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EFFECTS OF MONOCYTOPENIA AND ANTICOAGULATION IN EXPERIMENTAL *STREPTOCOCCUS SANGUIS* ENDOCARDITIS

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Summary.—The role of blood monocytes in the attachment of streptococci to endocardial vegetations was investigated in an experimental *Streptococcus sanguis* endocarditis by depletion of blood monocytes with the cytostatic drug VP 16-213 alone and combined with anticoagulant treatment with warfarin sodium. The numbers of streptococci in the vegetations of control, monocytopenic, and monocytopenic/anticoagulated rabbits were comparable. In the vegetations streptococci were found mainly in areas free of phagocytic cells. It is concluded that streptococci do not have to be phagocytosed by monocytes in the circulation before being deposited on the surface of endocardial vegetations.

Even the vegetations of intensively anticoagulated/monocytopenic rabbits showed colonies of streptococci embedded in polymerized fibrin and cellular material, this matrix possibly being held together by streptococcal dextran.

During the development of bacterial endocarditis, blood-borne bacteria colonize endocardial vegetations. It seems likely that in this process both bacterial properties and such host factors as phagocytic cells and the clotting mechanism are involved (Angrist and Oka, 1963; Archer, 1977; Durack and Beeson, 1972; Durack, 1975; Weinstein and Rubin, 1973; Weinstein and Schlesinger, 1974). For instance, dextran production by certain strains of streptococci (*e.g. mutans* and *sanguis*) facilitates their adherence to endocardial vegetations (Pelletier, Coyle and Petersdorf, 1978; Ramirez-Ronda, 1978; Scheld, Valone and Sande, 1978). An involvement of phagocytic cells could be deduced from Durack's observation that during the early stage of infection most of the streptococci on the surface of endocardial vegetations were inside mononuclear phagocytes (Durack, 1975). He suggested that the streptococci have to be phagocytosed by blood monocytes before they are deposited on the vegetations. However, from his findings it is not clear whether phagocytosis occurs before the deposition of the bacteria or after they have settled on the surface of the vegetations.

After attachment of the bacteria, the next important step in the colonization of the vegetations is the covering of these micro-organisms by a fibrin layer, a process probably initiated by the local generation of tissue thromboplastin by monocytes after phagocytosis of bacteria on the surface of the vegetations (van Ginkel *et al.*, 1979). This might explain why in a previous study we were unable to demonstrate an effect of treatment with the anticoagulant warfarin on the induction or course of a *Streptococcus sanguis* infection of endocardial vegetations (Thompson *et al.*, 1976).

Thus it is evident that monocytes could be involved in several steps of the development of streptococcal endocarditis. The aim of the present study was to investigate the effect of depletion of blood monocytes alone or combined with anticoagulant treatment on the induction and course of an experimental *Streptococcus sanguis* endocarditis.

MATERIALS AND METHODS

Experimental design.—The study was done in male Chinchilla rabbits weighing 2–2·5 kg, raised in the Central Institute for the Breeding of Laboratory Animals, Bilthoven, The Netherlands.

NBTE was induced according to Durack and Beeson (1972), by inserting a plastic catheter *via* the left carotid artery into the left ventricle. Three days later, the rabbits were injected with live streptococci to induce a bacterial endocarditis. The experiments were done in monocytopenic rabbits, anticoagulated/monocytopenic rabbits, and control rabbits. In all experiments at least 4 rabbits were used per time-point.

Cytostatic drug.—For the induction of monocytopenia, VP 16–213 (kindly donated by Sandoz Ltd, Basle, Switzerland) was chosen because the most striking action of this compound is a cytostatic effect on acute monocytic leukaemia and on the monocytic component of acute myelomonocytic leukaemia (Dombernowsky, Nissen and Larsen, 1972; Rosencweig *et al.*, 1977). This drug is a semisynthetic derivative of podophyllotoxin. The commercial preparation contains per 5-ml ampoule, 20 mg VP 16–213, 80 mg Tween 80, 650 mg polyethylene glycol 300, 30 mg benzyl alcohol, 2 mg citric acid anhydrous, and 1 ml absolute ethanol. This solution was diluted 1:1 with saline before use. VP 16–213 was injected i.v. into a marginal ear vein in a daily dose of 20 mg per rabbit for 7 days.

