Once within the bottle, the rat cannot turn around, sits comfortably with his nose in the neck of the bottle thus breathing freely, and is handled easily without being agitated or aroused. Intraperitoneal injections can be given easily through a port cut in the side of the bottle. If the experiment is terminal, the bottle cap, containing a piece of cotton saturated with chloroform, may be screwed on, to kill the animal. The device is sufficiently inexpensive so that both rat and bottle may be disposed.

Received February 24, 1967. P.S.E.B.M., 1967, v125.

Granulocytopoiesis in Germfree Mice.* (32084)

D. R. BOGGS,† P. A. CHERVENICK, J. C. MARSH, H. I. PILGRIM, G. E. CARTWRIGHT, AND M. M. WINTROBE
Departments of Medicine and Surgery, University of Utah College of Medicine, Salt Lake City

The only well delineated function of the neutrophilic granulocytic leukocyte is that of a phagocyte serving to prevent and combat infection. Human or canine subjects, in the absence of clinically apparent infection, produce enough neutrophils to replace the total number found in the blood an average of 3 times each day(1,2). The quantitative aspects of the fate of this massive number of neutrophils leaving the blood each day are unknown but neutrophils are routinely demonstrable in the bronchial secretions, in the gut and in the urine(3). Since bacteria form part of the normal ecology of the body orifices and of the gut, the outflow of neutrophils to these areas may play a role in the prevention of invasion by the "normal bacterial flora". The "normal flora" appear to be the source of many infections occurring in neutropenic patients(4).

This study was designed to determine if the absence of micro-organisms in the environment would result in a reduced rate of production of neutrophils. The total number of neutrophils and neutrophil precursors in the marrow of germfree and conventional mice was compared. A similar comparison was made with respect to the mitotic rate of neutrophil precursors and the number of neutrophils in blood. The ability of the neutrophil system of germfree mice to respond in a normal fashion was tested by determining the effect of endotoxin injection upon the rate of release of neutrophils from the marrow.

Materials and methods. Mice were males of the C3H/Pi strain, 7-10 weeks of age at the time of removal from the germfree environment. Germfree mice weighed an average of 20 g as compared to 25 g for control conventional mice of the same age, maintained on the same diet.

Germfree mice were reared and maintained in aluminum isolators(5), 10 mice per isolator and conventional controls were placed in isolators at 3 to 5 weeks of age. Both groups were given an identical diet which was autoclaved for 50 minutes and consisted of ground liver (750 g), corn oil (190 g), instant powdered milk (85 g), wheat germ (100 g), red beans (830 g), iodized salt (18 g), calcium propionate (4 g), water (1,550 ml), thiamin (30 mg), riboflavin (30 mg), pyridoxine hydrochloride (15 mg), nicotinamide (150 mg), pantothenic acid (75 mg), vitamin B_{12} (3 μg), ascorbic acid (450 mg) and desiccated liver (0.9 g). The food for the conventional animals was kept refrigerated to prevent spoilage, and was fed to the animals as needed. The food for the germfree mice was kept in the isolator at room temperature.

Mice removed from the germfree environment were then maintained 5 to 10 per cage in close proximity to a colony of normal mice. During this time they were given food

* This investigation was supported by a research grant (AM-04489) and a graduate training grant (2A-5098) from Nat. Inst. of Arthritis & Metab. Dis. and by a research grant (CA-05771) from Nat. Cancer Inst. Nat. Inst. Health, Bethesda, Md.

† Faculty Research Associate of Am. Cancer Soc.

(Purina Lab Chow) and water *ad libitum*.

The method for determining absolute numbers of nucleated cells per humerus is an adaptation of that developed by Fruhman(6) for the mouse tibia. The humerus is cleaned of all surrounding tissue, the proximal epihyseal cap is pulled off and the extreme tip of each distal epicondyle is cut off. Five ml of 1% EDTA in saline (measured in a volumetric pipet) is placed in a vial and aspirated into a 6 ml disposable plastic syringe. The needle is inserted into the humerus through the proximal end and fluid is forced through the humerus into the original vial. The position of the needle is adjusted until fluid is flowing freely from both ends of the bone. After 5 ml of fluid has been forced through the bone, the bone is immersed in the vial and approximately 2.5 ml is aspirated through and is again forced back through the humerus. This is repeated, so that a total of approximately 15 ml of fluid has passed through the bone. A 19 gauge needle is then mounted on the syringe and the marrow suspension is aspirated into the syringe and forced back into the vial 10 times. This serves to disperse all macroscopic cell clumps and provides an apparent single cell suspension. If the bone is broken or if any fluid is lost at any time during the procedure, that preparation is discarded. Both humeri from each mouse were studied.

