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# INTESTINAL FLORA AND HAEMOPOIESIS

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## INTRODUCTION

The relationship between the intestinal flora and haemopoiesis can be studied in several ways. One of the possibilities is to associate germfree animals with several well-defined bacterial strains and to study the effects on haemopoiesis. In a series of recent experiments we have, however, chosen for another approach. We administered non-absorbable antibiotics to conventional mice in order to either totally decontaminate (*van der Waaij* and *Sturm*, 1968) or selectively decontaminate (SD) conventional animals (*van der Waaij* and *Berghuis-de Vries*, 1974). SD was applied to selectively suppress facultatively anaerobic Gram-negative bacteria. We focussed on the Gram-negative bacteria, because it was known from the literature that these bacteria might release a cell-wall-component which is commonly known as endotoxin. It is also known from the literature that haemopoiesis is extremely susceptible to this bacterial component (*Joshi* et al., 1969; *Staber* et al., 1978). Therefore, it appears likely that intestinal Gram-negative bacteria are involved in the regulation of haemopoiesis by releasing endotoxin. Free endotoxin may pass through the intestinal epithelium, particularly if the intracellular junctions are affected by stress or radiation treatment (*Walker* et al., 1978; *Gans* and *Matsumoto*, 1974).

In Table 1 the different steps in which we studied the relationship between the intestinal flora and haemopoiesis is depicted. The first step is intestinal flora modulation by administration of non-absorbable broad-spectrum antibiotics. The second step is the relationship between intestinal Gram-negative bacteria and intestinal endotoxin and the third step is intestinal flora associated endotoxin and haemopoiesis. The last part of this communication will deal with the effect of intestinal flora modulation on haemopoietic recovery.

In Table 2 information is provided about the experimental protocol. Non-absorbable antibiotics were orally ad-

**Table 1**: Study-design (relatyionship between intestinal flora and haemopoiesis)

- Intestinal flora modulation
- Intestinal Gram-negative bacteria and intestinal endotoxin
- Intestinal flora associated endotoxin and haemopoiesis
- Haemopoietic recovery after cytotoxic treatment and intestinal flora associated endotoxin

**Table 2**: Experimental protocol

- Oral administration of non-absorbable antibiotics to C3H/Law mice
- Intestinal contents assayed for Gram-negative bacteria and endotoxin
- Haemopoiesis:
  - Femoral nucleated cell content
  - Femoral CFU-S content
  - Femoral CFU-GM content
  - Proliverative state of CFU-S and CFU-GM, hydroxyurea-kill

ministered to C3H/Law mice. The intestinal contents were then daily assayed for the presence of gram-negative bacteria and the free endotoxin level in intestinal contents, i.e. the endotoxin which is released by Gram-negative bacteria during growth and death (*Rothfield* and *Pearlman-Kothencz*, 1969). The haemopoietic parameters followed, were the femoral nucleated cell content, the CFU-s content, CFU-GM content as well as the proliferative state of the CFU-s and the CFU-GM by hydroxyurea-kill. Hydroxyurea is an S-phase specific drug. Therefore, the susceptibility of certain cell types to this drug may be an indication for the proliferative state of these cells. To clarify the abbreviations used, they will briefly be discussed. All blood cells originate from the CFU-s, i.e. the pluripotent haemopoietic stem cell. This cell is called a CFU-s on basis of the spleen colony assay for this cell (*Till* and *McCulloch*, 1961). This cell forms colonies, i.e. spleen colonies, in lethally irradiated recipients. In case of the granulocytes, these CFU-s differentiate into the CFU-C or the CFU-GM. This is the progenitor cell of the granulocytes and macrophages. This cell can be cultured *in vitro* and therefore is called colony-forming-unit (CFU-C) culture or colony-forming-unit-granulocyte-macrophage (CFU-GM).