Anticoagulant drug.—Warfarin sodium (kindly donated by ENDO laboratories Inc., New York) was used as anticoagulant. A daily dose of 8 mg per rabbit was given i.m. as described elsewhere (Thompson *et al.*, 1976). 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 specified limits.

Micro-organisms.—The *Streptococcus sanguis* certified as dextran-producing was kindly provided by Dr H. Engel (Rijks Instituut voor de Volksgezondheid, Bilthoven, The Netherlands). It was the same micro-organism as was used in our previous studies (Thompson *et al.*, 1976; van Ginkel *et al.*, 1979). For infection, overnight cultures in Todd–Hewitt medium were used.

Blood leucocytes.—At various time-points, 2 peripheral blood samples were taken from a marginal ear vein with leucocyte pipettes and diluted 1:20 in Türk's solution containing 6% acetic acid. The total leucocyte counts were done in duplicate in a Bürker haemocytometer. The total numbers of monocytes, granulocytes, and lymphocytes per mm^3 were calculated from the total number of leucocytes per mm^3 and differential counts of 400 leucocytes in 4 blood smears.

Quantitative bacteriology.—In general, the methods used were the same as those described in detail elsewhere (Thompson *et al.*, 1976). Briefly, endocardial vegetations were isolated, weighed, and homogenized in glucose broth, after which serial 10-fold dilutions of the homogenate were made, and 0·1-ml samples were plated on sheep blood agar plates and incubated for 24–48 h at 37°. The degree of infection of the vegetations was expressed as the number of bacteria per gram of vegetation.

Microscopical examination of endocardial vegetations.—For light microscopy, endocardial vegetations were removed together with the underlying aortic valve leaflets and/or heart muscle. The vegetations were then fixed in 4% formaldehyde or 96% ethanol, and embedded in Paraplast. Histological slides were stained with haematoxylin and eosin, Gram stain for bacteria, periodic acid–Schiff (PAS) stain for polysaccharides, or Lawson/Martius scarlet blue (MSB) stain (Lindeman, 1976) for fibrin.

Parablast sections of the vegetations from control and monocytopenic rabbits were investigated with respect to total cellularity, and the relative amounts of monocytes and granulocytes were determined by one of the authors (F.E.) without prior knowledge of the experimental group. The findings were scored according to a semi-quantitative scale from 0 to 4. In addition, the location of streptococci in relation to phagocytic cells was investigated.

Frozen sections of vegetations from control and anticoagulated/monocytopenic rabbits were examined for fibrin, polysaccharides, and bacterial location. For further characterization of fibrin-like material, frozen sections were incubated at room temperature for 15–60 min in 5M urea or 2% acetic acid and then stained for fibrin (Bleyl, Sebening and Kuhn, 1969).

Transmission electron microscopy to study the structure of the vegetation was performed in freshly removed vegetations which were immediately cut into blocks of 1 mm^3 and fixed for 2 h in 1·5% glutaraldehyde in 0·1M phosphate-buffered saline (PBS), pH 7·4, at 4°. After rinsing in PBS (1 h), the preparations were post-fixed in 1% OsO_4 in PBS at 4° for 1 h, dehydrated in graded solutions of ethanol. cleared in propylene oxide, and embedded in Epon 812. Semithin sections were stained with toluidine blue, ultra-thin sections with uranyl acetate and lead citrate. All sections were examined in a Philips EM 200.

Statistical analysis.—The results obtained in the various groups were compared on the basis of a two-tailed Student's t test.

RESULTS

Effect of VP 16-213 *on the number of peripheral blood leucocytes*

The effect of catheterization for the induction of NBTE on the number of leucocytes in the peripheral blood was studied in control and VP 16-213-treated rabbits. Figs 1 and 2 give the mean numbers of peripheral blood monocytes and granulocytes per mm^3 in at least 11 animals.

