NEUTROPHIL PRODUCTION IN DRUG-DEPLETED RAT BONE MARROW

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ABSTRACT

NEUTROPHIL PRODUCTION IN DRUG-DEPLETED RAT BONE MARROW

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The present study was designed to determine the capacity of neutrophil precursors to regenerate in drug-depleted rat bone marrow.

Myleran (15 mg/Kg body weight) was introduced by stomach tube into 200 g male albino rats (day zero). Five daily intraperitoneal injections of Hydrocortone Acetate (5 mg/Kg body weight) was administered to five day post-Myleran treated rats. Replicate serial qualitative and quantitative sampling procedures were carried out on the left femoral bone marrow of experimental and control animals at 13, 15, 17, and 18 days post-Myleran injection. Tritiated thymidine (.5 μc/g) was injected intraperitoneally 30 minutes before sampling time. Autoradiography which was used is described. The labeling index and absolute numbers of myeloblasts, promyelocytes, myelocytes, metamyelocytes, lymphocytes, and transitional cells were determined.

The labeling index of myeloblasts-promyelocytes and myelocytes-metamyelocytes increase significantly at days 17 and 15 respectively in the experimental animals. Absolute numbers of non-segmented and segmented neutrophils in the experimental were significantly lower than control animals. There are no significant differences within experimental animals for both cell types.

The labeling index of lymphocytes are significantly higher in experimental than control animals at days 17 and 18. Absolute numbers of these cells in experimental animals are significantly lower than control animals for all days sampled. There are no significant differences within each group. No significant increase in either labeling index or absolute numbers of transitional cells occurred.

These results indicate that for neutrophil precursors there is a significant increase in the labeling index reflects an actual increase in the absolute numbers of dividing cells. The data also indicates that bone marrow lymphocytes do not function as stem cells for neutrophil production.

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TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I

CHAPTER III

LIST OF TABLES

CHAPTER I

INTRODUCTION

Different experimental systems have been used to study the mechanisms of granulocyte production. Common to the design of these systems is the suppression of granulopoiesis with subsequent loss of mature cells. After termination of suppression, the increased demand for granulocytes magnifies the processes of granulopoiesis. It is during this period that mechanisms of granulopoiesis can best be studied. The present study utilizes drug-induced depression as a method of studying granulopoiesis.

A model of the lifespan of the neutrophilic granulocyte is shown in modified form in Fig. 1 (Athens, 1970). It can be divided into three phases: (1) the marrow phase, (2) the blood phase and (3) tissue phase. The marrow phase is divided into three general pools: (1) the stem cell pool containing colony forming units and primitive precursor cells, (2) the mitotic pool containing difinitive precursor cells capable of cell division, i.e., myeloblasts, promyelocytes and myelocytes, and (3) the neutrophil reserve or maturation pool, which is composed of cells which are undergoing maturation. This phase includes metamyelocytes, nonsegmented and segmented neutrophils. Cells move through these developmental stages sequentially in bone marrow and are then released into the blood, where they equilibrate rapidly between the circulating and the marginal (non circulating) granulocyte pools. These two pools constitute the total blood granulocyte pool. In normal subjects there