## INTESTINAL TOTAL DECONTAMINATION AND SELECTIVE GRAM-NEGATIVE ELIMINATION: INTESTINAL FLORA MODULATION

In Table 3, four different antimicrobial regimens are shown as well as the effect of these antimicrobial regimens on the colonisation pattern of faecal aerobic Gram-negative bacteria. As shown in this table, all Gram-negative strains were eliminated after 4 days of treatment by these different antimicrobial regimens, i.e. the faecal cultures *Escherichia coli*, *Enterobacter* spp. and *Klebsiella* spp. The difference between the four antimicrobial regimens is in the fraction of the intestinal flora that is suppressed. The first three antimicrobial regimens differ in this respect from the last one. Polymyxin, aztreonam and temocillin were used for selective elimination (SE) of Gram-negative strains, while the combination of cephalotin/neomycin was used for total decontamination (TD) of the intestinal tract.

In Figure 1 the effects of antimicrobial treatment on the faecal endotoxin concentration is shown. The faecal endotoxin concentration is given as a percentage of the initial control during treatment with the four antimicrobial

**Table 3**: Effect the antimicrobial regimens on the colonisation pattern of faecal aerobic Gram-negative bacteria

| Antimicrobial drug(s) | Days of treatment 0 | 1 | 2 | 4 | 8 |
|---|---|---|---|---|---|
| Polymixin | ●■▲ | - | - | - | - |
| Aztreonam | ●■▲ | ●▲ | ●▲ | - | - |
| Temocillin | ●■▲ | ▲ | - | - | - |
| Cephalotin/neomycin | ●■▲ | - | - | - | - |

●: *Escherichia coli*
■: *Enterobacter cloacae* >$10^2$ bacteria/g faeces
▲: *Klebsiella pneumoniae*

regimens. By SE of Gram-negative bacteria with polymyxin, aztreonam or temocillin the faecal endotoxin concentration decreased to 10% of the control level. Following TD with cephalotin and neomycin however, the faecal endotoxin concentration was decreased in two steps: In the first two days, concomitant with the disappearance of aerobic Gram-negative bacilli the faecal endotoxin concentration was reduced in a similar way as found during SE. However, after 2 days of TD an additional reduction of the faecal endotoxin concentration followed to 1% of the control.

One of the control experiments required for this type of study concerns the question whether these antibiotics, which were administered orally to mice, interfere with the Limulus assay for endotoxin (*Jorgensen* and *Smith*, 1974; *Gardi* and *Arpagaus*, 1980). Therefore, these antibiotics were administered to