VP 16-213 treatment alone resulted in a significant reduction of the number of peripheral blood monocytes and of 50% of the lymphocytes ($P < 0{\cdot}001$; not shown), whereas the number of granulocytes remained roughly constant.

Formation of catheter-induced vegetations in non-VP 16-213-treated rabbits coincided with a significant ($P < 0{\cdot}001$) increase in the number of peripheral blood monocytes, whereas the numbers of granulocytes and lymphocytes were not influenced.

In VP 16-213-treated animals monocytosis did not develop after catheterization, and the granulocyte numbers did not differ significantly from those in the controls (Figs. 1 and 2).

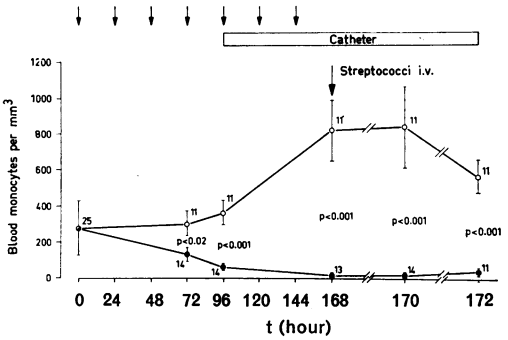


FIG. 1.—Changes in the number of peripheral blood monocytes per mm^3 in VP 16-213-treated and control rabbits before and during the development of streptococcal endocarditis. Each value is the mean ± s.e. of at least 11 animals. The control group (○——○) received no treatment, the VP 16-213 group (●——●) was injected (↓) with 7 daily doses of 20 mg of this drug. To induce NBTE in both groups a catheter was introduced at 96 h; and 72 h later both the control and the VP 16-213-treated rabbits were infected with streptococci. P values concern the number of monocytes of both groups at each time-point.

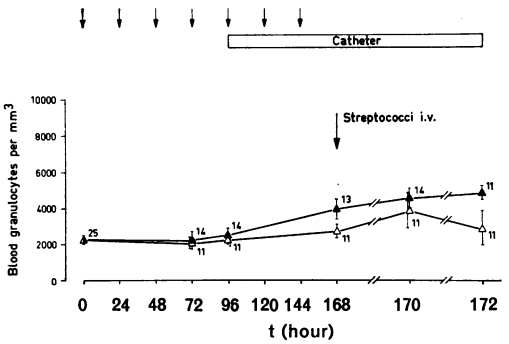


FIG. 2.—Changes in the number of peripheral blood granulocytes per mm^3 in VP 16-213-treated (▲——▲) and control (△——△) rabbits. For details, see Fig. 1. The differences between the two groups did not reach significance at any time-point.

Effect of VP 16-213 *on the induction and course of* Streptococcus sanguis *endocarditis*

To find out whether monocytopenia influences the induction and course of infection of sterile endocardial vegetations, control and VP 16-213-treated rabbits were infected with streptococci. We found that VP 16-213 treatment had no effect on the induction of *Strep. sanguis* endocarditis as measured by the number of rabbits with infected vegetations 1 day after infection with variously sized inocula of streptococci (Table I). The degree of infection of the vegetations on the first and second days of the infection was not influenced either (Table II).

Effect of VP 16-213 *on cellularity of endocardial vegetations*

The effect of depletion of blood monocytes on the total cellularity and relative numbers of monocytes and granulocytes

TABLE I.—*Effect of VP* 16-213 *on induction of* Streptococcus sanguis *endocarditis**

Pretreatment	I.v. streptococcal inoculum 10^6	10^4	10^3
None	4/4	3/4	0/5
VP 16-213	4/4	3/4	0/5

* Expressed as number of rabbits with infected vegetations per number of rabbits used. The animals were killed 1 day after the injection of streptococci.

in infected vegetations was assessed by comparison of cell numbers in 3-day-old vegetations of control and VP 16-213-treated rabbits 30 min after the injection of 10^{10} live streptococci (Table III). On the basis of semiquantitative scoring, no significant increase ($P > 0{\cdot}1$) was found in the total cellularity or the number of monocytes or granulocytes on the surface of the vegetations of infected non-VP 16-213-treated rabbits (Group II) 30 min after injection of bacteria as compared with the vegetations (NBTE) in non-infected animals (Group I). There was a significant reduction ($P < 0{\cdot}01$) of the number of monocytes on the vegetations of VP 16-213-treated and infected rabbits (Group III) as compared with Group II. The total cellularity and the number of granulocytes in these animals did not differ significantly between Group II and Group III.

