INFECTION AND IMMUNITY, Nov. 1985, p. 437–441
0019-9567/85/110437-05$02.00/0

Vol. 50, No. 2

Myelopoiesis in Experimentally Contaminated Specific-Pathogen-Free and Germfree Mice during Oral Administration of Polymyxin

HENK GORIS,* FROUKJE DE BOER, AND DIRK VAN DER WAAIJ
Laboratory for Medical Microbiology, 9713 EZ Groningen, The Netherlands

Received 29 April 1985/Accepted 22 July 1985

Oral administration of polymyxin to specific-pathogen-free C3H/Law mice which were previously contaminated with gram-negative bacteria resulted in complete suppression of cecal gram-negative bacteria. Suppression of cecal gram-negative bacteria was accompanied by reduction of the cecal endotoxin concentration from 10 to 1 μg/g of cecal content as measured with a microtechnique for the *Limulus* amebocyte lysate assay. Endotoxin determination by this assay appeared to be unaffected by the amount of polymyxin present in cecal preparations after oral administration of this antibiotic. In experimentally contaminated specific-pathogen-free mice, the femoral concentration of progenitor cells forming granulocyte-macrophage colonies in vitro (CFU-GM) decreased significantly ($P < 0.001$) to 66% of the initial control after 4 days of polymyxin treatment. However, the femoral CFU-GM concentration in germfree mice and splenic CFU-GM concentration in experimentally contaminated specific-pathogen-free and germfree mice was not affected by polymyxin treatment. The kinetic behavior of femoral and splenic CFU-GM in experimentally contaminated specific-pathogen-free and germfree mice was expressed as the in vivo sensitivity to the S-phase-specific cytostatic drug hydroxyurea, i.e., the hydroxyurea kill. Administration of polymyxin to experimentally contaminated specific-pathogen-free mice significantly diminished the hydroxyurea kill of femoral CFU-GM from 29 to 13% ($P < 0.02$) and of splenic CFU-GM from 53 to 27% ($P < 0.005$). The hydroxyurea kill of femoral CFU-GM in germfree mice was not significantly affected by polymyxin treatment. On basis of these results we conclude that the effect of polymyxin treatment on myelopoiesis is most likely due to elimination of intestinal gram-negative bacteria and may indicate a significant role of intestinal gram-negative bacteria in the regulation of myelopoiesis.

The microbial composition of the intestinal microflora, particularly (aerobic) gram-negative bacteria (GNB), appears to be of importance in myelopoiesis. Alterations of the level of intestinal GNB either by administration of antibiotics (21; H. Goris, S. Daenen, D. van der Waaij, and M. R. Halie, Exp. Hematol. **12**:382, 1984) or by association of germfree (GF) animals with GNB (6, 18) have been shown to affect myelopoiesis. These observations argue for a role of intestinal GNB in the regulation of myelopoiesis. Injection of endotoxin, a cell wall component of GNB, is known to influence hemopoiesis at the stem cell level (1, 9, 29). Since endotoxin is released by GNB into the surrounding medium (17, 27), myelopoiesis is supposed to be affected by leakage of endotoxin through the mucosa of the intestinal tract (5, 11, 16). It has been suggested that myelopoiesis is regulated by the host's own microflora via intermittent endotoxemia (25).

Selective elimination of GNB from the intestinal tract of C3H/Law mice by oral administration of the nonabsorbable drug polymyxin (14, 32) has been shown to reduce the fecal ("free") endotoxin concentration (H. Goris, F. de Boer, and D. van der Waaij, Scand. J. Infect. Dis., in press) and to affect hemopoietic stem cell kinetics at different stages (Goris et al., Exp. Hematol. **12**:382, 1984).