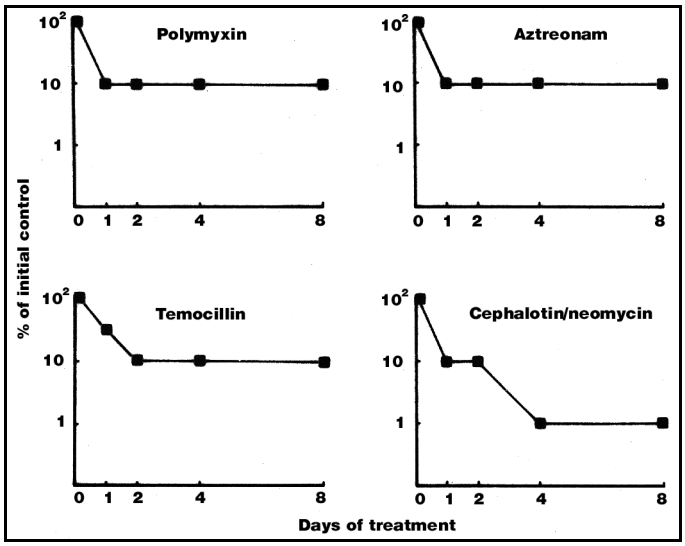


**Figure 1**: Effect of antimicrobial treatment on the faecal endotoxin concentration.

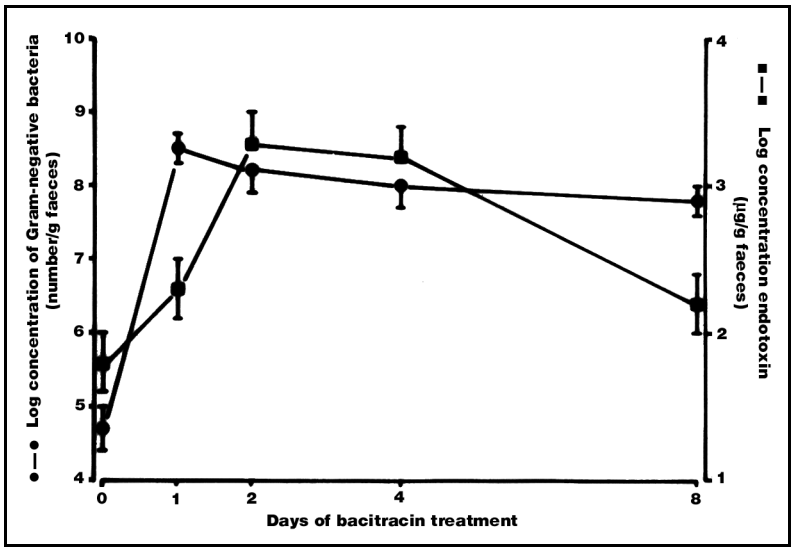


**Figure 2**: Faecal concentration of Gram-negative bacteria and endotoxin during bacitracin treatment.

faecal supernatants of control mice in concentrations similar to the concentrations which were measured after oral administration. The antibiotics used in the antimicrobial regimens did not influence the outcome as we found 100% recovery of endotoxin. On the basis of these results we could conclude that there is no interference of the antibiotics with the Limulus assay.

The SE experiments, therefore, indicate that 90% of the faecal free endotoxin is due to continuous release of endotoxin by aerobic Gram-negative bacteria. Secondly, on the basis of the total decontamination experiment it can also be concluded that 1% of intestinal endotoxin in mice could be ascribed to oral intake with food and water, while 9% of the faecal endotoxin is most probably due to release by anaerobic Gram-negative bacteria.

## SELECTIVE GROWTH STIMULATION

We did not restrict our studies to the elimination of particular strains. We also selectively stimulated growth of Gram-negatives in the intestines of our mice. The results of these experiments are given in Figure 2. To accomplish Gram-negative growth enhancement we administered bacitracin orally via the drinking water. This antibiotic has small spectrum anti Gram-positive activity and was administered to the drinking water to reduce the colonisation resistance (*van der Waaij* et al., 1971). Gram-negative strains are essentially resistant to this antimicrobial regimen. Soon after the start of bacitracin treatment an immediate increase occurs of the level of Gram-negative bacteria in the faeces. Already after 1 day of oral bacitracin treatment a 4-log increase in the level of Gram-negatives was observed. The Gram-negatives remained at a high concentration level during the entire treatment period of eight days of bacitracin treatment. In these mice the faecal endotoxin concentration was also determined. During the first two days of bacitracin treatment, we observed an