Effect of VP 16-213 *on the location of streptococci in endocardial vegetations*

Light microscopy of vegetations isolated from control and monocytopenic rabbits 30 min after bacterial challenge showed that the streptococci were located free in the vegetations or occasionally in the proximity of phagocytic cells.

Transmission electron microscopy showed small groups of streptococci encapsulated in areas of cellular material and fibrin in these vegetations. Occasionally, bacteria were associated with

TABLE II.—*Bacterial endocarditis after i.v. injection of* Streptococcus sanguis

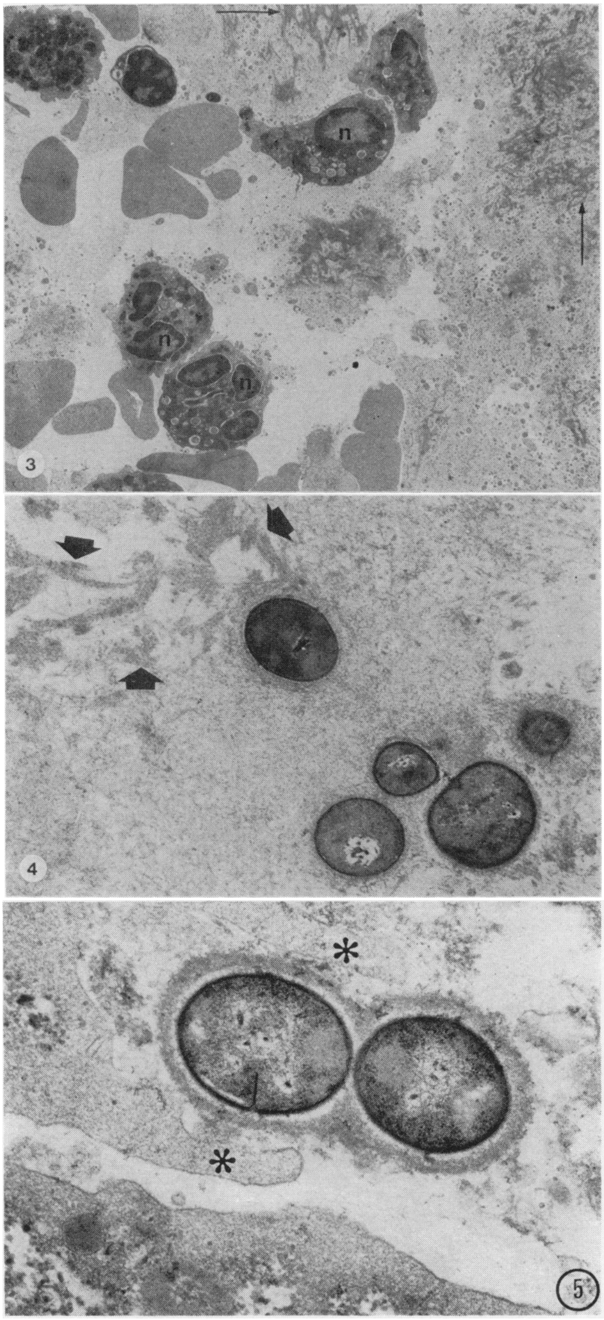
Days after infection§	Bacteria per g vegetation* Control	VP 16-213†	VP 16-213 + warfarin‡
1	$2{\cdot}2 \times 10^9$	$6{\cdot}3 \times 10^9$	$3{\cdot}3 \times 10^9$
	($3{\cdot}7 \times 10^8$–$1{\cdot}1 \times 10^{10}$)	($3{\cdot}5 \times 10^9$–$9{\cdot}3 \times 10^9$)	($3{\cdot}1 \times 10^8$–$1{\cdot}8 \times 10^{10}$)
2	$4{\cdot}8 \times 10^9$‖	$5{\cdot}5 \times 10^9$	$6{\cdot}5 \times 10^8$‖
	($1{\cdot}2 \times 10^9$–$1{\cdot}9 \times 10^{10}$)	($1{\cdot}7 \times 10^9$–$8{\cdot}7 \times 10^9$)	($2{\cdot}3 \times 10^6$–$2{\cdot}2 \times 10^{10}$)