To establish the involvement and contribution of the intestinal microflora, particularly GNB, in the regulation of myelopoiesis, experimentally contaminated specific-pathogen-free (EC-SPF) and GF C3H/Law mice were orally treated with polymyxin. In both types of mice the effect of polymyxin treatment on the cecal level of GNB, polymyxin, endotoxin, and level of bone marrow and splenic progenitor cells forming granulocyte-macrophage colonies in vitro (CFU-GM) was established. Interference of polymyxin with endotoxin determination in cecal supernatants by the *Limulus* amebocyte lysate (LAL) assay (12, 19) was estimated by incubation of cecal supernatants of untreated control mice with polymyxin. The kinetics of bone marrow and splenic CFU-GM were followed by determination of the susceptibility of these progenitor cells to the S-phase-specific cytostatic drug hydroxyurea (HU).

MATERIALS AND METHODS

Mice. Specific-pathogen-free (SPF) and GF female C3H/Law mice between 8 and 12 weeks of age and weighing 20 to 25 g were used.

Housing. The SPF mice were kept three per cage in a conventional animal room and fed ad libitum with sterilized pelleted food (Hope Farms, The Netherlands) and autoclaved water. The GF mice were maintained in plastic germfree isolators (positive pressure isolator 1020; Metall & Plastic GmbH, Radolfzell, Federal Republic of Germany).

Contamination. To prevent variability in the level and occurrence of intestinal GNB, all SPF animals were orally contaminated with 10 μl of a 10-fold dilution in saline of overnight cultures of *Escherichia coli* (API profile 5144572), *Enterobacter cloacae* (API profile 3305573), and *Klebsiella pneumoniae* (API profile 5205773) in brain heart infusion broth (Difco Laboratories, Detroit, Mich.). Each SPF mouse received a mixture of approximately 10^6 bacteria of each of these bacterial strains, which were isolated from the feces of

* Corresponding author.

TABLE 1. Cecal concentration of GNB, endotoxin, and polymyxin

Days of polymyxin treatment	EC-SPF mice			GF mice		
	GNB/g of cecal content[a]	Endotoxin concn (μg/g of cecal content)[a]	Polymyxin concn (μg/g of cecal content)[b]	GNB/g of cecal content[a]	Endotoxin concn (μg/g of cecal content)[a]	Polymyxin concn (μg/g of cecal content)[b]
0	$>10^2$	10	<16	$<10^2$	0.1	<15
4	$<10^2$	1	71 ± 6	$<10^2$	0.1	55 ± 2

[a] Cecal concentration of GNB and endotoxin of one experiment. This experiment was repeated twice in EC-SPF and GF mice with essentially the same results.
[b] Means ± standard error of the means of three separate experiments.

"clean" conventional C3H/Law mice. SPF mice contaminated with these bacterial strains were called EC-SPF mice.

Polymyxin treatment. One week after oral contamination of SPF mice, polymyxin E (Dumex, Denmark) was added to the drinking water (1 g/liter of drinking water) (10). Just before the initiation of polymyxin treatment, the GF mice were transferred from the plastic germfree isolators to germfree laminar flow units (Bioflow Chamber; the Germfree Laboratories Inc., Miami, Fla.).

Culturing of cecal contents. After 0 and 4 days of polymyxin treatment, groups of three mice were killed by cervical dislocation. The cecal contents were collected, pooled per experimental group, suspended in pyrogen-free saline (1:4, wt/vol), and serially diluted in 10-fold dilution steps in brain heart infusion broth. After overnight incubation (37°C), the dilutions were subcultured onto MacConkey agar (Oxoid Ltd., United Kingdom) to check for the presence of aerobic GNB. The detection level of aerobic GNB was 10^2 bacteria per g of cecal content. Below the detection level aerobic GNB are supposed to be completely suppressed. The sterility of the cecal contents of GF mice was checked by overnight incubation (37°C) of these contents in brain heart infusion broth.

Biotyping of aerobic GNB. After subculturing, pure cultures were made of the aerobic GNB. They were identified with the API 20E system (API Benelux b.v., The Netherlands) (4, 24).