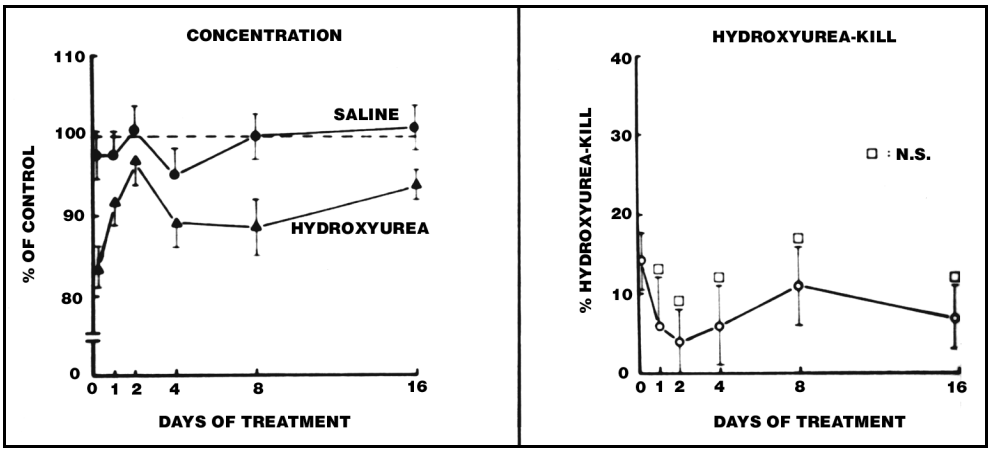


**Figure 3**: Femoral CFU-S and hydroxyurea-kill during polymyxin treatment.

increase of the faecal endotoxin concentration from 60 $\mu$g per g of faeces to 2000 $\mu$g per g of faeces. This is a very strong and significant increase in the faecal endotoxin concentration. Remarkably, after eight days of bacitracin treatment, however, we observed a gradual decline of the faecal endotoxin concentration. Several days after this relatively short observation period, the faecal endotoxin level was no longer significantly different from the control level; i.e. before bacitracin treatment. On basis of these results we may conclude that *in vivo* there is no strict relationship between the concentration of aerobic Gram-negative bacteria and the level of faecal endotoxin. It supports the conclusion that endotoxin is predominantly a product of highly proliferative Gram-negative bacteria.

## THE EFFECT OF INTESTINAL FLORA MODULATION ON THE PLURIPOTENT AND THE COMMITTED HAEMOPOIETIC STEM CELL

Polymyxin was administered to SE mice and the femoral CFU-s were followed to determine the pool size of the CFU-s and the hydroxyurea-kill. As can be seen in Figure 3 we did not observe a change in the femoral CFU-s pool size during polymyxin treatment. Upon administration of hydroxyurea, a reduction of the hydroxyurea-kill was observed in these mice in comparison to controls. However, this reduction did not reach significance, possibly because of the relatively high initial value and because of the variance of the assay.

In Figure 4 the effects of polymyxin treatment on the femoral CFU-GM are shown as well as the CFU-GM pool size and the CFU-GM hydroxyurea-kill. In contrast to what was found in the CFU-s determination, there was a significant reduction in the femoral CFU-GM pool size to approximately 60% of the control after 4 days of polymyxin treatment. Also following hydroxyurea-kill, a significant reduction was found. The hydroxyurea-kill was reduced from 30% to approximately 10% already after one day of polymyxin

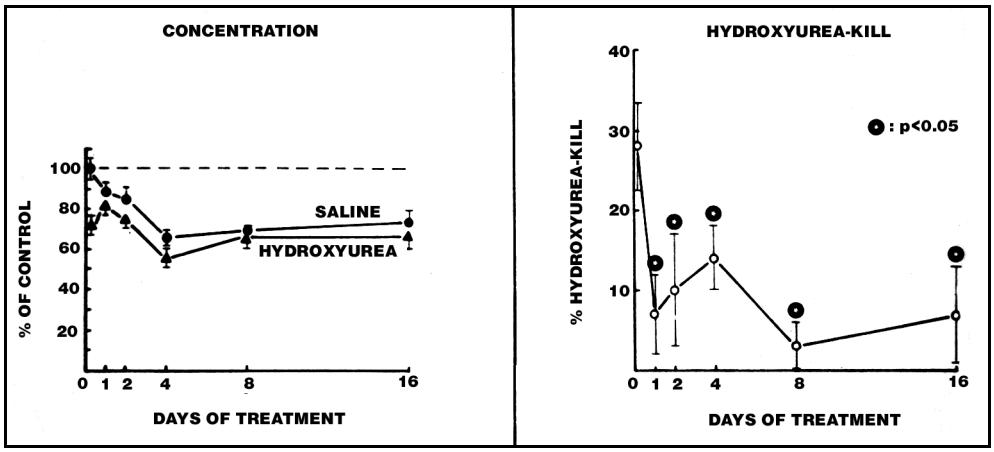


**Figure 4**: Femoral CFU-GM and hydroxyurea-kill during polymyxin treatment.

treatment. We have shown that polymyxin treatment reduces the proliferative state of haemopoietic stem cells. However, it is not certain after the experiment that the polymyxin effect represents a flora-mediated effect. This was studied in a subsequent experiment, during which we administered polymyxin also to germfree mice. In the germfree mice, this treatment did not cause a reduction of the CFU-GM during oral polymyxin treatment. Furthermore, there was no significant change in the hydroxyurea-kill in comparison to what was found in the polymyxin treated conventional mice.