* Expressed as geometric means and ranges (between parentheses) for at least 4 rabbits per group.
† Rabbits were given 20 mg VP 16-213 daily for a period of 7 days.
‡ Warfarin treatment was started 24 h after catheterization, in a daily dose of 8 mg.
§ With 10^6 streptococci administered i.v.
‖ $P > 0{\cdot}2$.

TABLE III.—*Effect of VP*-16-213 *on cellular composition of endocardial vegetations*

Group	Pre-treatment	Strepto-coccal† inoculum	Endocardial vegetations* Cellularity	Granulocytes	Monocytes	
I	None	None	$2{\cdot}5 \pm 0{\cdot}5$	$2{\cdot}3 \pm 0{\cdot}4$	$1{\cdot}1 \pm 0{\cdot}4$	(8)
II	None	10^{10}	$2{\cdot}0 \pm 0{\cdot}3$§	$2{\cdot}1 \pm 0{\cdot}3$‖	$1{\cdot}6 \pm 0{\cdot}2$‡	(15)
III	VP 16-213**	10^{10}	$1{\cdot}5 \pm 0{\cdot}3$§	$1{\cdot}5 \pm 0{\cdot}3$‖	$0{\cdot}6 \pm 0{\cdot}2$‡	(13)

* Expressed as the mean ± s.e. for at least 4 rabbits per group; number of vegetations between parentheses. Cellularity and relative numbers of monocytes and granulocytes were scored according to a scale of 0 to 4.
† Rabbits were killed 30 min after i.v. injection of live streptococci.
‡ $P < 0{\cdot}01$.
§ $P > 0{\cdot}1$.
‖ $P > 0{\cdot}1$.
** 7 daily injections, each 20 mg per rabbit.

