Effect of Warfarin on the Induction and Course of Experimental *Staphylococcus epidermidis* Endocarditis

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The effect of warfarin treatment on an experimental *Staphylococcus epidermidis* endocarditis was studied. Warfarin was found to affect both the induction and course of the infection of catheter-induced endocardial vegetations. In warfarin-treated rabbits, larger bacterial inocula were needed to induce an infection, and the degree of infection of the vegetations was also significantly lower, eventually resulting in the total elimination of the bacteria from the vegetations. Thus, warfarin treatment seems to have an inhibitory effect on the induction and development of an *S. epidermidis* infection of the endocardium. The results differ from previous findings in studies done with *Streptococcus anguis*, where warfarin was found to have no effect on the induction or course of the infection of endocardial vegetations, which suggests that different mechanisms are involved in the pathogenesis of endocarditis caused by these two species of bacteria.

Although recent experimental studies have provided some new insight into the pathogenesis of bacterial endocarditis, many factors remain elusive (12, 13). However, according to one currently held hypothesis, sterile platelet-fibrin thrombi (the so-called nonbacterial thrombotic endocarditis [NBTE]) are formed on endocardial lesions prior to infection (1). Blood-borne bacteria adhere to the surface of these vegetations, become covered by a fibrin layer, and grow out to form colonies (3, 6). Since this process (surface attachment, covering by fibrin, and outgrowth to colonies) seems to be continuous during the development of the infection (4), fibrin formation might play an important role in the pathogenesis of bacterial endocarditis, and anticoagulant treatment could therefore influence both the induction and the course of this disease.

However, recently Hook and Sande (10) and Thompson et al. (11) failed to demonstrate an effect of treatment with the anticoagulant warfarin sodium on the induction and course of a *Streptococcus sanguis* infection of sterile endocardial vegetations. These studies were done in an experimental model in rabbits in which the NBTE was induced by insertion of a plastic catheter into one of the cardiac ventricles (6). Warfarin treatment was, however, found to significantly shorten survival and to significantly accelerate the progress of bacteremia in anticoagulated rabbits (10, 11).

The mechanisms by which the bacteria settle on the vegetations are largely unknown, but several reports suggest that they may not be the same for different species of bacteria (8, 13). In a recent study on the very early phase of the infection Durack (2) found adhering streptococci mainly inside phagocytic cells, whereas staphylococci adhered directly to the surface of the vegetations. The possibility that a defective fibrin cover resulting from anticoagulant treatment might promote the removal of the more loosely attached staphylococci from the vegetations led us to investigate the effect of treatment with warfarin sodium on the induction and course of a *Staphylococcus epidermidis* endocarditis. There was an additional reason to perform this study: as a preliminary finding in a study on host factors involved in bacterial endocarditis, we observed that rabbits with endocardial vegetations infected by *S. epidermidis* often had negative blood cultures or only a low-grade bacteremia. Thus, it was of interest to find out whether warfarin treatment would provoke or increase bacteremia as had been observed in rabbits infected with *S. sanguis*.

MATERIALS AND METHODS

In general the methods used were the same as described previously (11).

Animals. The study was done with 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 (4). After intravenous (i.v.) administration of 1 to 1.2 ml of pentobarbital sodium (Nembutal), a sterile polyethylene 17-gauge saline-filled cath-

eter was introduced into the left ventricle via the left carotid artery. The catheter was kept in place for the duration of the experiments.

Anticoagulant treatment. Warfarin sodium (kindly made available by ENDO Laboratories Inc., New York) was used as an 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 performed according to the manufacturer's instruction (Nyegaard, Oslo, Norway). No attempt was made to keep blood coagulability within certain limits.

Microorganisms. The microorganism used was *S. epidermidis* type S6, according to Baird-Parker, maintained in agar medium. Overnight cultures in glucose broth, usually giving an average 1.5×10^7 colony-forming units (CFU) per ml (range 0.8×10^7 to 5.5×10^7 CFU per ml), were washed twice with saline and suspended 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. For each inoculum, the number of CFU per milliliter was determined. Tenfold serial dilutions were made, and 0.1-ml samples were plated on DST agar plates. Bacterial numbers per milliliter were determined as described in the next section.