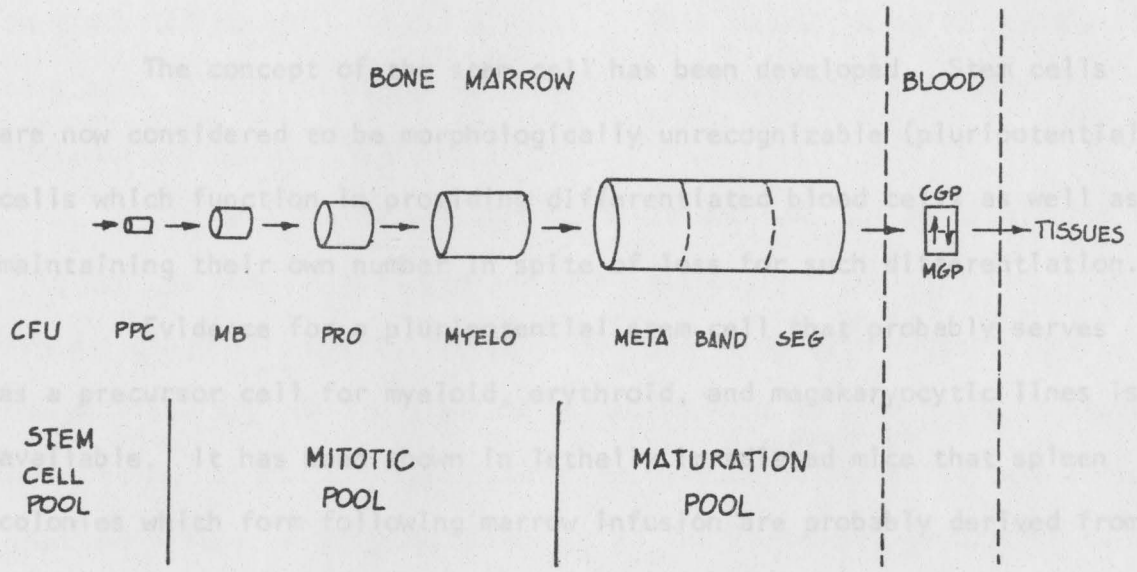

Fig. 1. Model of neutrophil life span

CFU	=	colony forming unit
PPC	=	primitive precursor cell
MB	=	myeloblast
PRO	=	promyelocyte
MYELO	=	myelocyte
META	=	metamyelocyte
BAND	=	band neutrophil
SEG	=	segmented neutrophil
CGP	=	circulating granulocyte pool
MGP	=	marginal granulocyte pool

is no appreciable pool of granulocytes in the tissues, and granulocytes do not return from the tissues to the blood. The large pool of cells in the bone marrow serves as a reserve from which cells can be obtained rapidly in the event of an increased demand in the periphery.

Stem Cell Pool

The concept of the stem cell has been developed. Stem cells are now considered to be morphologically unrecognizable (pluripotential) cells which function in providing differentiated blood cells as well as maintaining their own number in spite of loss for such differentiation.

Evidence for a pluripotential stem cell that probably serves as a precursor cell for myeloid, erythroid, and megakaryocytic lines is available. It has been shown in lethally irradiated mice that spleen colonies which form following marrow infusion are probably derived from a single precursor cell (Becker et al., 1963). Each colony appears as a cluster of hematopoietic cells, many of which are dividing and differentiating into cells of erythrocytic, granulocytic and megakaryocytic series (Till and McCulloch, 1961). The common ancestry of these cells was indicated by the fact that all three types of cells had a similar marker chromosome (Wu et al., 1967). Also, in chronic myelocytic leukemia, the Ph_1 chromosome is present in nucleated erythrocytic, granulocytic, and megakaryocytic cells in such patients (Whang et al., 1963). This finding has been interpreted as being compatible with the hypothesis that chronic myelocytic leukemia is the result of an abnormality in a pluripotential stem cell compartment. Further evidence of the pluripotential character of the hemopoietic stem cell was obtained from lethally irradiated mice in which erythropoiesis had been stimulated.

In these animals the granulopoietic capacity of infused bone marrow was depressed. Erythropoiesis was stimulated which demonstrated that an increased demand for red blood cell production reduced the ability of transplanted syngeneic stem cells to produce granulocytic progeny. This experiment seems to indicate that competing proliferative demands on a pool of common stem cells exists. This implies a multipotent stem cell able to produce either erythrocytic or granulocytic progeny (Hellman and Grate, 1967).

The identity of this stem cell is unknown, but the reticulum cell and the small lymphocyte are prime suspects. The fact that infused blood leukocytes protect irradiated animals from death suggests that normal blood may contain stem cells (Goodman and Hodgson, 1962). Since, among normal blood leukocytes, only the lymphocyte can be stimulated to grow and divide, it or some component of the lymphocyte population seems a likely candidate. In recent years, the tendency has been to regard the small lymphocyte as the pluripotential stem cell in the bone marrow (Yoffey, 1957; 1966). It has been suggested that small lymphocytes are capable of enlarging, as represented by transitional lymphocytes, to become blast cells, which are capable of differentiating into the myeloid or erythroid lineages (Harris and Burke, 1957). This idea received support in the demonstration that peripheral blood small lymphocytes enlarged into blast cells and divided when incubated in vitro with phytohaemagglutinin, a mitosis-stimulating agent (Elves and Wilkinson, 1962; Yoffey et al., 1965). Study of the haemopoietic potential of marrow lymphocytes requires the separation of large number of these cells, in viable form, from the other cellular elements of bone marrow. Morrison (1967) was able to obtain purer preparations of bone

