At first sight, suggesting that the endogenous bacterial microflora in the gut can have effects outside the intestinal tract might seem fanciful. At the same time, supposing that antibiotics can modulate such effects and thus have actions in addition to their antibacterial activity could sound rather unexpected. However, theoretical considerations concerning the production and absorption of biologically active substances, and a growing number of experimental data support the notion that intestinal bacteria are an integral constituent of a living organism and that they can affect several physiological processes. It is important also from a clinical perspective to unravel the underlying mechanisms in order to avoid untoward effects of giving antibiotics (e.g. in case of malignancy) and to be able in the future to manipulate them therapeutically. It is the **basic premise of this thesis** that the GI flora has an influence on hematopoiesis and that antibiotics which affect the GI bacteria can modulate hematopoiesis. The **purpose of this thesis** is to find evidence for these processes in animal models of normal and malignant hematopoiesis and to search for possible mechanisms which mediate the effects.

The first introductory chapter describes the composition of the intestinal microflora, which is a strictly defined, well-balanced and stable ecosystem in which hazardous events do not occur. This in itself suggests an important role in normal physiology. The mechanisms regulating the interactions between the bacteria as well as between bacteria and mammalian host are reviewed extensively. Subsequently, substances from bacterial origin with known activity towards hematopoietic cells *in vitro* or *in vivo* are enumerated. It is obvious that these should be absorbed from the gut before they can have a "physiological" effect - i.e. under normal circumstances, via natural routes, and with doses which are attainable *in vivo* without reverting to unnatural manipulations such as parenteral injection. Although formal proof is still lacking for most of these substances, there seems to be enough circumstancial evidence for their regular absorption from the gut. Finally,

the biological literature is screened for experiments with bacterial products and/or antibiotics which demonstrate their possible influence on malignancy.

In chapter II the effect of oral treatment with the non-absorbable polymyxin on the kinetics of stem cells - CFU-S, the relatively immature spleen colony-forming units, and CFU-GM, stem cells committed to the myeloid lineage - was studied in the mouse. Polymyxin suppresses the Gram-negative bacteria in the gut and by doing so markedly reduces the intestinal endotoxin content. Although it was known for some time that antibiotic treatment or a germfree state resulted in a reduction of the pool size of CFU-GM in bone marrow, and of colony stimulating activity in serum, it was shown for the first time that elimination of intestinal Gram-negatives caused a significant decrease of the percentage of CFUs in S phase of the cell cycle. There was a strong suspicion that this decrease was mediated by a reduction of the amount of endotoxin absorbed from the gut.

Chapter III and IV demonstrate in the same murine model that oral nonabsorbable antibiotics can affect the susceptibility of hematopoietic cells to cytostatic drugs *in vivo*. In chapter III bacitracin was used. In contrast to polymyxin, oral bacitracin suppresses the Gram-positive bacteria; this causes an increase in intestinal Gram-negative bacteria and endotoxin content. In accordance with the results from chapter II, this should lead to an increase of S phase cells (but in these experiments, the percentage of stem cells in S phase was not specifically measured). The response to the cytostatic drug cytosine arabinoside (Ara-C) and the subsequent recovery of bone marrow and blood were dependent on the timing of Ara-C in relation to bacitracin. The outcome was compatible with a model in which bacitracin induced an increase of stem cells in S phase of the cell cycle, resulting in an enhanced susceptibility of hematopoietic stem cells to the cytotoxic action of Ara-C and at the same time an accelerated recovery of the

remaining stem cells.

The findings of chapter III were extended and confirmed in a new series of experiments (chapter IV) in which both polymyxin and bacitracin were used, thus in situations with low versus high load of intestinal Gram-negative bacteria and endotoxin. One day after Ara-C, hematopoietic parameters differed only slightly between the groups of animals, but the speed and height of recovery of CFUs in spleen and bone marrow were markedly affected by the antibiotic treatment. The differences in recovery patterns between polymyxin and bacitracin also suggested that stem cells migrate extensively between bone marrow and spleen during regeneration after cytostatic drugs. This phenomenon is firmly established now by other investigators and proved to have important clinical consequences in the practice of harvesting circulating stem cells for autologous bone marrow transplantation.

