Role of Granulocytes in Experimental *Streptococcus sanguis* Endocarditis

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We investigated the role of granulocytes during the induction and course of experimental *Streptococcus sanguis* endocarditis in rabbits by depleting blood granulocytes with nitrogen mustard. The induction of the endocarditis was not influenced by granulocytopenia: the 50% infectious dose was $5.4 \times 10^4$ colony-forming units in normal and granulocytopenic rabbits. However, granulocytopenia influenced the course of the endocarditis, as shown by a significant increase in the number of colony-forming units per gram of vegetation ($P < 0.02$) from 24 to 72 h after the injection of $10^5$ colony-forming units of *S. sanguis*. This rise did not occur in the control rabbits. Furthermore, bacteremia was significantly higher in the granulocytopenic rabbits ($P < 0.05$) during the first 48 h compared with the control rabbits. This was not because of altered clearance of the streptococcus inoculum or seeding of streptococci from extracardiac bacterial foci. We concluded that granulocytes have no measurable effect on the induction of *S. sanguis* endocarditis, but during the course of the endocarditis, granulocytes keep the endocardial infection in check.

Bacterial endocarditis develops when endocardial vegetations are colonized by blood-borne microorganisms (1). The influence of host factors on the process of colonization is not completely understood (3, 5, 15). For example, specific antibodies against the causative microorganism may have a protective effect, as some experimental studies (5, 10) have shown; however, another study failed to demonstrate such an effect (14).

Monocyte depletion does not prevent the deposition of bacteria on endocardial vegetations (13) but probably affects the pathogenesis, since the phagocytosis of bacteria on the vegetation surface stimulates these cells to generate tissue thromboplastin. This in turn triggers the formation of fibrin, an important feature in the development of bacterial endocarditis (15).

The role of granulocytes in the pathogenesis of bacterial endocarditis is not clear. These cells might have a protective effect at the surface of the vegetation by removing bacteria before they can be covered by fibrin and form colonies. Freedman and Valone (7) suggested that local agranulocytosis permits bacteria to grow unhindered inside the vegetations. In agreement with this hypothesis, Durack and Beeson (4) rarely found granulocytes in the proximity of bacterial colonies. However, in an earlier study (13), we observed bacteria ingested by granulocytes in the fibrillar matrix of the vegetations, which suggests that these cells play an active role by inhibiting the proliferation of settled bacteria.

The present study was performed to investigate the role of granulocytes in the development and course of experimental *S. sanguis* endocarditis in rabbits made granulocytopenic with nitrogen mustard (HN2).

**MATERIALS AND METHODS**

**Experimental design.** The study was done in the male chinchilla rabbits, weighing 1.8 to 2.3 kg, raised in the Central Institute for Breeding of Laboratory Animals, Bilthoven, The Netherlands.

Nonbacterial thrombotic endocarditis was induced by inserting a plastic catheter via the left carotid artery into the left ventricle by the method of Durack and Beeson (4). The catheter was left in situ during the experiment. Immediately after catheterization, the animals were divided into two groups; one group served as controls and the other was given HN2 to induce granulocytopenia. Three days later, 1 ml of a suspension of live streptococci was injected intravenously in a marginal ear vein. The animals were killed 1, 2, or 3 days after this injection.

In all experiments, there were at least four rabbits in each group.

**Induction of granulocytopenia.** HN2 was used to induce granulocytopenia because this compound gives a significant reduction of the number of peripheral blood granulocytes (8). HN2 is an alkylating agent that acts by cross-linking guanine bases in DNA, thus arresting cell division. One vial containing 10 mg of lyophilized HN2 (mustine hydrochloride, ACF, Che-
cyte counts were done in duplicate in a Farma cytometer. The total numbers of granulocytes, monocytes, and lymphocytes per cubic millimeter were calculated from the total number of leukocytes per cubic millimeter and differential counts of 400 leukocytes in four blood smears.

Microorganism. The strain of S. sanguis was the same as that used in previous studies (12-14). This strain is known to produce dextran. Bacteria from an overnight culture in Todd-Hewitt medium were washed three times in saline and diluted with saline to the appropriate concentration before injection.

Quantitative bacteriology. The methods used were generally the same as those described in detail elsewhere (13). Briefly, endocardial vegetations were isolated aseptically, weighed, and homogenized in glucose broth, after which serial 10-fold dilutions of the homogenate were made, and 100-μl samples were plated on sheep blood agar plates and incubated for 24 to 48 h at 37°C.