marrow lymphocytes using combined glass-wool filtration and dextran density gradient centrifugation. The resulting preparation contained about 80 per cent small lymphocytes. These preparations were capable of promoting survival when injected into lethally irradiated rats (Morrison and Toepfer, 1967). Due to the fact that if stem cells other than lymphocytes do exist in bone marrow, their role is obscured by the presence of lymphocytes in large numbers. Toepfer (1969) determined the capacity of drug-depleted rat bone marrow to regenerate myeloid and erythroid cells in the presence of large number of bone marrow lymphocytes. The result suggested that complete regeneration of heterophil granulocytes and erythroid cells depends on the presence of lymphocytes.

Marrow Mitotic Pool

The marrow mitotic pool consists of myeloblasts, promyelocytes, and myelocytes. These cells have been shown to be capable of mitotic division by direct observation in cell cultures (Boll and Kuhn, 1965) and by virtue of their uptake of tritiated thymidine (H^3-thymidine) (Patt and Maloney, 1959). Sin and Sainte-Marie (1965) observed mitosis in metamyelocytes of rat thymuses which agrees with the observations of Harris and Kugler (1963) that guinea-pig metamyelocytes can become labelled within one hour after an injection of H^3-thymidine. However, this is in conflict with the conclusion of Cronkite et al. (1960) that human metamyelocytes do not divide because they do not become labelled until 3 - 4 hours after H^3-thymidine injection. Maloney et al. (1963), studying dog metamyelocytes, reached a similar conclusion to that of Cronkite et al.. This difference of opinion could be a matter of

nomenclature, since as the metamyelocytes closely resemble the mature forms of myelocytes the classification of these cells may differ with different authors. It is thus conceivable that some cells termed metamyelocytes by Harris and Kugler (1963) and by Sin and Sainte-Marie (1965) were considered as myelocytes by Cronkite et al. (1960) and by Maloney et al. (1963).

It is generally agreed, but it has not been proven, that cells in the marrow mitotic compartment move from myeloblast to promyelocyte to myelocyte. The number of cell divisions that occur at each morphologic stage is also unknown. From the exponential decrease in radioactivity during Phase III of the in vivo radioactive diisopropylfluorophosphate (DFP^{32}) curve, it has been suggested that there must be at least three divisions at the myelocyte stage (Warner and Athens, 1964). If a single division is postulated for the myeloblast and promyelocyte stages, a minimum of five divisions occur during myelopoiesis in man. As many as seven divisions have been postulated for the rat (Sin and Sainte-Marie, 1965).

There are several theoretical kinetic models of granulopoiesis based on the studies with DFP^{32} as a label for granulocytes, and nuclear DNA labeling by means of radiophosphorus and tritiated thymidine. Warner and Athens (1964) have proposed a "semiclosed" model (Fig. 2). In this model, cells in the bone marrow are divided into two populations (A and B). Population A consists of cells which will divide at least once before entering the blood, while population B consists of metamyelocytes and more mature granulocytes which are not capable of cell division. It is assumed that cells move through population A in a sequential fashion to a point M where cell division occurs. When a cell