Quantitative bacteriology. Blood cultures were made from samples taken from an ear vein or, immediately after the animal had been 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 not earlier than the time of the experiment in previously uncatheterized rabbits. 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 were added to agar pour plates. When high bacterial numbers were expected, a 10-fold dilution was also made, and 1-ml samples 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 means of two consecutive dilutions.

In addition to these quantitative determinations, bacteremia was assessed qualitatively at the time of sacrifice. For this purpose, 2 ml of blood was added to 60 ml of Trypticase soy broth and held at 37°C for 14 days. When the blood culture became positive during this period, the animal was considered to have bacteremia at the time of death.

In the clearance experiments a K-value was computed for the first 15 min according to the formula $K_{15} = \log N_0 - \log N_{15}$, in which N_0 and N_{15} are the number of bacteria in the blood at 15 s and 15 min after injection of 10^7 bacteria, respectively.

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 homogenizer containing 5 ml of

glucose broth. Tenfold serial dilutions of the homogenate were made, and 0.1-ml samples were plated on DST 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.

Morphology. For morphological studies, hearts removed 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 location of bacterial colonies in relation to the vegetation surface was determined.

Experimental design. In the warfarin-treated rabbits, anticoagulant treatment was started 24 h after catheterization for the induction of NBTE, and staphylococci were injected 48 h after that.

In the control rabbits, staphylococci were injected 72 h after the induction of NBTE. Warfarin-treated and control animals were usually paired.

RESULTS

Effect of warfarin on survival. The mean survival of four catheterized infected rabbits was 26 (range 17 to 35) days. The duration was significantly shorter in warfarin-treated rabbits (Table 1), whose mean survival time was 3.5 (range 2 to 5) days.

All control rabbits were found to have strongly infected vegetations at the time of death, the mean number of bacteria being 1.4×10^8 (range 2.9×10^7 to 6.7×10^8) per g of vegetation. Of the warfarin-treated rabbits, only one had bacteria-bearing vegetations at the time of death. In the period prior to death, all animals became progressively dyspnoeic and apathic. At autopsy both groups showed pulmonary edema, but the warfarin-treated rabbits also showed extensive pulmonary hemorrhages.

As an additional control, four uncatheterized rabbits were treated with the usual warfarin regimen and on day 3 were injected with 10^7

TABLE 1. *Effect of warfarin on survival of rabbits with Staphylococcus epidermidis endocarditis[a]*

Days after infection[b]	Control[c]	Warfarin treated
1	4/4	7/7
2	4/4	6/7
3	4/4	3/7
4	4/4	1/7
5	4/4	0/7

[a] Expressed as number of surviving animals per total number of rabbits infected with 10^7 staphylococci.

[b] With 10^7 staphylococci administered i.v.

[c] In this group the mean survival time was 26 days (range 17 to 35).

staphylococci. All these animals were alive and well 7 days after the injection of bacteria.

Effect of warfarin on the induction of bacterial endocarditis. To determine whether warfarin treatment influences the induction of the infection of sterile vegetations, control and warfarin-treated rabbits were infected with staphylococci in amounts ranging from 10^3 to 10^7 (Table 2) and killed on the following day. All control rabbits given 10^6 staphylococci showed infected vegetations 24 h later, whereas those given 10^4 microorganisms all had sterile vegetations. The warfarin-treated rabbits required larger inocula for infection. In this group the injection of 10^7 staphylococci resulted in infection of vegetations in all animals, whereas 10^5 microorganisms failed to infect any of them (Table 2). The 50% infective dose, calculated with the Spearman-Kärber method (5), was 6.05 $\times$ 10^4 for the control and 1.95 $\times$ 10^6 for the warfarin-treated rabbits, respectively. The difference between these values is significant ($P <$ 0.01) (Table 2). Thus, warfarin treatment seems to have an influence on the induction of infection of sterile endocardial lesions.

Effect of warfarin on the infection of vegetations. To determine whether warfarin treatment has an effect on the course of the infection of the vegetations, control and warfarin-treated rabbits were injected with 10^7 staphylococci, and the animals were killed at 24-h intervals after infection. It is noteworthy that, macroscopically, warfarin treatment had no appreciable effect on the size of the vegetations. All control rabbits had infected vegetations within 24 h. No significant increase in the number of bacteria per gram of vegetation occurred over a 4-day period (Table 3).