Blood for leukocyte enumeration and for determining the volume of packed red blood cells was obtained from the orbital sinus. A microhematocrit tube containing heparin was inserted into the sinus and allowed to fill. Four consecutive tubes were filled from each mouse, the first 2 for duplicate leukocyte counts and the second 2 for duplicate determination of the volume of packed red blood cells. For leukocyte counts, blood from the tube was expressed onto a clean glass slide and aspirated into a volumetric pipet for dilution. Leukocyte counts from tail blood were done in the same fashion, and unwarmed tail vein being nicked and a microhematocrit tube filled therefrom.

Marrow counts and blood leukocyte counts were performed with a Coulter counter. Dilutions were made with saline containing cetrimide as the stromatolizing agent(7). Dilutions were adjusted so that the count on the fluid was not less than 5,000 nor more than 10,000 per mm^3. Threshold amplification and aperture current settings were obtained by experimental determination of the proper settings with the aid of an automatic particle size distribution plotter(7).

The morphological boundaries of the mitotic neutrophil compartment of the bone marrow were determined by injecting tritiated thymidine, killing the mice one hour later and noting which neutrophils or neutrophil precursors were labeled in radioautographs. Within the mitotic compartment, the following somewhat arbitrary cellular distinctions were made. Myeloblasts were considered to be cells with very fine chromatin, round nuclei with nucleoli but without a central hole and with sparse cytoplasm containing no granules. Promyelocytes were considered to be cells in which the chromatin was slightly courser than that of a myeloblast, which contained granules and nucleoli and which might or might not have a very tiny central hole in the nucleus. Myelocytes were considered to be all cells which were apparently more mature (coarser chromatin) than a promyelocyte up to and including the most mature cell observed to label with tritiated thymidine. This proved to be a cell whose nucleus was doughnut shaped with a hole diameter approximately 1/3 that of the total nuclear diameter but with smooth nuclear borders.

The post-mitotic compartment was divided as follows. Metamyelocytes were considered to be cells in which the nuclear hole was larger than that of a myelocyte and in which beginning nuclear indentations might or might not be evident. Metamyelocytes were not observed in normal blood except on rare occasion so these cells were considered to be in the maturation but not in the storage pool. Segmented neutrophils were required to have a thread like filament separating nuclear lobes and bands were considered to be all cells intermediate between metamyelocytes and segments.

Bone marrow differential cell counts were done on Wright's stained particles of femoral

marrow which were smeared between two coverslips after adding a tiny drop of autologous serum to the particle. At least 500 cells were differentiated in an area of the marrow smear in which at least 95% of the cells were judged identifiable. Two hundred cell differential leukocyte counts were done on Wright's stained blood smears prepared on coverslips.

All samples were obtained between 9 and 11 A.M.

Results. Cell counts from marrow and blood of 10 germfree and 10 conventional, age matched controls are summarized in the Table.

Total nucleated cells per humerus were slightly less in germfree than in conventional mice (line 1). This difference, if of any significance ($P = >.2$), reflected a difference in lymphocytes (line 2, $p = >.1$). Erythroblasts (line 3) and neutrophils (line 4) were virtually identical in number in the two groups. The number of neutrophil precursors in which nuclei were judged to be in some phase of mitosis was similar in the two groups (90,000/humerus in germfree, 85,000 in conventional). The differential count of marrow neutrophils was similar (lines 5-10) although the total percentage of cells in the mitotic pool was slightly but not significantly ($p = >.2$) greater in germfree than in conventional mice (31.7 and 26.8).

The volume of packed red cells and the total leukocyte count obtained from orbital sinus blood were identical in the two groups (lines 11 and 12). However, in germfree mice a slight but significant decrease in neutrophil concentration was observed (line 14, $p = <0.1$). The ratio of band to segmented neutrophils in the blood was similar in the two groups (.44 in germfree, .47 in conventional).

A further comparison of blood leukocyte concentration was made in a second group of 7 germfree and 5 conventional mice. In these, a count was obtained from a tail vein, then from the right orbital sinus and finally from the left orbital sinus. No significant difference was observed between total leukocyte counts from any site in germfree as compared to controls (tail, 6,440/mm^3, right orbital sinus 5,830/mm^3, left orbital sinus 5,160/mm^3). However, in each site, blood neutrophil concentration of germfree mice was approximately two thirds that of controls.

The removal of mice from a germfree environment proved to have an influence on marrow granulocytes (solid bars in the Figure).