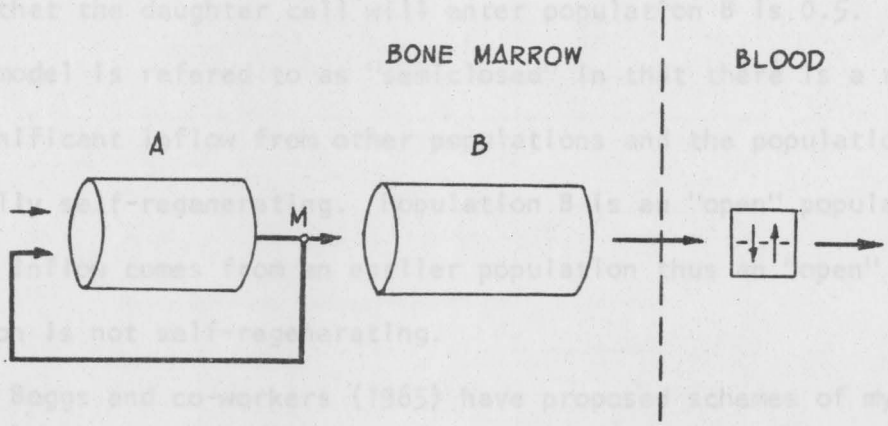

Fig. 2. "Semiclosed" Model of granulocyte kinetics
A. "Mitotic marrow pool" (myelocyte compartment): capable of certain degree of self-perpetuation and replenished, sequentially, by progenitor (stem cells).
B. "Post mitotic marrow pool" of metamyelocytes, non-segmented and segmented neutrophils.
Blood. Peripheral granulocytes composed of two distinct populations: marginal pool and circulating pool (vertical arrows). M = mitosis.

in population A divides, there is a given probability that one or both of the daughter cells may enter population B or may re-enter population A. It is postulated that the re-entry of myelocytes is the prime source of new cells for the myelocyte population. It seems obvious that some cells do enter population A from populations of cells which are less mature (promyelocytes and myeloblasts). In the steady state the probability that the daughter cell will enter population B is 0.5. This type of model is refered to as "semiclosed" in that there is a relatively insignificant inflow from other populations and the population is essentially self-regenerating. Population B is an "open" population in that its inflow comes from an earlier population thus an "open" population is not self-regenerating.

Boggs and co-workers (1965) have proposed schemes of myeloid proliferation on the basis of DFP^{32} labeling. Fig. 3 A is the classical scheme in which there is a "stem" cell not morphologically identified. This cell divides and half the daughter cells become myeloblasts while half remain stem cells to perpetuate the existence of the stem-cell pool. The myeloblasts are then thought to divide, both daughter presumed to become promyelocytes, which divide again, and so on. The last division is presumed to be in the myelocyte stage, and the cells then mature and provide the blood and tissues with mature neutrophils. Athens (1969) suggested another scheme (Fig. 3 B and C). In model B all cells, upto and including the myelocyte, may act as stem cells. In scheme C, there must be at least 3 or 4 divisions in the myelocyte population to match the *in vivo* DFP^{32} data curves.

From the fact that the blood neutrophil concentration:
(1) remains remarkably constant in the normal individual, (2) reacts in