Preparation of cecal supernatants. Cecal supernatants were prepared by centrifugation (5,000 × *g*, 10 min, 0 to 4°C) of cecal suspensions in pyrogen-free saline (1:4, wt/vol).

Determination of cecal endotoxin concentration. The cecal endotoxin concentration was estimated by a modification (Goris et al., in press) of a microtechnique (12) for the LAL test. Gel formation of a mixture of 1 μl of LAL (Mallinckrodt, Inc., St. Louis, Mo.) and 1 μl of the test sample in a 5-μl capillary tube (Omnilabo, The Netherlands) was investigated after incubation of the mixture for 45 min at 37°C by dipping of the capillary for 2 to 3 s in a tube containing 0.1% bromophenol blue (E. Merck AG, Darmstadt, Federal Republic of Germany) in ethanol-phosphate buffer. Starting from a 4-fold dilution of the cecal supernatants, 10-fold serial dilutions were made in pyrogen-free saline and tested in duplicate for the highest dilution step that still caused gel formation. The endotoxin concentration of a sample was calculated by multiplying the sensitivity of the LAL assay, as tested by 10-fold serial dilution of a reference solution of endotoxin (Mallinckrodt), by the reciprocal of the highest positive dilution of a sample.

Determination of the cecal polymyxin concentration. The concentration of polymyxin was estimated by the method of Goss and Cimijotti (13). Twofold serial dilutions of cecal supernatants in Trypticase soy broth (Becton and Dickinson, The Netherlands) were made in microtiter plates of 8 by 12 wells (U/Form; Thovadec Hospidex, The Netherlands). To prevent bacterial contamination, cephalothin was added to the Trypticase soy broth medium. The test was performed in duplicate with a strain of *Escherichia coli* (MIC of polymyxin, 0.3 μg/ml; MIC of cephalothin; >250 μg/ml) with triphenyltetrazolium chloride as the growth indicator.

Recovery of polymyxin and of endotoxin from cecal supernatants. To establish interference of polymyxin with endotoxin determination by the LAL assay, polymyxin (0, 20, 40, and 100 μg/ml) was added to cecal supernatants of untreated EC-SPF and GF mice. After incubation for 30 min at 37°C, the polymyxin and endotoxin concentrations were estimated.

Preparation of femoral bone marrow and spleen cell suspensions. A single femur from each animal was removed. Bone marrow cell suspensions were obtained by flushing the femurs with 1 ml of α-medium–2 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), pH 7.2 (Flow Laboratories, United Kingdom). Single-cell suspensions were made by repeated flushing through a 25-gauge needle. Spleens were removed, pressed through a stainless steel sieve, and suspended in α-medium–2 mM HEPES (pH 7.2)–5% fetal calf serum (GIBCO, United Kingdom) by flushing through 18- and 25-gauge needles, sequentially. Bone marrow and spleen cell suspensions per group of three mice were pooled. Nucleated cell counts of the bone marrow and spleen cell suspensions were determined with a Coulter Counter.

CFU-GM assay. CFU-GM were estimated with the methylcellulose method of Iscove and Sieber (15). Briefly, a 1-ml suspension of 5×10^4 nucleated femoral marrow or 5×10^5 nucleated spleen cells was incubated for 7 days at 37°C in a water-saturated atmosphere of 5% CO_2 in air. The culture medium consisted of 0.8% methylcellulose (Methocel MC 400mP a.s.; Fluka AG, Switzerland), 30% fetal calf serum, 10^{-4} M β-mercaptoethanol (Merck), 1% bovine serum albumin (GIBCO), 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Furthermore, an optimal amount of 10 μl of a pokeweed mitogen-stimulated spleen cell-conditioned medium (3) per ml of culture medium was added to provide the system with factor(s) essential for colony growth. Cellular aggregates consisting of 50 or more cells were scored as colonies by using an inverted microscope. For each experimental point four replicate cultures were counted. In all of the harvested colonies, macrophages, granulocytes, or both were found after staining with May-Grünwald-Giemsa.