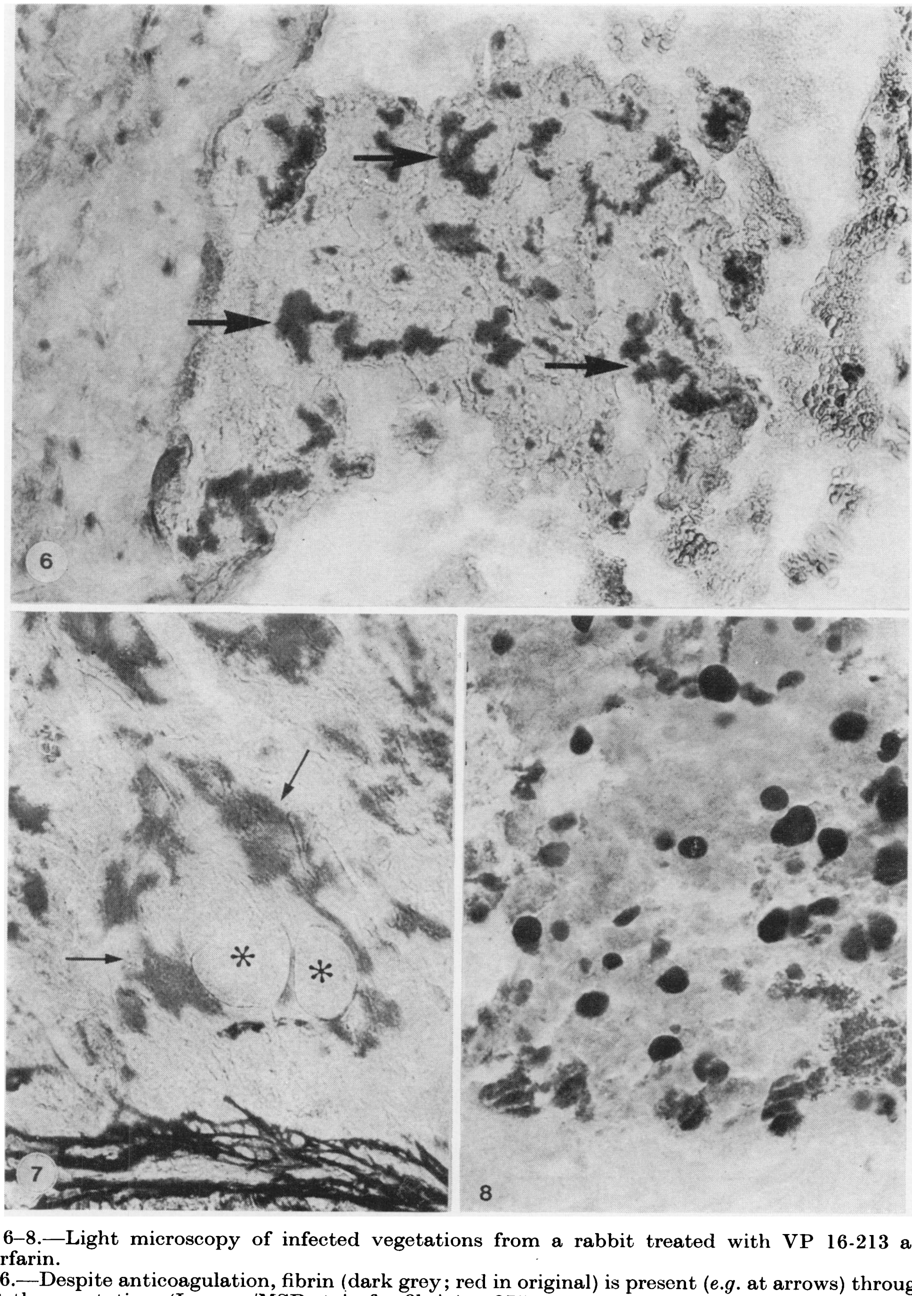


FIGS 3–5.—Electron micrographs showing infected vegetations from a rabbit treated with VP 16-213 and warfarin.

FIG. 3.—Neutrophil granulocytes (n) and erythrocytes embedded in an amorphous, occasionally fibrillar (arrows) matrix. × 1,666.

FIG. 4.—At higher magnification, fibrin-like fibrillar material (although without cross-striations) is seen (arrows) close to bacteria which are embedded in amorphous material, possibly dextran. × 16,666.

FIG. 5.—Two bacteria are surrounded by surface projections (asterisks) of a neutrophil granulocyte, suggesting that the bacteria are in the process of being endocytosed. × 30,000.



FIGS 6–8.—Light microscopy of infected vegetations from a rabbit treated with VP 16-213 and warfarin.

FIG. 6.—Despite anticoagulation, fibrin (dark grey; red in original) is present (*e.g.* at arrows) throughout the vegetation. (Lawson/MSB stain for fibrin) × 375.

FIG. 7.—Fibrin (arrows) is often seen in close proximity to bacterial colonies (asterisks). (Lawson/MSB stain for fibrin) × 750.

FIG. 8.—Bacterial colonies are intensely PAS-positive; the remaining components of the vegetation show moderate staining. (PAS stain) × 300.

phagocytes (Figs. 3–5). No streptococci could be definitively identified within monocytes.

Effect of VP 16-213 and warfarin treatment on the course of Streptococcus sanguis *endocarditis*

The effect of anticoagulant treatment on the induction and course of infection was investigated by comparing vegetations of control, VP 16-213-, and VP 16-213/warfarin-treated rabbits 1 and 2 days after the injection of 10^6 streptococci.

All rabbits had infected vegetations and the number of bacteria in the vegetations did not differ significantly between the 3 groups (Table II).