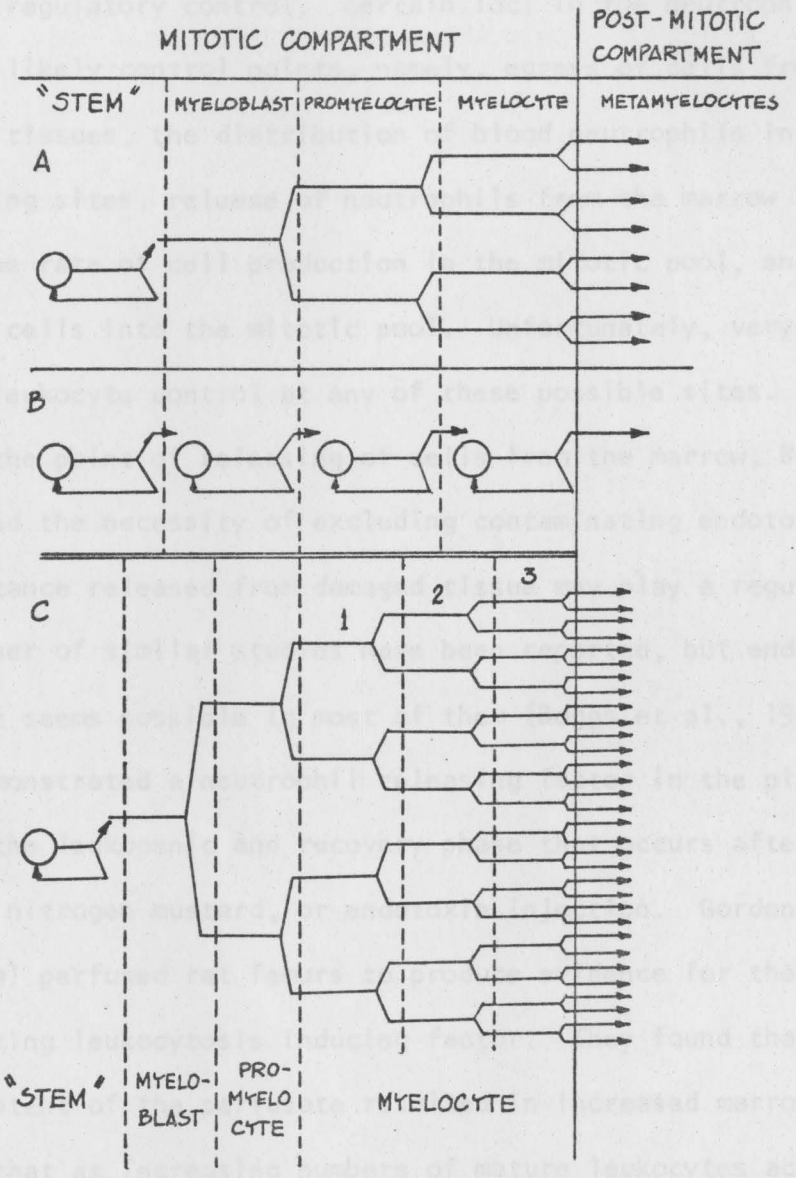

Fig. 3. Several models of granulopoiesis

a predictable manner to certain stimuli, and (3) then returns to the normal steady state, it seems likely that the neutrophil system is under some form of regulatory control. Certain loci in the neutrophil system appear to be likely control points, namely, egress of cells from the blood to the tissues, the distribution of blood neutrophils in marginal and circulating sites, release of neutrophils from the marrow reserve to the blood, the rate of cell production in the mitotic pool, and the inflow of stem cells into the mitotic pool. Unfortunately, very little is known about leukocyte control at any of these possible sites.

At the point of releasing of cells from the marrow, Boggs (1966) has emphasized the necessity of excluding contaminating endotoxin or a similar substance released from damaged tissue may play a regulatory role. A number of similar studies have been reported, but endotoxin contamination seems possible in most of them (Boggs et al., 1966). Boggs has demonstrated a neutrophil releasing factor in the plasma of dogs during the leukopenic and recovery phase that occurs after vinblastine, nitrogen mustard, or endotoxin injection. Gordon and coworkers (1964) perfused rat femurs to produce evidence for the existence of a circulating leukocytosis inducing factor. They found that low leukocyte content of the perfusate resulted in increased marrow cell release and that as increasing numbers of mature leukocytes accumulated in the perfusate further leukocyte release was suppressed. On this basis they postulated a negative feedback control, mediated by mature neutrophils, which may regulate marrow cell release. Many substances have been reported to alter neutrophil production but probably only neutrophil releasing factor can be accorded some physiologic significance as yet.

The above review of granulopoiesis brings out the fact that knowledge of the process is incomplete and often conflicting. Notably, much confusion exists concerning the contribution of the different neutrophil precursors in the production of mature progeny. The present investigation, therefore, has been designed to determine the capacity of bone marrow neutrophil precursors to repopulate drug-depleted rat bone marrow.

CHAPTER II

MATERIALS AND METHODS

Preparation and Administration of Drugs

Fifteen mg/Kg Myleran[1] was administered by stomach tube into 200 ± 5 g male albino rats of the Wisconsin Holtzman strain. Myleran suspensions for injection were prepared by crushing and mixing two mg pellets of the drug with gum tragacanth powder using a mortar and pestle. Water was then added to the fine powder so that a final concentration of 3 mg Myleran and 20 mg gum tragacanth were contained in 1 ml of water. Experimental animals received 1 ml of this preparation. Control animals received 20 mg gum tragacanth in 1 ml of water.