Warfarin treatment was found to influence the infection of the vegetations in two ways. Firstly, after 1 day all warfarin-treated rabbits showed infected vegetations, but the numbers of bacteria were significantly lower ($P < 0.025$) than in control rabbits. Secondly, over the next 3 days many warfarin-treated rabbits were found

TABLE 2. *Effect of warfarin on induction of bacterial endocarditis[a]*

Experimental condition	Intravenous staphylococcal dose				
	10^7	10^6	10^5	10^4	10^3
− Warfarin	4/4	4/4	3/4	0/4	0/4
+ Warfarin	4/4	2/4	0/4	0/4	0/4

[a] Expressed as number of rabbits with infected vegetations per number of rabbits sacrificed. The animals were sacrificed 1 day after the injection of staphylococci.

TABLE 3. *Effect of warfarin on infection of vegetations after i.v. injection of 10^7 staphylococci*

Days after in-fection	Bacteria per g of vegetation[a]	
	− Warfarin	+ Warfarin
1	2.3×10^{7b}	2.3×10^{3b}
	$(2.1 \times 10^5$–$3 \times 10^9)$	$(2.6 \times 10^2$–$5.9 \times 10^3)$
2	6.3×10^5	—[c]
	$(1.3 \times 10^4$–$1.9 \times 10^7)$	
3	1.5×10^6	—[c]
	$(1.9 \times 10^3$–$1.6 \times 10^8)$	
4	1.4×10^6	—[c]
	$(7.3 \times 10^3$–$2.3 \times 10^8)$	

[a] Expressed as geometric means and ranges (in parentheses).
[b] $P < 0.025$.
[c] Since at these time points fewer than four rabbits had infected vegetations (Table 4), mean bacterial numbers were not computed.

to have sterile vegetations (Table 4). Thus, warfarin treatment results in staphylococci disappearing from the vegetations.

The effect of warfarin treatment on the survival of infected rabbits (Table 1) made it impracticable to study the effect of anticoagulant treatment on the degree of infection of the vegetations for a period of more than 4 days.

Effect of warfarin treatment on bacteremia. To determine the effect of warfarin treatment on bacteremia, qualitative and quantitative cultures were made from blood collected at the time of sacrifice from rabbits infected with 10^7 staphylococci (Table 5). None of the blood cultures of control rabbits was positive on day 1, and a positive blood culture was only found incidentally on subsequent days. Positive blood cultures were found more frequently for the warfarin-treated rabbits, especially during the first few days after infection. Thereafter, the blood cultures of this group also showed a tendency to become negative. Thus, warfarin treatment did not result in a rapidly progressive bacteremia shortly before the death of the rabbits. It should be noted that in this group positive blood cultures occurred in rabbits with sterile endocardial vegetations. When the numbers of bacteria in the blood could be determined quantitatively, they were invariably low in both groups, ranging from 2 to 8 bacteria per ml.

To exclude a bactericidal effect of rabbit serum on staphylococci as a cause of the negative blood cultures, the microorganisms were cultured overnight at 37°C in whole serum from both normal and warfarin-treated rabbits. These cultures showed normal growth, i.e., 10^7 CFU per ml.

Effect of warfarin on bacterial clearances. Since the increased frequency of bacteremia in warfarin-treated rabbits could be due

to impaired clearance of bacteria from the circulation, the clearance of 10^7 staphylococci was determined in warfarin-treated and control rabbits without induction of NBTE. As can be seen from Fig. 1, warfarin had no effect on bacterial clearance. The mean of K_{15} values was 3.515 ± 0.318 for the control rabbits and 3.113 ± 0.219 for the warfarin-treated rabbits. The difference

TABLE 4. *Effect of warfarin on number of rabbits with infected vegetations[a]*

Days after infection[b]	− Warfarin	+ Warfarin
1	4/4	4/4
2	4/4	2/4
3	4/4	1/4
4	4/4	0/2

[a] Expressed as number of rabbits with infected vegetations per number of rabbits sacrificed.

[b] A total of 10^7 staphylococci was injected i.v.

TABLE 5. *Effect of warfarin on number of rabbits with bacteremia[a]*

Days after injection[b]	− Warfarin	+ Warfarin
1	0/4	3/4
2	1/4	4/4
3	1/4	2/4
4	0/4	1/2

[a] Expressed as number of rabbits with bacteremia when killed per total number of rabbits sacrificed.