Determination of the HU kill. At day 0 and day 4 of polymyxin treatment, groups of three animals were injected intravenously with 900 mg of HU (Sigma Chemical Co., St. Louis, Mo.) per kg of body weight (injection volume, 0.25 ml per 20 g of body weight) in pyrogen-free saline 2 h before sacrifice (26). The corresponding control group of three mice was injected with pyrogen-free saline. The HU kill of femoral and splenic CFU-GM, i.e., the difference in concentration between the saline and HU groups, was expressed as a

TABLE 2. Polymyxin and endotoxin concentration in cecal supernatants after incubation

Addition of polymyxin (μg/ml)	EC-SPF mice		GF mice	
	Polymyxin concn (μg/ml)[a]	Endotoxin concn (μg/ml)[b]	Polymyxin concn (μg/ml)[a]	Endotoxin concn (μg/ml)[b]
0	<3	2	<3	0.02
20	3 ± 0	2	10 ± 1	0.02
40	10 ± 1	2	19 ± 1	0.02
100	27 ± 2	2	46 ± 0	0.02

[a] Mean polymyxin concentration ± standard error of the mean of three separate experiments in EC-SPF and GF mice.
[b] Results of one experiment. This experiment was repeated twice with essentially the same results.

percentage of the saline control during corresponding periods of polymyxin treatment.

Statistical evaluations. The mean and the standard error of the mean were calculated. A two-tailed Student *t*-test was used for comparison of two means. The level of significance was set at $P = 0.05$.

RESULTS

Cecal concentration of aerobic GNB, endotoxin, and polymyxin. Before oral administration of polymyxin to EC-SPF mice, 10^4 to 10^6 *E. coli* organisms, 10^4 to 10^6 *Enterobacter cloacae* organisms, and 10^2 to 10^4 *Klebsiella pneumoniae* organisms were cultured per gram of cecal content. After 4 days of polymyxin treatment, aerobic GNB were found completely suppressed ($<10^2$ bacteria per g of cecal content) (Table 1). From the cecal contents of GF mice no aerobic GNB were cultured before and after polymyxin treatment. Before initiation of polymyxin treatment the cecal endotoxin concentration of EC-SPF mice was found to be 100 times higher than the endotoxin concentration in the cecal content of GF mice before initiation of polymyxin treatment. No difference was observed between the cecal endotoxin concentration in SPF and EC-SPF mice. Suppression of aerobic GNB in EC-SPF mice by oral administration of polymyxin was accompanied by a reduction of the cecal endotoxin concentration from 10 to 1 μg/g of cecal content. In GF mice polymyxin treatment did not affect the low cecal endotoxin concentration. Despite the similarity in daily intake of polymyxin (3 to 4 ml/day per mouse) by EC-SPF and GF mice, the cecal concentration after 4 days of treatment in EC-SPF mice was found to be significantly ($P < 0.05$) higher than the cecal concentration in GF mice.

Polymyxin and endotoxin concentration in cecal supernatants after incubation. After incubation of cecal supernatants of untreated EC-SPF and GF mice with different amounts of polymyxin, a significant reduction of the polymyxin concentration was observed (Table 2). From cecal supernatants of GF mice 2 to 3 times more polymyxin was recovered than from cecal supernatants of EC-SPF mice. Polymyxin concentrations established after incubation of cecal supernatants of EC-SPF and GF mice with this antibiotic, which were similar to the polymyxin concentration after oral administration (Table 1), did not affect endotoxin determination in cecal supernatants by the LAL assay.