The degree of infection of the vegetation was expressed as the number of colony-forming units (CFU) per gram of vegetation. For quantitative blood cultures, 2-ml blood samples were taken daily (starting 24 h after injection of the streptococci) and immediately after the rabbits were killed. These samples were diluted in 1 ml of Liquoid, plated in 4 ml of glucose agar broth, and incubated for 24 to 48 h at 37°C. Bacteremia was expressed as the number of CFU per milliliter of blood.

Microscopic examination of endocardial vegetations and spleen tissue. For light microscopy, endocardial vegetations were removed together with the underlying aortic valve leaflets, heart muscle, or both. After weighing, the spleens were cut into pieces measuring approximately 10 by 5 by 3 mm3, which were then fixed in 4% formaldehyde and embedded in Paraplast. Histological slides were stained with hematoxylin and eosin. Spleen tissue embedded in plastic by the method of te Velde et al. (11) was also examined after gallamine Giemsa and Gomorri reticulin staining.

Statistical analysis. Results were compared by a parametric two-tailed Student’s t test (effect of HN2 treatment on the number of CFU per gram of vegetation, number of blood leukocytes, weight of spleen), a paired t test (bacteremia and blood cell counts), the nonparametric Mann-Whitney U test (bacteremia), and covariance analysis (differences in clearance). The 50% infective dose was calculated by the method of Spearmann-Kärber as reported by Finney (6). Multiple regression analysis (2) was used to assess correlation between various individual factors and the degree of infection.

RESULTS

Effect of HN2 treatment on the number of peripheral blood leukocytes. In control rabbits, catheterization resulted in a significant increase in the number of blood granulocytes (P < 0.005) after 72 h. After the injection of 105 CFU of S. sanguis at 72 h to induce bacterial endocarditis, the granulocyte counts returned to normal within 1 day and remained at that level for the next 2 days (Fig. 1). This course of the blood granulocyte count was not influenced when the number of streptococci injected was varied from 104 to 106 CFU (not shown).

When HN2 was injected immediately after catheterization, the granulocyte counts decreased significantly (P < 0.001), i.e., to almost zero after 72 h. When streptococci were injected at that time, the granulocyte counts remained at this low level for at least 3 days. The difference between granulocyte counts in the two groups is highly significant (P at least <0.025) for all time points after the injection of streptococci.

The course of the blood monocyte counts was initially similar to that of the granulocytes (Fig. 2), but monocytopenia persisted for only 2 days after induction of bacterial endocarditis. On day 3, blood monocyte counts were at the level of the controls (P > 0.5).

Effect of HN2 treatment on the induction of S. sanguis endocarditis. To investigate the effect of granulocytes on the induction of S. sanguis endocarditis, HN2-treated rabbits were injected with various numbers of streptococci. As shown in Table 1, the 50% infective dose was similar for controls and HN2-treated rabbits. Thus, granulocytes had no measurable effect on the induction of S. sanguis endocarditis.

Effect of HN2 treatment on the course of bacteremia. In control rabbits injected with 105 CFU of streptococci, the number of bacteria in

FIG. 1. Course of the number of peripheral blood granulocytes per cubic millimeter in control and HN2-treated rabbits before and during the course of S. sanguis endocarditis. All rabbits were inoculated with 106 CFU of S. sanguis at 72 h. The vertical bars represent the standard error of the mean; the number at each time point represents the total number of rabbits in the control (○) and HN2-treated (●) groups.
the blood rose slowly during the first 48 h (Fig. 3) and increased significantly between 48 and 72 h \((P < 0.05)\), whereas in the HN2-treated rabbits the number increased significantly during the first 48 h \((P < 0.02)\) and remained at a constant level during the next 24 h, the differences with the controls at 24 and 48 h being statistically significant \((P < 0.002\) and \(P < 0.02\), respectively). At 72 h, no significant differences were found between the two groups \((P > 0.9)\).

Thus, HN2 treatment resulted in a higher number of bacteria per milliliter of blood during the first 48 h. Since an impaired clearance of bacteria from the circulation because of the effect of HN2 treatment on cells of the mononuclear phagocyte system could contribute to this effect, we measured the clearance of \(10^5\) CFU of streptococci in uncatheterized control rabbits on day 3 or 4 after the injection of HN2.

Analysis of covariance, with the \(\log_{10}\) of the number of bacteria per milliliter of blood taken as a linear function of \(\log_{10}\) time \((r^2 > 0.98)\), showed no differences in this respect between the control and the HN2-treated groups \((P > 0.1)\). Three rabbits were used in each group.

**Effect of HN2 treatment on the number of streptococci in the endocardial vegetations.**
To find out whether granulocytes influence the number of bacteria in the vegetations, HN2-treated and control rabbits were infected with \(10^5\) CFU of streptococci and were killed 24, 48, and 72 h after inoculation (Fig. 4).