Five mg/Kg Hydrocortone Acetate[2] was injected intraperitoneally into 5 days post Myleran-treated rats. Injections were administered once daily for five days. Control animals were given an intraperitoneal injection of .2 ml of sterile saline.

0.5 μc/g of tritiated thymidine was injected intraperitoneally into both experimental and control animals 30 minutes before sampling time.

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[1] Donated through the courtesy of Dr. George H. Hitchings, Ph.D., Burroughs, Williams and Co.

[2] Donated through the courtesy of Dr. Ashton C. Cuckler, Ph.D., Merck, Sharp and Dohme Co.

Sampling consisted of quantitative and qualitative procedures carried out on the left femoral bone marrow. At each sampling time, 13, 15, 17, and 18 days post-Myleran injection, a total of five experimental and five control animals were accumulated.

Bone Marrow Quantitative Technique

All glassware in this procedure was siliconized. The left femoral bone was isolated and all remaining attached tissue removed. The proximal end of the bone was removed and then shaft split lengthwise with fine iris scissors. The exposed marrow was scraped onto a glass slide. Bone marrow was then taken up into a stem of a 500 µl pipet, which had been calibrated to contain 4 µl and expelled into a 1 ml volumetric flask calibrated to contain .2 ml homologous serum. The marrow was suspended 300 times using a pipet with an attached rubber bulb. It was then diluted 100 times with 3 per cent acetic acid in red blood cell diluting pipet. This suspension was then shaken by pipet shaker for one and a half minutes. Nucleated cell counts were made using a hemocytometer. Knowing the dilution factor, the concentration of nucleated bone marrow cells was expressed as the number of cells per mm^3 of bone marrow. Marrow cells were deposited on a subbed slide, which had been previously dipped in a solution containing 0.5 gm gelatin and .05 gm chrome alum in 100 ml water, using a cyto-centrifuge. Slides were fixed in absolute methanol for 30 minutes and dried.

Autoradiographic Technique

Dipping and Developing

The autoradiographic procedures were carried out as described by Everett and Tyler[3]. In the darkroom, slides were dipped into melted Eastman Kodak NTB 3 bulk emulsion, diluted in 1 ml of dupanol solution (Dodecyl Sodium Sulfate) to 3 ml of emulsion, at 40° C. They were left in 45° position to dry for 2 hours and then placed for exposure in sealed plastic boxes containing Drierite. They were stored in a refrigerator at 5° C. for 2 weeks. After exposure, the slides then were developed for 2 minutes in D 19 developer, rinsed in tap water, and fixed for 10 minutes in Kodak Acid Fixer. Thus procedures were carried out at 18 - 20° C. Slides were washed for 45 minutes in running tap water to remove all acid, then dried overnight on a warming plate at 37-40° C. The slides were immersed in 70 per cent alcohol for 3 minutes to harden emulsion, dried, then placed in pH 6.4 phosphate buffer for 5 minutes. They were then rinsed throughly with tap water and dried on warming plate overnight.

Staining

A mixture of 1 ml MacNeal's Tetrachrome stain and 2 ml of phosphate buffer pH 6.4 were poured onto the slides completely covering the slide for 4 minutes, then washed from the slides with 150 ml of distilled water. The slides were blotted with Kimwipes, dried on a warming plate over night, and coverslips mounted with permount.

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[3] Obtained through personal communication with Dr. N. B. Everett and Dr. R. W. Tyler.

Differential counts of smears were made to determine the per cent of various cell types. These percentages multiplied by the absolute nucleated cell counts gave an expression of absolute numbers of each cell type present in bone marrow.

Morphological Criteria

The morphological criteria for the classification of rat marrow cells, both the neutrophilic granulocytic maturation series comprised of myeloblasts, promyelocytes, myelocytes, metamyelocytes, non-segmented, and segmented rings, and the lymphoid series comprised of lymphocytes and transitional cells was in accordance with Ramsell and Yoffey (1961), and Harris and Burke (1957).