Femoral nucleated cell content, CFU-GM concentration, and HU kill. Oral administration of polymyxin and injection of HU did not affect the femoral nucleated cell content of EC-SPF and GF mice (Table 3). The femoral cellularity of GF mice was found to be higher than the cellularity of EC-SPF mice. Four days of polymyxin treatment of EC-SPF mice decreased the femoral CFU-GM concentration after saline and also after HU injection from 93 to 61 and 66 to 53 CFU-GM per 5×10^4 nucleated cells significantly ($P < 0.001$ and $P < 0.005$), respectively. However, in GF mice, the CFU-GM concentration after saline or HU injection was not significantly affected by polymyxin treatment. Comparison of corresponding data for the femoral CFU-GM concentration of EC-SPF and GF mice revealed a significant difference ($P < 0.01$) at day 0 of polymyxin treatment for the saline control. Only in EC-SPF mice both the S-phase and non-S-phase fraction were decreased by oral administration of polymyxin. The susceptibility of femoral CFU-GM to HU in EC-SPF mice during polymyxin treatment, i.e., the decrease of the S-phase fraction, was reflected by a significant decrease ($P < 0.02$) of the HU kill from 29 to 13%. However, the HU kill of GF mice was not significantly affected by polymyxin treatment.

Obviously, oral administration of polymyxin to EC-SPF mice during a period of 4 days reduced the HU kill of femoral CFU-GM to a level which was similar to the level in GF mice.

Splenic nucleated cell content, CFU-GM concentration, and HU kill. The splenic nucleated cell content of EC-SPF and GF mice was not affected by oral polymyxin treatment and injection of HU (Table 4). The spleens of EC-SPF mice contained 2 to 5 times more CFU-GM (total and non-S-phase) than did the spleens of GF mice. Polymyxin treatment of EC-SPF and GF mice for 4 days did not affect the splenic CFU-GM concentration of the saline control. However, the CFU-GM concentration in EC-SPF mice after HU injection, i.e., the non-S-phase fraction, increased significantly ($P < 0.001$) from 87 to 130 CFU-GM per 5×10^5 nucleated cells after 4 days of polymyxin treatment.

Before initiation of polymyxin treatment the splenic CFU-

TABLE 3. Femoral nucleated cell content, CFU-GM concentration, and HU kill

Days of polymyxin treatment	Addition	EC-SPF mice			GF mice		
		NC content ($\times 10^6$)[a]	CFU-GM/5 $\times 10^4$ NCs[a]	HU kill (%)[b]	NC content ($\times 10^6$)[a]	CFU-GM/5 $\times 10^4$ NCs[a]	HU kill (%)[b]
0	Saline	7.5 ± 0.9	93 ± 5	29 ± 5	9.1 ± 0.4	74 ± 4	14 ± 6
	Hydroxyurea	7.6 ± 0.2	66 ± 3		9.2 ± 0.3	64 ± 4	
4	Saline	7.4 ± 0.5	61 ± 3	13 ± 4	9.2 ± 0.3	71 ± 5	20 ± 6
	Hydroxyurea	7.3 ± 0.3	53 ± 3		9.9 ± 0.3	57 ± 3	

[a] Mean ± standard error of the mean of the femoral nucleated cell (NC) content and CFU-GM concentration of three separate experiments in EC-SPF and GF mice.
[b] Mean HU kill ± standard error of three separate experiments.

TABLE 4. Splenic nucleated cell content, CFU-GM concentration and HU kill

Days of polymyxin treatment	Addition	EC-SPF mice			GF mice		
		NC content ($\times 10^6$)[a]	CFU-GM/5 × 10^4 NCs[a]	HU kill (%)[b]	NC content ($\times 10^6$)[a]	CFU-GM/5 × 10^5 NCs[a]	HU kill (%)[b]
0	Saline	93 ± 5	184 ± 4	53 ± 6	101 ± 4	37 ± 2	14 ± 2
	Hydroxyurea	81 ± 3	87 ± 4		107 ± 3	32 ± 2	
4	Saline	86 ± 2	178 ± 4	27 ± 5	99 ± 13	35 ± 2	28 ± 2
	Hydroxyurea	84 ± 10	130 ± 3		101 ± 8	26 ± 1	

[a] Mean ± standard error of the mean of the splenic nucleated cell (NC) content and CFU-GM concentration of three separate experiments in EC-SPF and GF mice.