On macroscopic examination, HN2 treatment did not appreciably influence the size of the vegetations. Also, the weight of the total removed vegetation mass per rabbit was not significantly different at 24, 48, or 72 h after the injection of \(10^5\) CFU of streptococci in control and HN2-treated rabbits \((P \geq 0.2\) at each time point).

In the control rabbits, the number of CFU per gram of vegetations remained at the same level from 24 to 72 h after inoculation. In the HN2-treated rabbits, the degree of infection of the vegetations showed a different course. At 24 h, the number of CFU per gram of vegetation did not yet differ significantly from that in the control rabbits, but increased significantly from 24 to 72 h \((P < 0.001)\) and became significantly higher at 48 and 72 h \((P < 0.05\) and \(P < 0.01\), respectively).

**TABLE 1. Effect of HN2 on the induction of S. sanguis endocarditis**

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Inoculum (CFU)</th>
<th>ID(_{50})^a (CFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(10^5)</td>
<td>(10^4)</td>
</tr>
<tr>
<td>Control</td>
<td>0/4b</td>
<td>1/4</td>
</tr>
<tr>
<td>HN2 treated</td>
<td>0/4</td>
<td>1/4</td>
</tr>
</tbody>
</table>

^a ID\(_{50}\), 50% infective dose.

^b expressed as the number of rabbits with infected vegetations per number of rabbits used. The rabbits were killed 48 h after the induction of the infection.

^c Values in parentheses are twice the standard deviation (on a geometric basis by the method of Spearman-Kärber).
respectively) in the granulocytopenic rabbits compared with the control rabbits.

Influence of HN2 treatment on the formation of extracardiac bacterial foci. The higher number of bacteria in the blood in HN2-treated rabbits during the first 48 h after infection with 10³ CFU of *S. sanguis* could also have been due to seeding of bacteria from extracardiac foci formed by the bacteria injected to infect the vegetation. To investigate this possibility, we injected 10⁵ CFU of streptococci (the inoculum was also used to infect the vegetation) in three uncatheterized rabbits and found that the blood cultures taken daily on 7 consecutive days remained sterile. Subsequently, the same rabbits were treated with HN2, and 3 days later they were again injected with 10³ CFU of streptococci. Again, these new granulocytopenic rabbits did not develop bacteremia during the next 7 days. Thus, injection of 10⁵ CFU of streptococci does not lead to the formation of extracardiac septic foci that give rise to bacteremia under either control or granulocytopenic conditions.

Correlation of various factors with the degree of infection of the vegetation by multiple regression analysis. The number of CFU per gram of vegetation can be influenced by different factors. Multiple regression analysis makes it possible to investigate the relationship between a given factor and the number of CFU per gram of vegetation. Five parameters were analyzed in this way: granulocyte count, blood monocyte count, blood lymphocyte count (mean of all values, determined daily during infection in each rabbit) number of bacteria per milliliter of blood (log_{10} CFU/ml), and the duration of infection (i.e., the period between the injection of 10⁵ CFU of streptococci and the killing of the animals, expressed in days).

The data for control and HN2-treated rabbits were analyzed both separately and in combination (Table 2). The analysis of the combined data indicated that the number of granulocytes and of CFU per milliliter of blood were the only parameters showing significant correlation with the number of CFU per gram of vegetation (*P* < 0.05 and *P* < 0.001, respectively).

The equation for control plus HN2-treated rabbits shows that the granulocyte count is correlated negatively and the degree of bacteremia is correlated positively with the number of CFU per gram of vegetation. This means that an increasing number of CFU per gram of vegetation is correlated quantitatively with decreasing

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>y</th>
<th>Multiple regression (y = a + bx₁ +/− cx₂)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>log_{10} CFU/g veg =</td>
<td>7.40 + 0.52 × log_{10} bacteremia</td>
</tr>
<tr>
<td>HN2 treated</td>
<td>log_{10} CFU/g veg =</td>
<td>7.39 + 0.46 × log_{10} bacteremia + 0.33 × duration of infection</td>
</tr>
<tr>
<td>Control + HN2 treated</td>
<td>log_{10} CFU/g veg =</td>
<td>7.95 + 0.52 × log_{10} bacteremia − 0.16 × granulocyte count</td>
</tr>
</tbody>
</table>

* The relationship between one parameter and the number of CFU per gram of vegetation is not dependent on the other parameters.

* CFU/g veg (number of CFU per gram of vegetation), degree of infection of the vegetation.

* Bacteremia is expressed as CFU per milliliter of blood.

* Duration is expressed in days.