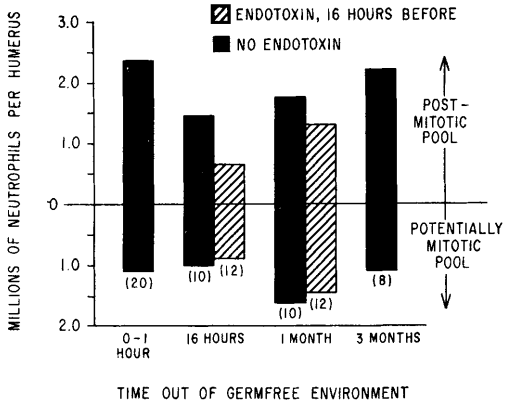

FIG. 1. Response of neutrophils in the humerus of germfree mice to removal from the germfree environment and to injection of endotoxin. Endotoxin was injected as mice were removed from germfree environment and again 4 weeks after removal. Sixteen hours after injection of endotoxin, neutrophils per humerus were determined and compared to those of uninjected but otherwise identical mice. The number of humeri examined to construct the mean figure represented by each bar on the graph is shown in parentheses under each bar. Values for size of the post-mitotic pool (metamyelocytes, band and segmented neutrophils) at 16 hours and after one month out of germfree environment differ significantly ($P = <.05$) from those for germfree controls. Endotoxin induced a further significant decrease at 16 hours ($p = < .001$) and at one month ($p = < .05$). The mitotic pool (myeloblasts, promyelocytes and meylocytes) was significantly increased ($p = <.05$) one month after removal from the germfree environment as compared to germfree controls. Endotoxin injection had no significant ($p = < .5$) effect on the mitotic pool.

Within 16 hours of removal from the germfree environment some 30% of post-mitotic neutrophils had left the marrow. After the mice had lived one month in a conventional environment the post-mitotic pool was still modestly reduced and at this time the potentially mitotic pool was increased in size. After 3 months, both pools had returned to a size similar to that of mice just removed from the germfree environment. Examination of 8 humeri from control animals (not illustrated in Figure) in which the same cage and dietary changes were carried out as with the germfree

TABLE I. Comparison of Marrow and Blood of 10 Germfree and 10 Conventional Mice.

Line		Germfree $\bar{X} \pm$ S.E. (10 mice)	Conventional $\bar{X} \pm$ S.E. (10 mice)
	Humerus (millions of cells)		
1.	Total nucleated cells:*	7.77 ±.17	8.15 ±.27
2.	Lymphocytes	1.68 ±.06	1.98 ±.15
3.	Erythroblasts	2.28 ±.16	2.27 ±.13
4.	Neutrophils:	3.29 ±.22	3.38 ±.21
5.	Myeloblasts (%)	.8	1.0
6.	Promyelocytes (%)	4.1	3.6
7.	Myelocytes (%)	26.8	22.2
8.	Metamyelocytes (%)	35.7	38.7
9.	Bands (%)	28.0	26.8
10.	Segmented (%)	4.5	7.7
	Blood		
11.	VPRC (%)	51.1 ±.6	51.1 ±.5
12.	WBC (1000's/mm^3)	4.80 ±.39	4.72 ±.30
13.	Lymphocytes	3.54 ±.31	2.88 ±.26
14.	Neutrophils	1.12 ±.12†	1.71 ±.14

* Figures based on both right and left humerus from each mouse, 20 values from germfree, 19 from control.

† Differs from conventional mice by $p = <.01$ by t test. All other values in table are not significantly different ($p = >.05$).

mice, disclosed no significant changes after one month.

The effect of injecting 10 μg of endotoxin (*S. typhosa,* 0901, lot 3946-2s, Difco) intraperitoneally upon the total number of neutrophils in the humerus, measured 16 hours after injection, is also illustrated in the Figure. A significant reduction in the post-mitotic pool was induced by endotoxin when administered as mice were removed from the germfree environment or one month after removal. However, the reduction of the size of the post-mitotic pool was twice as large at the former time.

Discussion. The number of myeloblasts, promyelocytes and myelocytes (the neutrophil production pool) in the humerus of germfree mice was not significantly different from that found in conventional mice of the same age, sex and strain. The number of cells which were judged to be neutrophil precursors in mitosis and the number of neutrophils in the post-mitotic maturation and storage pools were also very similar in the two groups. Therefore, it is apparent that in this experimental model neither microorganisms nor their products can be considered to play a major role in regulating the production of neutrophils in the absence of apparent infection.

Fliedner, Fache and Adolphi(8) recently reported studies of certain qualitative aspects of granulocytopoiesis in germfree mice. The curve of blood neutrophil radioactivity following injection of tritiated thymidine was examined. The time at which the maximum number of heavily labeled neutrophils appears in the blood approximates the time required for a myelocyte to complete the generative cycle, mature and enter the blood(9). This point was reached at about 82 hours in germfree mice but approximately 10 hours earlier in conventional mice. This difference, if significant, suggests that either the storage pool is larger or that the average maturation time is longer in germfree as compared to conventional mice. In our mice a slight but statistically insignificant ($p = >0.4$) difference was observed in the size of the post-mitotic pool (2.24 million cells in germfree as compared to 2.47 million cells in conventional mice). This suggests that, if there is a difference in this compartment, the compartment is smaller in germfree mice than in conventional mice.