When it was thus shown in healthy mice that the GI flora - or at least its modulation by antibiotics - could influence the number of stem cells in cell cycle, their susceptibility to cytostatic drugs, and the hematopoietic recovery after cytostatics, we wondered if the GI flora could have comparable effects on malignant hematopoietic cells. It was feared that the extensive use of antibiotics in patients with malignancies might adversily affect the behaviour of the malignant cells. We found that the sole use of a nonabsorbable oral antibiotic with activity against Gram-positive bacteria caused reduction of a leukemic tumor cell load by about 40% (chapter V). For practical reasons, however, a switch had to be made from a murine to a rat model (and from bacitracin to vancomycin which has fairly comparable antibiotic activity). It appeared afterwards that not only the animal species but the whole system was different since Gram-negative bacteria and endotoxin (which correlated with the effect on normal hematopoiesis in the mouse) contributed only marginally to the antileukemic effect. Gram-positive bacteria obviously were responsible for

the production of new substances which were essential for stimulation of myeloid leukemia in this model. Although the demonstration of at least one additional mechanism complicated the object of investigation thoroughly, it strengthened our basic premise that the GI microflora can modulate (patho-) physiological processes, among them hematopoiesis.

Reducing a leukemic tumor load by 40% unfortunately is a relatively minor effect since it corresponds to a cell kill of less than one log. The cell kinetic mechanism(s) involved were not easy to detect: a small decrease by a few percentages of the leukemic growth fraction was sufficient to explain the reduction. In order to understand how the GI flora produced its influence and to be able to enhance this by future manipulations, we examined if antibiotic modulation induced changes in the expression of cytokines. Somewhat arbitrarily a number of cytokines with mainly hematopoietic potential were chosen for detection of messenger RNA (mRNA) in a semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR). The results are reflected in chapter VI and VII.

Upon absorption in the gut, the (still hypothetical) factors originating in the intestinal tract would be transported to the liver via the portal vein. Therefore, changes in cytokine expression were searched for in the large bowel wall and the liver. As mentioned earlier, oral vancomycin causes a strong reduction of Gram-positive bacteria in the gut (both aerobes and anaerobes) and an increase of Gram-negatives and endotoxin. This correlated with an increase of mRNA for stem cell factor (SCF), interleukin 1 beta (IL-1ß), transforming growth factor beta (TGFß), and tumor necrosis factor alpha (TNFα) in the bowel wall and liver of healthy rats, without affecting IL-4, IL-6, TGFα, or granulocyte colony-stimulating factor (G-CSF) (chapter VI). This led us to believe that the observed effects of oral antibiotics were mediated by changes in cytokine expression in the body; an increase or decrease of cytokines could then influence the hematopoiesis directly or

indirectly. Proving that bacteria in the digestive tract (or products derived from them) do interact with the cytokine network in the body would mean that we touched upon some basal mechanism of cellular metabolism. The GI flora could then contribute to the base-line expression of cytokines; modulation of the GI flora by antibiotics or otherwise could bring about many secondary events of which the effects on hematopoiesis was only an example.

In chapter VII, preliminary results are given from recent investigations in which we tried to reproduce these data *in vitro*. This proved to be very difficult, partly because of problems in finding adequate *in vitro* correlates. As a substitute for the liver, hepatoma cells were examined for cytokine expression. In the rat-derived FAO line, fecal suspensions of vancomycin-treated rats did cause an altered mRNA expression for TNFα and TGFß, but in contrast to the *in vivo* model, a downregulation was found. The differences could be due to the peculiarities of the FAO cells and to a change in balance between activating and inhibitory compounds in the fecal suspensions. The LT12 cell line which is directly derived from BNML with which it shares most characterisitics, was not inhibited by those cytokines which were upregulated *in vivo*, either when used alone or in combination. We must conclude that the anti-leukemic effect was not due to these cytokines, at least in the *in vitro* system; possibly other cytokines were involved or their action was indirect, e.g. via activation of the immune system.

In conclusion, the GI flora and antibiotics which modulate it do have effects on hematopoiesis in healthy and leukemic animals. Several bacterially-derived and/or bacteria-modified substances are possibly involved but their nature is not defined yet. Endotoxin from Gram-negative bacteria is one of them: it seems to stimulate myelopoiesis at least in mice. Peptidoglycan and (lipo-)teichoic acid could be others; they probably stimulate leukemic growth in the rat BNML model. These effects

of intestinal bacteria on hematopoiesis could be mediated directly or indirectly by interactions with the cytokine network in the liver and in the colon wall.

If the mechanisms could be elucidated in detail they could possibly be enhanced for therapeutic benefit. Further investigations could be directed at finding additional responsive cytokines in the liver and gut. The exact cell types (i.e. Kupffer cells, hepatocytes, endothelial cells, or even colonic epithelial cells) which are responsible for the cytokine upregulation could be defined by *in situ* hybridization techniques. The increased mRNA level is believed to correspond to an increased production of the relevant cytokines, but this should to be proven, e.g. by ELISA assays or Western blotting. Also, the active intestinal substances are to be identified and the bacterial species which produce them. Then it could be examined if the antileukemic effect in Brown-Norway rats can be enhanced by contaminating the animals with these bacteria, or by administering the bacterial products enterally or parenterally. Only if such interventions can be performed successfully and without major toxicity, the procedure could possibly be extended to the human situation.