[b] Mean HU kill ± standard error of three separate experiments.

GM pool in GF mice was found to be less susceptible to HU than that in EC-SPF mice; the HU kill was 14 and 53%, respectively. The S-phase fraction of splenic CFU-GM in EC-SPF mice, presented as the HU kill, was decreased significantly ($P < 0.005$) by polymyxin treatment. In contrast to the effect of polymyxin on the HU kill of splenic CFU-GM in EC-SPF mice, in GF mice the HU kill of CFU-GM increased significantly ($P < 0.05$) from 14 to 28%.

DISCUSSION

The significance of the intestinal microflora in relation to myelopoiesis was demonstrated by comparison of the level and HU kill of myelopoietic progenitor cells (CFU-GM) in the bone marrow and spleen of EC-SPF and GF C3H/Law mice. The EC-SPF mice resembled clean conventional (i.e., pathogen-free) mice with respect to level and occurence of GNB. However, in comparison with conventional mice, from which *Proteus* and *Pseudomonas* species can be often isolated (28), pathogens were absent.

The absence of the intestinal microflora in GF mice was associated with a reduction of the level and HU kill of both bone marrow and splenic CFU-GM. Regarding the difference in CFU-GM level between EC-SPF and GF mice, a reduced level has also been reported for GF CBA mice (6, 21). In Swiss mice, on the other hand, no difference was observed between conventional and GF mice (2, 22, 23). This may indicate the mouse strain dependency of this phenomenon.

In the present study, however, polymyxin treatment of EC-SPF mice caused reduction of the number of bone marrow CFU-GM level and HU kill to a level similar to the level in GF mice. In contrast, in GF mice, bone marrow and splenic myelopoiesis was not affected by polymyxin treatment, with the exception of the HU kill of splenic CFU-GM. The increase of the HU kill of splenic CFU-GM in the GF mice during polymyxin treatment was most probably aspecific and may be due to stress. The GF mice were transferred into a laminar-air-flow bank just before polymyxin treatment. Nevertheless, polymyxin-induced alterations of myelopoiesis in EC-SPF mice were not accompanied by similar (parallel) alterations in GF mice. Therefore, the effect of polymyxin treatment on myelopoiesis in EC-SPF mice was most likely mediated by the intestinal flora.

The involvement of the intestinal microflora in canine granulopoiesis has also been observed in dogs after oral administration of a broad-spectrum gut-sterilizing combination of bacitracin and neomycin (20, 31). In contrast to polymyxin treatment, however, this antimicrobial regimen was not selective for GNB (31). Since polymyxin treatment has been shown to cause selective elimination of intestinal GNB in conventional mice (10, 14), polymyxin-induced alterations in myelopoiesis may be ascribed to the role (i.e., contribution) of intestinal GNB in myelopoiesis.

Selective elimination of GNB by polymyxin treatment was accompanied by a reduction of the cecal endotoxin concentration to 10% of the initial (normal) value. Reduction of the cecal endotoxin level cannot be ascribed to inactivation of endotoxin (7, 8, 30) or inhibition of the LAL assay by the presence of polymyxin in cecal supernatants after oral administration. The present study has shown that cecal polymyxin levels similar to the level after oral administration do not interfere with endotoxin determination in cecal supernatants by the LAL assay. Therefore, reduction of the cecal endotoxin concentration by polymyxin treatment should be due to elimination of the major source (i.e., GNB) of cecal endotoxin.