A slight but statistically significant decrease in neutrophil concentration of the blood was observed in germfree mice as compared to controls. Gordon(10) noted that neutrophil concentration of germfree rats was but one-half that of conventional rats but observed no difference in neutrophil concentration in the blood of germfree and conventional chickens. Godwin(11) also observed decreased neutrophil concentration in germfree rats.

A difference in blood neutrophil concentration may not be representative of a difference in the total neutrophils of the blood(1). In man(1) or dog(12) approximately one-half of all neutrophils within the blood are not freely circulating but are marginated. The observed difference in blood neutrophil concentration in germfree and conventional mice might reflect a difference in the ratio of circulating to marginal cells rather than a difference in the total number of neutrophils in the vascular compartment. Assuming

that the difference in concentration does reflect a difference in total blood neutrophils, then germfree mice may have a more rapid turnover of blood neutrophils. Alternatively, this difference may reflect a difference in production rate which is to subtle to be detected by the technics employed herein.

The response of the neutrophilic system to removal from the germfree environment was of interest. A rapid decrease in the size of the postmitotic pool was followed by an increase in size of the mitotic pool, following which the system returned to normal. We assume that these changes reflect a response to infection. It has been reported that, when the strain of mice used in our studies is removed from the germfree environment, examination of the gut reveals pathological evidence of severe infection within a few hours(13). Dogs challenged with intrabronchial pneumococci initially respond with an outpouring of neutrophils from the post-mitotic marrow pool to the blood(14). We have interpreted the changes observed in mice in response to removal from the germfree environment as follows.

An infection is rapidly initiated which results in a marked acceleration of the rate of release of neutrophils from marrow to blood. This is detectable by the decrease in size of the post-mitotic pool of the marrow, 16 hours after removal from the germfree environment. Since the storage pool was still reduced in size one month after removal from the germfree environment, it must be assumed that, in surviving mice, accelerated release persists for some time. This prolonged phase of accelerated release is sustained by hypertrophy of the mitotic pool; this was evident at one month. By 3 months the system had returned to normal.

Endotoxin induced an increase in the rate at which neutrophils were released from the storage pool of the marrow to the blood in germfree mice, as it does in normal mice (6,15). It is somewhat difficult to interpret the observation that the number of neutrophils released in response to endotoxin as mice were removed from the germfree environment was greater than those released one month later. This observation could signify a difference in the sensitivity of such mice to endotoxin or a difference in the sensitivity of their release mechanism. The sensitivity of germfree mice to endotoxin has been studied with somewhat conflicting results. Landy and co-workers(16) noted little difference in germfree and conventional mice in response to endotoxin with respect to lethal dosage range, enhancement of resistance to infection or enhancement of glycolysis of peritoneal macrophages and spleen cells. In contrast, Jensen, Mergenhagen and Fitzgerald (17) reported that germfree mice were more resistant to the lethal effects of endotoxin than were controls. Matsuzuwa(18) reported that the change in tissue oxygen tension induced by endotoxin, while qualitatively similar, was of greater degree in germfree than in conventional mice. Wilson and Matsuzawa (19) noted that endotoxin resulted in a proportionally greater increase in the LD50 of X-irradiation in germfree mice as compared to controls. Thus, excepting one study(17), when differences have been noted in the response of germfree and conventional mice to endotoxin, such differences have suggested that germfree mice are more sensitive than conventional mice to endotoxin.

Summary. A comparison of the total number of neutrophils and neutrophil precursors within the humerus of germfree and conventional mice revealed no statistically significant difference between the two groups. The distribution of marrow neutrophils with respect to morphologic type and the number of neutrophils in mitosis were similar in the two groups. There was, however, a slight, but significantly, lower concentration of blood neutrophils in germfree mice as compared to controls. Removal of mice from the germfree environment was associated with a rapid decrease in the number of mature and relatively mature neutrophils in the marrow and with a subsequent increase in potentially mitotic, immature neutrophils, following which the marrow returned to normal. Germfree mice responded to endotoxin administration with an increase in rate of release of neutrophils from the marrow, as do normal mice. It was concluded that in this model system the presence or absence of microorganisms in the environ-

ment could not be assigned a major role in regulating granulocytopoiesis.

We are indebted to Mrs. Vreni Bithell and Miss Lona Bindbeutel for technical assistance.

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Received March 1, 1967. P.S.E.B.M., 1967, v125.

Erratum. In Volume 124, No. 4, page 1070, the first two authors of Article 31926 should read: André Gerard and Jacques DeGraef.