[b] A total of 10^7 staphylococci was injected i.v.

between these values is not significant ($0.15 < P < 0.20$).

Morphology. Because increased frequency of sterile vegetations in warfarin-treated rabbits could have been due to a more superficial location of the bacteria in the vegetations, leading to their removal by the passing blood, an attempt was made to determine localization of bacterial colonies in relation to the surface of the vegetations in warfarin-treated and control rabbits 24 and 48 h after injection of 10^7 staphylococci. However, even though multiple sections of the vegetations occasionally showed bacterial colonies, their frequency in both groups of animals was too low to permit accurate determination of their location or to detect significant differences between the two groups.

DISCUSSION

The present results show that treatment with the anticoagulant warfarin sodium affects both the induction and the course of an *S. epidermidis* endocarditis in rabbits with a previously induced NBTE. However, before the results of warfarin treatment are considered, some data obtained in the control rabbits must be discussed. It was remarkable that although inoculation with 10^7 staphylococci gave infection of endocardial vegetations in all control rabbits, a positive blood culture was only found incidentally during the first 4 days of infection. The
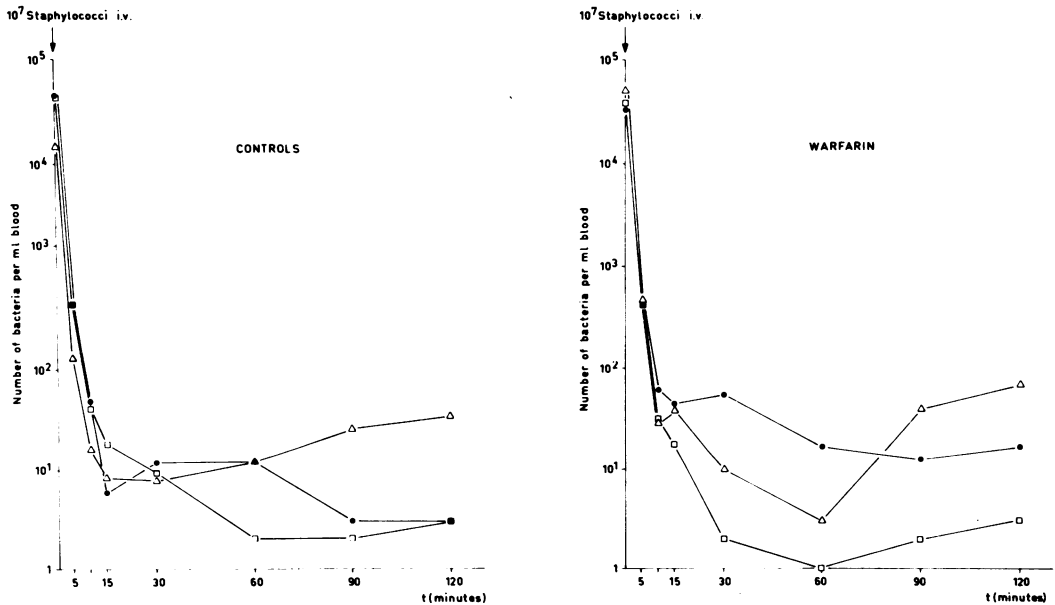

FIG. 1. *Clearance pattern in the blood of rabbits injected with 10^7 staphylococci, with and without administration of warfarin (started 48 h before the injection). Time point zero is 15 s after injection of staphylococci. Each curve represents the clearance in a single rabbit.*

normal outgrowth of the bacteria in rabbit se-
rum means that a bactericidal effect of rabbit
serum could not have been responsible for these
negative blood cultures. Therefore, it must be
concluded that either the bacterial colonies are
so effectively buried under their fibrin cover
that they have no access to the circulation or
that, after entering the circulation, they are so
rapidly and efficiently removed from the blood
that they cannot be detected there. Over the
first 4 days there was no significant increase in
the degree of infection of the vegetations. This
could mean that after the initial multiplication
the outgrowth of bacteria stops after 24 h. Al-
ternatively, it is possible that in the absence of a
sustained bacteremia no new colonies are
formed on the vegetations. Thus, the situation
for S. epidermidis must differ from that of S.
sanguis, which in the presence of a sustained
bacteremia showed a significant increase in the
degree of infection of the vegetations during the
first 4 days of the infection (11).