The rather low contribution of GNB to the total bacterial counts (10^6 GNB versus 10^{11} total bacteria per g of feces [28]) in the intestinal tract of EC-SPF mice was inversely related to the substantial contribution of these bacteria in the regulation of myelopoiesis. Moreover, myelopoiesis has been shown to be influenced by injection of endotoxin or structurally related cell wall components of GNB (1, 25, 29). Release of endotoxin (17, 27) by intestinal GNB and subsequent absorption of endotoxin by the intestinal mucosa (11, 16) may explain the relationship between intestinal GNB and myelopoiesis.

The nature of this relationship and significance to regeneration of myelopoiesis after cytotoxic reduction is under investigation.

ACKNOWLEDGMENTS

We thank W. Hofstra, Central Animal Laboratory, State University of Groningen, for breeding GF C3H/Law mice.

This investigation was supported by The Netherlands' Cancer Foundation.

LITERATURE CITED

1. **Apte, R. N., C. Galanos, and D. H. Pluznik.** 1975. Lipid A, the active part of bacterial endotoxins in inducing serum colony stimulating activity and proliferation of splenic granulocyte/macrophage progenitor cells. J. Cell. Physiol. **87**:71–78.
2. **Boggs, D. R., P. A. Chervenick, J. C. Marsh, H. I. Pilgram, G. E. Cartwright, and M. M. Wintrobe.** 1967. Granulocytopoiesis in germfree mice. Proc. Soc. Exp. Biol. Med. **125**:325–330.
3. **Burgess, A. W., D. Metcalf, S. H. M. Russell, and N. A. Nicola.** 1980. Granulocyte-macrophage, megakaryocyte, eosinophil and erythroid colony stimulating factors produced by mouse spleen cells. Biochem. J. **185**:301–314.
4. **Butler, D. A., C. M. Lobrefat, and T. L. Gavan.** 1975. Reproducibility of the Analytab (API 20E) system. J. Clin. Microbiol.

