial dilutions of the homogenate were made, and 0.1-ml samples were plated on sheep blood agar plates. After 24 to 48 h of incubation at 37°C, plates with 6 to 500 colonies were counted with an electric colony counter. Bacterial numbers per gram of vegetation were calculated from the means of two consecutive dilutions.

Penicillin levels. Serum penicillin levels were determined with a bioassay method according to Grove and Randall. *Bacillus subtilis* 6633 was used as the test organism (11).

Morphology. For the morphological studies, hearts from infected animals were fixed in formalin. Multiple sections of parts bearing vegetations were stained with hematoxylin/eosin and Gram stain. The preparations were examined by one of the authors (F.E.) without prior knowledge of whether the rabbit had been treated with warfarin. The presence of bacterial colonies in direct contact with the surface of the vegetations was determined.

Experimental design. In the warfarin-treated rabbits, anticoagulant treatment was started 48 h after the catheterization for the induction of NBTE. Streptococci were injected 24 h after the start of warfarin administration. In the control rabbits, streptococci were injected 72 h after the induction of NBTE. Generally, warfarin-treated and control animals were studied in pairs.

General remarks. The values in the tables are the means of results from at least four rabbits.

RESULTS

Effect of warfarin on the survival of infected rabbits. The mean survival time of NBTE rabbits injected 3 days after catheterization with 10^8 streptococci was 15.5 days (range, 7 to 24) after infection. This is in agreement with other observations (7, 12). The survival was significantly reduced in warfarin-treated rabbits: all animals died within 4 days after the infection, most of them between h 48 and 96 (Table 1). (Since groups of animals were killed at daily intervals to investigate the infection of vegetations, the mean survival time of the infected, warfarin-treated rabbits was not computed.) Six NBTE rabbits were still alive after 7 days of treatment with warfarin only.

Effect of warfarin on infection of vegetations. Warfarin-treated and control rabbits were infected with 10^8 streptococci. Table 2 shows that up to day 4 the degree of infection of vegetations was not influenced by anticoagulant treatment. An analysis of covariance of the logarithms of the bacterial number per gram of vegetation gave a difference that was not significant ($0.80 < P < 0.90$).

It was noteworthy that macroscopically, up to day 4 after infection, the size and extent of

TABLE 1. *Effect of warfarin on survival of rabbits with endocarditis*

Expt condition	Survival[a] on day after infection[b]:			
	1	2	3	4
−Warfarin	4/4	5/5	5/5	4/4
+Warfarin	4/4	5/5	3/7	0/5

[a] Number of rabbits surviving/number infected for determination of bacterial numbers in the vegetations at a given time point.

[b] A dose of 10^8 streptococci was given intravenously; warfarin treatment was started 24 h before infection.

the vegetations was not influenced appreciably by warfarin treatment. When the infecting dose was reduced to 10^5 microorganisms, all warfarin-treated rabbits still showed signs of infection at 24 h, and the degree of infection of the vegetations was comparable to that in control animals, the mean bacterial number being 2.0×10^8 (range,1.3×10^6 to 3.7×10^9) in the controls and 2.3×10^8 (range 6.7×10^7 to 1.6×10^9) in warfarin-treated rabbits. However, an infecting dose of 10^4 microorganisms failed to infect either the control or the warfarin-treated rabbits persistently (Table 3).

Effect of warfarin on bacteremia. In both control and warfarin-treated rabbits, blood cultures became positive 24 h after the injection of 10^8 bacteria. However, in the warfarin-treated rabbits the bacteremia was found to be rapidly progressive, and shortly before death bacterial numbers on the order of 10^4 per ml were observed (Table 4). Analysis of covariance of the logarithms of bacterial numbers per milliliter of blood against time showed a highly significant difference ($P < 0.001$) between warfarin-treated and control rabbits. The short survival time of the warfarin-treated animals made it impossible to culture blood later than 72 h after infection. In control rabbits, from which serial blood cultures were taken, bacterial numbers on the order of 10^4 per ml were only found shortly before death.

Effect of penicillin treatment on survival of warfarin-treated rabbits. Since it was possible that the rapid progress of the bacteremia contributed to the early death of the anticoagulant-treated rabbits, one group of six warfarin-treated rabbits with a bacterial endocarditis was treated twice daily with 300,000 U of procaine penicillin G, plus 100,000 U of sodium penicillin G, starting 24 h after infection. This resulted in peak penicillin levels of 6 to 45 μg/ml and residual levels of 0.6 to 10.0 μg/ml. Twenty-four hours after the start of this treatment, the blood cultures were sterile, but the

TABLE 3. *Effect of warfarin on the induction of bacterial endocarditis*[a]

Expt condition	Intravenous streptococcal dose[a] (no. of bacteria)			
	10^4		10^5	10^8
	24 h p.i.[b]	72 h p.i.	24 h p.i.	24 h p.i.
−Warfarin	1/4	0/4	4/4	4/4
+Warfarin	1/4	0/4	4/4	4/4

[a] Expressed as number of rabbits with infected vegetations per number of rabbits sacrificed.
[b] p.i., Postinfection.