The structure of the infected vegetations of the monocytopenic rabbits and anticoagulated/monocytopenic rabbits was comparable. Paraplast and frozen sections of the vegetations stained for fibrin, bacteria (Figs. 6 and 7), and polysaccharides (Fig. 8) showed in both groups dense colonies of streptococci in cell-free areas, intermingled with fibrin-containing areas held together by PAS-positive polysaccharides, possibly dextran. Non-infected vegetations were negative for the PAS stain.

Since non-polymerized fibrin is soluble in urea and acetic acid, frozen sections from vegetations of warfarin-treated rabbits were incubated with these 2 compounds to differentiate between polymerized insoluble fibrin and non-polymerized, so-called soluble fibrin. No differences were observed between incubated and control sections, which indicates that in warfarin-treated rabbits fibrin has been formed on the vegetations.

DISCUSSION

The results of the present study show that after the injection of live streptococci into rabbits with NBTE comparable numbers of bacteria are present in the vegetations of control rabbits, monocytopenic rabbits, and anticoagulated/monocytopenic rabbits.

Involvement of blood monocytes in the deposition of streptococci from the circulation on the surface of the endocardial vegetations has been postulated (Durack, 1975). Durack found many more monocytes or granulocytes containing streptococci on the surface of vegetations in rabbits that had been injected with 10^{10} streptococci and killed 30 min after the bacterial challenge than in rabbits with similar sterile vegetations. In our control rabbits (non-VP 16-213-treated) and in the monocytopenic rabbits as well, the injection of live streptococci did not lead to more phagocytic cells on the vegetations, and the colonies of bacteria were located outside phagocytic cells. The number of monocytes on the vegetations of VP 16-213-treated rabbits was significantly lower, whereas the number of animals with infected vegetations and the degree of infection of the vegetations in this group did not differ significantly from the non-VP 16-213-treated group. From these findings it may be concluded that streptococci do not have to be phagocytosed by monocytes before they are deposited on the surface of the vegetations.

Another way in which monocytes might be involved in the development of streptococcal endocarditis is the initiation of local fibrin formation on infected endocardial vegetations. In the vegetations of control and monocytopenic rabbits the colonies of streptococci were found intermingled in material staining positively for fibrin. The insolubility of this material in urea and acetic acid shows that it is not deposited fibrinogen or fibrin monomer but fully polymerized fibrin.

In another study we showed *in vitro* that monocytes are stimulated to generate tissue thromboplastin after phagocytosis of bacteria (van Ginkel *et al.*, 1979). This monocyte-derived thromboplastin could initiate the local formation of fibrin. However, we found that the induction and course of an experimental *Strep. sanguis* endocarditis was unaffected by anticoagulant treatment with warfarin, and anticoagulation did not lead to a defective fibrin formation on infected vegetations (Thompson *et al.*, 1976). In this connection it should be kept in mind that patients and animals under adequate anticoagulation can still develop deep venous thrombi, as indicated by *in vitro* tests (Hoak, Connor and Warner, 1966; Morris and Mitchell, 1976). One explanation for fibrin formation on infected vegetations in intensively anticoagulated rabbits might be that sufficient procoagulant activity is still generated in the micro-environment of the vegetations through local formation of thromboplastin by monocytes after phagocytosis of streptococci. However, depletion of blood monocytes and reduction of the number of monocytes on the vegetations did not diminish the number of streptococci in the vegetations of warfarin-treated rabbits, and colonies of streptococci were found embedded in fully polymerized fibrin. Thus, if the hypothesis concerning the local generation of thromboplastin by monocytes is valid, this would mean that even after anticoagulation and reduction of the number of monocytes enough of these cells must still be present on the vegetations to generate sufficient tissue thromboplastin to initiate fibrin formation. Another possible way in which fibrin formation could occur on the vegetations is through direct activation of the clotting mechanism by the streptococci. However, in our *in vitro* study we were unable to detect such activation (van Ginkel *et al.*, 1979). Finally, a factor contributing to the colonization of the vegetations by streptococci might be dextran produced by these micro-organisms. PAS staining showed that a polysaccharide, presumably dextran, was present throughout the vegetation. This dextran could contribute to the coherence of the vegetational matrix.

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