2:322–326.
5. **Caridis, D. T., R. B. Reinhold, P. W. H. Woodruff, and J. Fine.** 1972. Endotoxaemia in man. Lancet **i:**1381–1385.
6. **Chang, C. F., and M. Pollard.** 1973. Effects of microbial flora on levels of colony stimulating factor in serums of irradiated CFW mice. Proc. Soc. Exp. Biol. Med. **144:**177–180.
7. **Cooperstock, M. S.** 1974. Inactivation of endotoxin by polymyxin B. Antimicrob. Agents Chemother. **6:**422–425.
8. **Cooperstock, M. S., and L. Riegle.** 1981. Polymyxin B inactivation of lipopolysaccharide in vaccines of gram-negative bacteria. Infect. Immun. **33:**315–318.
9. **Eaves, A. C., and R. Bruce.** 1972. Endotoxin-induced sensitivity of hemopoietic stem cells to chemotherapeutic agents. Ser. Haematol. **2:**64–72.
10. **Emmelot, C. H., and D. van der Waaij.** 1980. The dose at which neomycin and polymyxin B can be applied for selective decontamination of the digestive tract in mice. J. Hyg. **84:**331–340.
11. **Gans, H., and K. Matsumoto.** 1974. The escape of endotoxin from the intestine. Surg. Gynaecol. Obstet. **139:**395–402.
12. **Gardi, A., and G. R. Arpagaus.** 1980. Improved microtechnique for endotoxin assay by the *Limulus* amebocyte lysate test. Anal. Biochem. **109:**382–385.
13. **Goss, W. A., and E. B. Cimijotti.** 1976. Evaluation of an automatic diluting device for microbiological applications. Appl. Microbiol. **16:**1414–1416.
14. **Hazenberg, M. P., M. van de Boom, M. Bakker, and J. P. van de Merwe.** 1983. Effect of antibiotics on the human intestinal flora in mice. Antonie van Leeuwenhoek J. Microbiol. Serol. **49:** 97–109.
15. **Iscove, N. N., and F. Sieber.** 1975. Erythroid progenitors in mouse bone marrow detected by macroscopic colony formation in culture. Exp. Hematol. **3:**32–43.
16. **Jacob, A. I., P. K. Goldberg, N. Bloom, G. A. Degenshein, and P. J. Kozinn.** Endotoxin and bacteria in portal blood. Gastroenterology **72:**1268–1270.
17. **Jorgensen, J. H., and R. F. Smith.** 1974. Measurement of bound and free endotoxin by the *Limulus* assay. Proc. Soc. Exp. Biol. Med. **146:**1024–1031.
18. **Joshi, J. H., M. A. Entringer, and W. A. Robinson.** 1979. Bacterial stimulation of serum colony-stimulating activity and neutrophil production in germ-free mice. Proc. Soc. Exp. Biol. Med. **162:**44–47.
19. **Levin, J., E. Poore, N. P. Zauber, and R. S. Oser.** 1970. Detection of endotoxin in the blood of patients with sepsis due to Gram-negative bacteria. N. Engl. J. Med. **283:**1313–1316.
20. **MacVittie, T. J., and R. I. Walker.** 1978. Canine granulopoiesis: alterations induced by suppression of Gram-negative flora. Exp. Hematol. **6:**639–647.
21. **Metcalf, D.** 1972. Effect of thymidine suiciding on colony formation *in vitro* by mouse hematopoietic cells. Proc. Soc. Exp. Biol. Med. **139:**511–514.
22. **Metcalf, D., and R. S. Foster.** 1969. *In vitro* colony forming cells in the bone marrow of germfree mice, p. 383. *In* Germfree biology: experimental and clinical aspect. Plenum Publishing Corp., New York.
23. **Metcalf, D., and S. Stevens.** 1972. Influence of age and antigenic stimulation on granulocyte and macrophage progenitor cells in the mouse spleen. Cell Tissue Kinet. **5:**433–446.
24. **Murray, P. R.** 1978. Standardization of the Analytab Enteric (API 20E) system to increase the accuracy and the reproducibility of the test for biotype characterization of bacteria. J. Clin. Microbiol. **8:**46–49.
25. **Quesenberry, P., A. Morley, F. Stohlman, K. Rickard, D. Howard, and M. Smith.** 1972. Effect of endotoxin on granulopoiesis and colony-stimulating factor. N. Engl. J. Med. **286:**227–232.
26. **Richard, K. A., R. K. Shadduck, D. E. Howard, and F. Stohlman.** 1970. A differential effect of hydroxyurea on hemopoietic stem cell colonies *in vitro* and *in vivo*. Proc. Exp. Biol. Med. **134:**152–156.
27. **Rothfield, L., and M. Pearlman-Kothencz.** 1969. Synthesis and assembly of bacterial membrane components. A lipopolysaccharide-phospholipid-protein complex excreted by living bacteria. J. Mol. Biol. **44:**477–492.
28. **Savage, D. C.** 1977. Microbial ecology of the gastrointestinal tract. Annu. Rev. Microbiol. **31:**107–133.
29. **Staber, F. G., L. Tarcsay, and P. Dukor.** 1978. Modulation of myelopoiesis *in vivo* by chemically pure preparations of cell wall components from Gram-negative bacteria: effects at different stages. Infect. Immun. **20:**40–49.
30. **Vaara, M.** 1983. Polymyxin B nonapeptide complexes with lipopolysaccharide. FEMS Lett. **18:**117–121.
31. **Walker, R. I., T. J. MacVittie, B. L. Sinha. P. E. Ewald, J. E. Egan, and G. L. McClung.** 1978. Antibiotic decontamination of the dog and its consequences. Lab. Anim. Sci. **28:**55–61.
32. **Wright, W. W., and H. Welch.** 1959/60. Chemical, biological, and clinical observations on colistin. Antibiot. Ann. **1959/60:** 61–74.