TABLE 2. *Effect of warfarin on infection of vegetations after 10^8 streptococci given intravenously*

Days after infection	Bacteria/g of vegetation[a]	
	−Warfarin	+Warfarin
1	5.8×10^7 ($1.7 \times 10^6 - 5.0 \times 10^8$)	8.4×10^8 ($2.6 \times 10^8 - 2.6 \times 10^9$)
2	3.2×10^9 ($8.7 \times 10^8 - 2.4 \times 10^{11}$)	7.8×10^9 ($1.0 \times 10^9 - 1.3 \times 10^{11}$)
3	4.4×10^9 ($8.3 \times 10^8 - 3.4 \times 10^{10}$)	1.2×10^9 ($2.4 \times 10^6 - 2.9 \times 10^{10}$)
4	2.5×10^{10} ($1.0 \times 10^{10} - 6.8 \times 10^{10}$)	1.2×10^{10} ($5.0 \times 10^9 - 2.0 \times 10^{10}$)

[a] Expressed as geometric mean and, in parentheses, range.

TABLE 4. *Effect of warfarin on bacteremia in rabbits with endocarditis*

Time after infection (days)[b]	Bacteria/ml of blood[a]	
	−Warfarin	+Warfarin
1	13 ($4 - 1 \times 10^2$)	67 ($11 - 3.5 \times 10^2$)
2	83 ($5 - 1.9 \times 10^3$)	6.4×10^2 ($4 \times 10^2 - 3 \times 10^3$)
3	66 ($2 - 4.9 \times 10^2$)	1.2×10^4 ($7.5 \times 10^3 - 2.3 \times 10^4$)
4	1.5×10^3 ($1.2 \times 10^2 - 5.5 \times 10^3$)	

[a] Expressed as geometric mean and, in parentheses, range.
[b] Dose was 10^8 streptococci given intravenously.

survival (3.5 days; range, 2 to 6 days) was not significantly prolonged. The numbers of bacteria in the vegetations at the time of death were greatly reduced (to between 10^3 and 10^6 per gram), but none of the vegetations was sterile.

Effect of warfarin on bacterial clearance. Since the mechanism underlying the rapid increase of the bacteremia in warfarin-treated rabbits could be an impaired clearance of bacteria from the bloodstream, the clearance of 10^8 streptococci was determined in uncatheterized rabbits. As can be seen from Fig. 1, bacterial clearance was not diminished by warfarin.

Effect of warfarin on the localization of the bacteria in the vegetation. Another factor in the rapidly progressive bacteremia in warfarin-treated animals could be a more superficial location of bacterial colonies in the vegetation, possibly making the circulation more accessible for the bacteria. Therefore, the localization of the colonies was determined in multiple sections of the vegetations 24 and 48 h after infection with 10^8 streptococci in warfarin-treated and control rabbits. At 48 h, both groups of animals showed superficial colonies in contact with the circulation, but also colonies buried in fibrin. With this screening no obvious differences between the two groups were found.

Autopsy findings in warfarin-treated rabbits. In the period before death, the rabbits became progressively dyspneic and apathic. At autopsy, pulmonary edema was found in both groups of animals, but the warfarin-treated animals also showed very striking extensive pulmonary hemorrhages. No gross hemorrhages were found elsewhere (Fig. 2).

DISCUSSION

In the present study, warfarin treatment had no effect on the number of rabbits with infected vegetations or the number of bacteria in the vegetations when various doses of streptococci were injected in rabbits with a previously induced NBTE. The number of bacteria that can be recovered from the vegetations after hematogenous infection is the result of the number of bacteria adhering to the surface of the vegetations and the subsequent outgrowth of these bacteria. Diminished adherence would be reflected in a lower number of animals with infected vegetations after warfarin treatment, and such an effect would be especially prominent when a low infecting dose is applied. However, the results obtained with infecting doses of 10^4 and 10^5 streptococci gave no indication of an effect of warfarin treatment. If the outgrowth of bacteria were influenced, this would be reflected in alterations in both the numbers of bacteria in the vegetations and the time course of the infection of the vegetations. However, 24 h after infection with 10^5 streptococci, bacterial numbers were the same in the warfarin-treated and control animals. The increase in the number of bacteria per gram of vegetation after an infection with 10^8 streptococci was also unaffected by warfarin administration over a period of 4 days. It may therefore be