* Count is expressed as the number per cubic millimeter × 10⁹.
granulocyte counts and also with an increasing number of bacteria in the blood.

Analysis of the data for HN2-treated rabbits alone revealed a positive correlation of bacteremia and duration of infection with the number of CFU per gram of vegetation ($P < 0.001$ and $P < 0.02$, respectively). Analysis of the data for the control rabbits alone indicated that only bacteremia had a significant relation with the number of CFU per gram of vegetation.

A similar relationship between the number of bacteria in the blood and the number of CFU per gram of vegetation was found for the control group, the HN2-treated group, and these groups combined. Therefore, HN2 treatment influenced only the number of the bacteria in the blood. The parameter duration of infection only showed significant correlation with the number of CFU per gram of vegetation in the HN2-treated rabbits, which means that the numbers of CFU per gram of vegetation increased independently of the correlation between the number of CFU per gram of vegetation and bacteremia. This can be explained by proliferation of the streptococci within the vegetation. No statistically significant correlation between the number of CFU per gram of vegetation and the duration of infection was found for the control rabbits or the combined group (control and HN2-treated rabbits).

**Correlation between the number of blood granulocytes and the degree of bacteremia during the course of an infection.** To investigate a possible relationship between the number of blood granulocytes and the degree of bacteremia, these parameters were measured daily in four control rabbits with bacterial endocarditis during the course of the disease (Fig. 5). The linear regression was found to be $\log_{10}$(CFU per milliliter of blood) = $3.23 - 0.50 \times$ granulocyte counts, $r = 0.687$; this correlation was statistically significant ($P < 0.005$). The negative regression shows that an increase in the number of granulocytes correlates with a decrease of the number of bacteria per milliliter of blood. Thus, a quantitative relationship exists between the number of Streptococcus in the blood and the naturally occurring variation in the number of blood granulocytes.

**Effect of HN2 treatment on the morphology of infected vegetations.** Histologically, two effects of HN2 treatment were found in vegetations taken from the aortic wall, aortic valves, or mural endocardium 72 h after injection of $10^5$ CFU of streptococci.

Vegetations of the control rabbits contained many granulocytes (Fig. 6A), whereas those in the HN2-treated rabbits contained almost none (Fig. 6B). Fibroblast proliferation was seen in the underlying tissue in both control and HN2-treated rabbits. Also, in the vegetations of granulocytic colonies, more and larger bacterial colonies were found, and confluence of colonies was observed frequently.

**Effect of HN2 treatment on the spleen after induction of S. sanguis endocarditis.** After the animals were killed, the spleen was examined macroscopically. On the first 2 days after infection, the organ appeared normal; on day 3, however, it was enlarged in control rabbits but not in the HN2-treated rabbits: the difference in spleen weight between these two groups was significant ($P < 0.005, 2.38 \pm 0.68$ g [$n = 4$] and $0.74 \pm 0.18$ g [$n = 4$], respectively [mean $\pm$ standard deviation]). Histologically, the spleens of the controls showed increased numbers of granulocytes including younger forms, which suggests active granulocytosis, edema, erythrophagocytosis, and accumulation of cells in T-cell areas, probably as a response to the infection. This picture was much less pronounced in the HN2-treated rabbits.

**DISCUSSION**

In the present study, depletion of blood granulocytes did not affect the number of bacteria needed to induce S. sanguis endocarditis. Furthermore, the clearance of injected bacteria from the circulation, taking approximately 10 min, was not affected by granulocyte depletion.
Thus, the time during which the injected streptococci could colonize the vegetation was the same in both groups. Apparently, the granulocytes did not prevent the initial settling of the streptococci on nonbacterial thrombotic endocarditis or remove the streptococci from the surface of the vegetation. It seems possible that the streptococci were covered by fibrin very quickly and thus were protected against phagocytosis.

Once bacterial endocarditis has been induced, the number of streptococci in the endocardial vegetations became significantly higher under granulocytopenia. Concomitantly, more bacteria appeared in the circulation. In the control rabbits, the number of CFU per gram of vegeta-
tion remained at approximately the same level during the first 72 h, as was also found by other investigators (4). In HN2-treated rabbits, however, the number of CFU per gram of vegetation increased after 24 h, and differences relative to the control rabbits became statistically significant at 48 and 72 h after induction. A positive correlation was found between the number of CFU per gram of vegetation and the number of CFU per milliliter of blood. This correlation was not influenced by HN2 treatment, as shown by multiple regression analysis. This analysis does not allow one to conclude whether this correlation is due to seeding of bacteria into the circulation, reinfection of the vegetation from the circulation, or both.