In the model used in this study, attachment
of bacteria to the surface of the endocardial veg-
etations is the initial event in the development
of bacterial endocarditis. These attached bacte-
ria are subsequently covered by fibrin and then
grow out to colonies. Thus, warfarin treatment
could affect both attachment and outgrowth of
the bacteria.

An impaired attachment of the bacteria
would be reflected in the need for larger bacte-
rial inocula to cause an infection in all rabbits.
Indeed we found that in warfarin-treated rab-
bits, significantly higher staphylococcal inocula
were needed to induce an infection. An effect on
the outgrowth of the bacteria would be reflected
in an altered numerical course of the bacteria
recovered from the vegetations. Such an effect
was indeed demonstrated. Not only were bacte-
rial numbers in the vegetations 24 h after infec-
tion significantly lower in warfarin-treated rab-
bits, but over the next 3 days the bacteria disap-
peared entirely from the vegetations in several
of the warfarin-treated animals.

Thus, warfarin treatment seems to have an
inhibitory effect on the induction and develop-
ment of an S. epidermidis infection of the endo-
cardial vegetations. How this effect is brought
about is not entirely clear, but two possible
explanations can be mentioned. In the first
place, the bacterial colonies in the warfarin-
treated rabbits might be located more superfi-
cially in the vegetations, thus making them
more accessible to phagocytic cells. Alterna-
tively, a deficient fibrin cover might promote
the removal of the rather loosely attached
staphylococci from the surface of the vegeta-
tions by the bloodstream. The finding that war-

farin-treated rabbits had positive blood cultures
more frequently seems to support the latter pos-
sibility. Unfortunately, the morphological find-
ings do not help to solve this problem, but the
data obtained by Durack (2) on the location of
S. epidermidis on the surface of the vegetation
during the early phase of infection might sup-
port this possibility. The normal clearance of
10^7 bacteria from the circulation in warfarin-
treated and control rabbits excludes a direct
effect on bacterial clearance as the cause of the
increased frequency of bacteremia in warfarin-
treated rabbits. Again, there is a difference be-
tween S. epidermidis and S. sanguis, since for
the latter warfarin treatment was found to have
no effect on the induction or the course of the
infection of the vegetations (11).

The divergent effects of warfarin treatment
on the induction and course of endocarditis
caused by S. sanguis and S. epidermidis sug-
gest that different mechanisms are involved in
the development of endocarditis due to these
two bacteria. Clearly it is easier for S. sanguis
to settle and maintain itself on the endocardial
vegetations under unfavorable conditions than
it is for S. epidermidis. Whether this difference
is due to surface properties of the former species
promoting tighter adherence to certain surfaces
(7), or to an ability of these bacteria, after ad-
hering to the surface of the vegetation, to acti-
vate locally the clotting mechanism and so pro-
mote their definitive settlement on the vegeta-
tions (9), remains to be determined. In this con-
nection it should be kept in mind that even
under intense anticoagulation colonies of strep-
tococci were found in vegetations buried in fi-
brin (11).

The mean survival of 26 days found for rab-
bits with S. epidermidis endocarditis is much
longer than the mean survival of 15.5 days for
rabbits with S. sanguis endocarditis (11). How-
ever, warfarin treatment also significantly
shortened the survival of rabbits with S. epider-
midis endocarditis: the mean survival of 3.5
days was comparable to that of 3 days for war-
farin-treated S. sanguis-infected rabbits. How-
ever, the S. epidermidis rabbits did not show
rapidly progressive bacteremia shortly before
their death. As observed in streptococcal endo-
carditis, the autopsy findings in rabbits dying
prematurely from the combination of S. epider-
midis infection and warfarin treatment showed
not only severe pulmonary edema but also ex-
tensive pulmonary hemorrhages. It should be
kept in mind that we showed previously (11)
that a group of six catheterized uninfected rab-
bits survived warfarin treatment for more than
7 days. Thus, rapid valvular destruction by bac-
terial endocarditis seems to provoke severe pul-

monary congestion that, combined with anticoagulant treatment, results in extensive pulmonary hemorrhages that precipitate the early death of warfarin-treated rabbits.

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