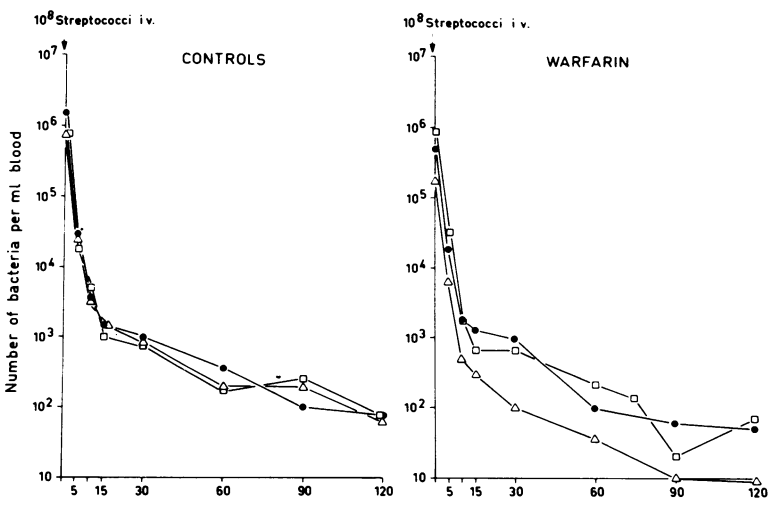

FIG. 1. *Clearance pattern in the blood of rabbits injected with 10^8 streptococci, with and without administration of warfarin (started 48 h before the injection). Time point zero is 15 s after injection of streptococci. Each curve represents the clearance in a single rabbit.*

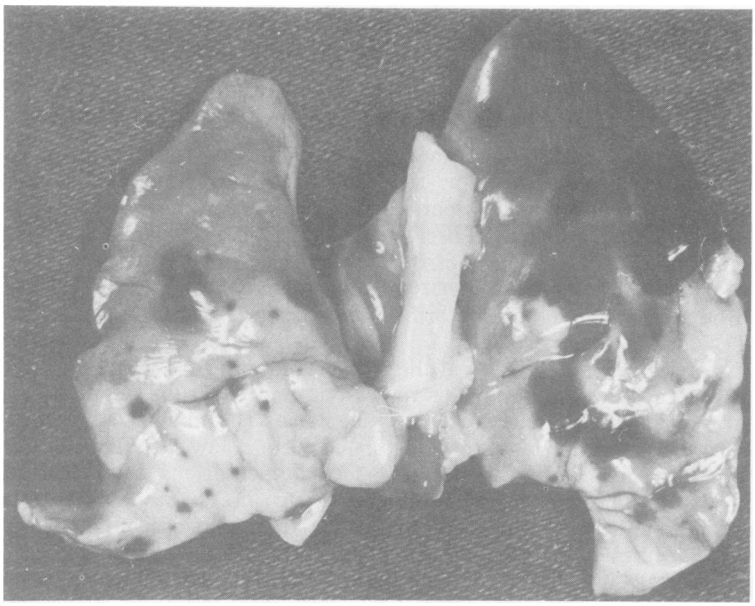

FIG. 2. *Lungs of a warfarin-treated rabbit that died 3 days after the onset of streptococcal endocarditis. Both lungs show multiple hemorrhages, some of which are very extensive.*

concluded that warfarin treatment does not influence the induction or course of the *S. sanguis* infection of the endocardium. However, histological examination showed bacterial colonies lying under the surface of the vegetations in warfarin-treated rabbits. Apparently, even under intense anticoagulation fibrin formation still can be activated during the second phase of the infection.

The warfarin-treated rabbits showed a rapidly progressive bacteremia. This raises two questions. The first concerns the cause of the rapid increase in the number of circulating bacteria. Defective fibrin formation might lead to a more superficial location of the bacterial colonies and thus in turn to an easier and more massive invasion of the bloodstream. However, the morphology of the vegetations, which did not differ appreciably between control and warfarin-treated animals, provided no evidence pointing in this direction. Impaired clearance of bacteria could also account for the effect. The normal clearance of 10^8 streptococci in warfarin-treated rabbits excludes a direct effect of warfarin on the clearance mechanism, in which phagocytes and thrombocytes are involved (4). This is in agreement with previous observations (12). However, a circulatory impairment resulting from valvular damage could lead to a decreased clearance in such organs as the lungs, liver, and spleen. The warfarin-treated rabbits showed not only massive pulmonary edema but also extensive hemorrhages, which could further impair bacterial clearance in the lung, an important organ for the removal of circulating microorganisms in the rabbit (2). A third possibility is that warfarin treatment provokes the formation of multiple metastatic septic foci that contribute to the bloodstream infection. This possibility deserves further investigation.

The second question concerns the role of the rapidly progressive bacteremia in the early death of the warfarin-treated rabbits. Since penicillin treatment eliminated the microorganisms from the circulation but had almost no effect on the survival of the rabbits, the rapidly progressive bacteremia seems to be a consequence of the rapidly deteriorating state of the animals rather than a factor contributing to their early death. What causes the early death in warfarin-treated infected animals? The survival for at least a week of the catheterized, warfarin-treated rabbits excludes anticoagulant treatment as single cause. However, Corrigan et al., in a study on the influence of heparin on gram-negative septicemia, found that this anticoagulant treatment increased mortality in young rabbits (3), and Hook and Sande found that warfarin treatment decreased the survival of rabbits with endocarditis (12). Therefore, the combination of infection and anticoagulant treatment seems to have a detrimental effect. How this is brought about is not clear. Our animals, however, were observed to become intensely dyspneic shortly before death. At au-

topsy the lungs of anticoagulated rabbits showed not only extensive pulmonary edema caused by infectious valvular destruction but also extensive hemorrhages, and it seems likely that these pulmonary lesions had contributed to the early death of the anticoagulant-treated rabbits.

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