HN2 treatment depletes blood monocytes and granulocytes. However, in a previous study (13), we showed that selective depletion of blood monocytes does not affect the induction or course of S. sanguis endocarditis. In the present study, too, blood monocyte counts returned to normal after day 2 of the infection in HN2-treated rabbits, whereas the degree of infection continued to increase.

Although not specifically studied, HN2 treatment could also reduce serum antibody levels. However, since an earlier study indicated that the induction and course of S. sanguis endocarditis were not influenced by serum antibody levels (14), it is unlikely that the effect of HN2 treatment is attributable to depressed serum antibody levels. On this basis, the effects of HN2 treatment can be attributed solely to granulocytopenia.

HN2 treatment by a reduction of the number of circulating platelets might affect the structure and consequently the degree of infection of the vegetations. Although platelet numbers were not systematically followed during the course of the disease, the macroscopic sizes of the vegetations were not appreciably influenced by HN2 treatment, and also the weight of the removed vegetation mass was not significantly lower in HN2-treated rabbits at any time point. Also, in view of the role currently attributed to platelets in the pathogenesis of bacterial endocarditis, one would expect a reduced number of platelets to result primarily in an impaired adherence of bacteria to the vegetational surface and consequently in an increased 50% infective dose (12). This effect was not observed. For these reasons, an effect of HN2 treatment on the platelets seems to be a less probable explanation for the higher degree of infection in the granulocytopenic rabbits.

The moderate but significant granulocytosis found in the control rabbits after catheterization could be caused by either the inflammation due to the inserted catheter or by the surgery required to insert the catheter. Despite these stimuli which lead to granulocytosis, HN2 treatment resulted in a profound granulocytopenia.

The influence of the granulocytes on the number of bacteria in the vegetations during the course of the endocarditis can be explained by three possible mechanisms. The first mechanism could be phagocytosis of the bacteria by granulocytes within the vegetation. Contrary to Durack (3), who only rarely observed granulocytes in the immediate vicinity of bacterial colonies, we found granulocytes in close proximity. These granulocytes might control proliferation of bacteria and prevent them from emerging at the surface. This granulocyte function could explain the correlation between the number of granulocytes and the number of CFU per gram of vegetation shown by the multiple regression analysis. It also could explain the finding that the duration of infection showed a statistically significant correlation with the number of CFU per gram of vegetation only after HN2 treatment. This might indicate proliferation of bacteria in the vegetation that might be inhibited by granulocytes in the vegetation.

The second mechanism could be phagocytosis of bacteria by granulocytes on the surface of the vegetation during the course of bacterial endocarditis. These bacteria could reach the surface either by emerging from the inside of the vegetation or by settling on it once a bacteremia has developed. With the aid of scanning and transmission electron microscopy, McGowan and Gillet (9) showed that bacteria reappear on the surface of the vegetation between 18 and 24 h after induction of an infection, and concomitantly they saw granulocytes appearing just under the surface. Presumably, these were cells from the circulation that had settled on the surface of the vegetation and were subsequently covered by fibrin. Since the vegetations of HN2-treated rabbits contain almost no granulocytes, presumably bacteria emerging at the surface can freely enter the circulation without being attacked by granulocytes. This could also contribute to the higher number of CFU per milliliter of blood found in the HN2-treated rabbits at 24 h when the number of CFU per gram of vegetation did not differ significantly from the control values. It might be argued that extracardiac bacterial foci formed during the induction of endocarditis in the granulocytopenic rabbits might have been responsible for the higher number of CFU per milliliter of blood. However, injection of 10^7 CFU of streptococci into uncatheterized rabbits which initially had normal numbers of blood granulocytes and subsequently were granulocytopenic gave no indication of the formation of such foci. Therefore, in all probability, the bacteremia seen at 24 h after infection originated...
from the vegetations, and the absence of granulocytes on the surface of the vegetation in HN2-treated rabbits resulted in the higher number of bacteria per milliliter of blood.

The third mechanism could be phagocytosis of bacteria by granulocytes outside the vegetation. This granulocyte function could influence the number of bacteria per milliliter of blood and consequently the number of bacteria available to reinfect the vegetations. Granulocytopenia would result in a higher bacteremia, which could contribute to the higher degree of infection of the vegetations. An indication that this mechanism is operative might be found in the negative correlation between the number of granulocytes and the number of bacteria per milliliter of blood in control rabbits during the course of the infection. Another indication could be the increased number of granulocytes, including younger forms found histologically in the spleens of control rabbits, which suggests active granulocytopenia. This suggests a high turnover of granulocytes, since at that time no granulocytosis was found in the peripheral blood.

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LITERATURE CITED