Based on these findings in the germfree mice it can be concluded that the aerobic Gram-negative intestinal bacteria play an obvious role in the regulation of haemopoiesis.

## HAEMOPOIETIC RECOVERY

Mice were treated with ARA-C, i.e. 3 sequential injections in conventional mice treated with polymyxin or bacitracin treatment. Because the former experiment had indicated that polymyxin decreased haemopoietic stem cell activity, and that bacitracin would enhance growth of Gram-negatives in the first week and therewith would increase the proliferative state of stem cells because of the high endotoxin level associated with Gram-negative proliferation, we decided to study the effect of these different flora modulating regimens. Just before ARA-C injection as a result of polymyxin, the endotoxin level was present in our mice as a low faecal endotoxin level. In contrast, the bacitracin-treated mice were found to have a very high endotoxin faecal level, as was to be expected. Figure 5 shows the effect of these pre-treatment regimens on the recovery of the femoral nucleated cell content and the CFU-GM content. In case of bacitracin treatment as well as in case of polymyxin SE, a bi-phasic recovery of the femoral CFU-GM was found after ARA-C injection. Following a first rebound of the femoral CFU-GM content on day 2, the second rebound started on day 6 or day 8. It is clear from these data that, in case of bacitracin treatment (i.e. in case of a high intestinal endotoxin level), the first re-

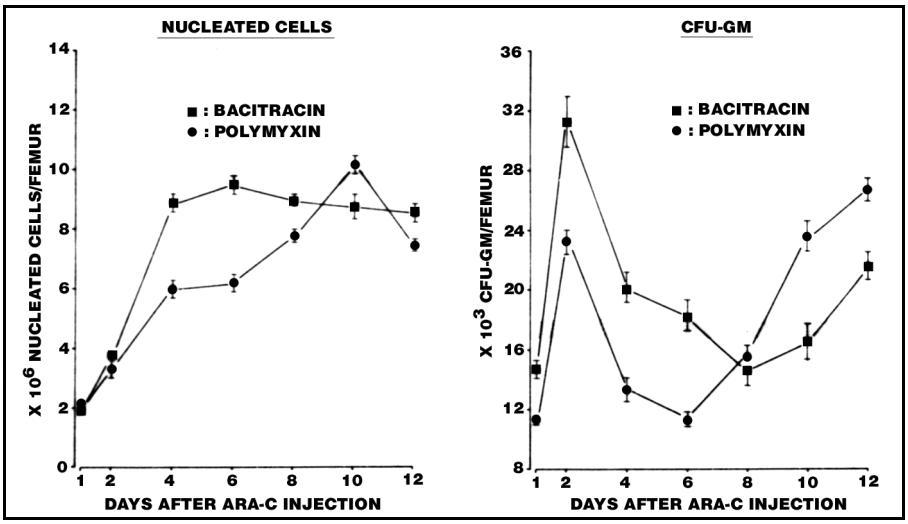


**Figure 5**: Effect of bacitracin and polymyxin treatment on femoral nucleated cells and CFU-GM after ARA-C injection.

bound increase of the femoral CFU-GM was significantly higher than in case of polymyxin treatment. These effects of endotoxin were also reflected in the recovery of the femoral nucleated cell content. In case of polymyxin pre-treatment it took 10 days after ARA-C injection before the femoral nucleated cell content was restored to the control value. In case of bacitracin pre-treatment, however, the nucleated cells were already recovered after 4 days. This means most probably, that at a high intestinal endotoxin level the recovery of the femoral nucleated cell content is accelerated with as much as 6 days.

## CONCLUSION

Elimination of Gram-negative bacteria by antimicrobial treatment, which reduces the intestinal endotoxin level, may cause a delayed haemopoietic recovery after cytotoxic insult.

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