Myeloblasts and Promyelocytes

The cells are usually 15 - 30 μ in diameter and were characterized by the presence of azurophil granules located in an abundant cytoplasm. The cytoplasm was basophilic. The nucleus was large, round, with fine chromatin network, and a definite nuclear border with often an indentation on one side. There was often a well defined, unstained golgi zone, located in the nuclear indentation, which will progressive become wider and deeper.

Myelocytes and Metamyelocytes

These cells were characterized by development of a small hole usually the center of the nucleus, giving a doughnut-shaped appearance. As maturation proceeded, this hole became progressively larger. The nuclear chromatin was more clumped than the preceding stage and was

located against the thin, regular nuclear membrane. The cytoplasm was characterized by the two main changes during maturation: (1) a gradual increase in specific neutrophil granulation and (2) a progressive loss of cytoplasmic basophilia.

Non-segmented Rings

The nucleus at this level of maturation displayed a prominent ring configuration. The distribution of chromatin is less homogeneous, appearing as clumps along the thin, regular nuclear membrane. There was no nuclear segmentation. The cytoplasm of this cell is faintly acidophilic and appears to have a full complement of specific granules.

Segmented Rings

The last stage in nuclear maturation is characterized by extensive heteropycnosis of the chromatin and beginning segmentation at one or more points along the ring nucleus. In the most mature forms, the nucleus is segmented into five to eight distinct lobes interconnected by thin chromatin strands.

Lymphocytes

This category contained small, round mononuclear cells that ranged from 7 - 14 μ in diameter. The nuclear/cytoplasmic (N/C) ratio was large. The nucleus was the prominent feature of these cells and stained pink to light purple. The cytoplasm was always scantly and occupied a polar position on one side of the nucleus. Staining of cytoplasm was also variable, some cells exhibiting a distinct basophilia and other showing no dye uptake at all.

Transitional Cells

These are a series of cells morphologically intermediate between lymphocytes and blast cells. They were characterized by a decreasing N/C ratio. The size of the cell increased. The nucleus became increasingly leptochromatic, and the cytoplasm progressively enveloped the nucleus as maturation proceded. Also, the cytoplasm became more basophilic.

Other Cells

This category included all the maturation stages of eosinophilic granulocytes and erythroid cells. In addition, damaged cells, representing nuclear fragments resulting from the smear procedure, megakaryocytes, plasma cells, monocytes, and unidentifiable cells were included in this category.

Labeling Study

The labeling indices (the total number of labeled cells, cells in DNA synthesis, divided by the total number of cells in the population) were determined for myeloblasts-promyelocytes, myelocytes-metamyelocytes, lymphocytes, and transitional cells in both experimental and control animals. Positive labeling was recorded only if three or more silver grains were located over each nucleus. In view of the low density of background grains, the results probably do not reflect maximal value.

Statistical Treatment

Mean, standard error of labeling indices and absolute numbers of each cell type of experimental and control animals were calculated.

A three factor analysis of variance was used to test the effects of drug treatment on the labeling indices and absolute numbers of each bone marrow cell category. Following significant F values, the Tukey (a) multiple-range test was used. This test is particularly useful in investigating the nature of differences between several treatment means. All statements of significance in this study were made at the .05 probability level.

CHAPTER III

RESULTS

Neutrophilic Granulocyte Maturation Series

Cells of this lineage have been divided into two groups, the myeloblasts-promyelocytes and myelocytes-metamyelocytes. They are recognized as granulocyte precursors and are capable of incorporating tritiated thymidine into the cells that are synthesizing DNA in preparation for mitosis. Non-segmented and segmented rings are recognized as mature cells and are unable to divide.

Table 1 (APPENDIX) presents means (± S.E.) of labeling indices of bone marrow cells following treatment of rats with Myleran and cortisol. Means (± S.E.) of absolute values (cells x $10^3/mm^3$ of bone marrow) and percentages of the granulocyte maturation lineage cells in control and experimental animals are given in Table 2 through Table 5 (APPENDIX).

Myeloblasts-Promyelocytes

Changes in labeling indices of these cell at different time intervals following combined drug treatment with Myleran and cortisol are shown graphically in Fig. 1. The labeling index of these cells are depressed at 13 days post Myleran treatment. After this time they progressively increase and reach a maximum at day 17 which is significantly higher than controls at the same day.

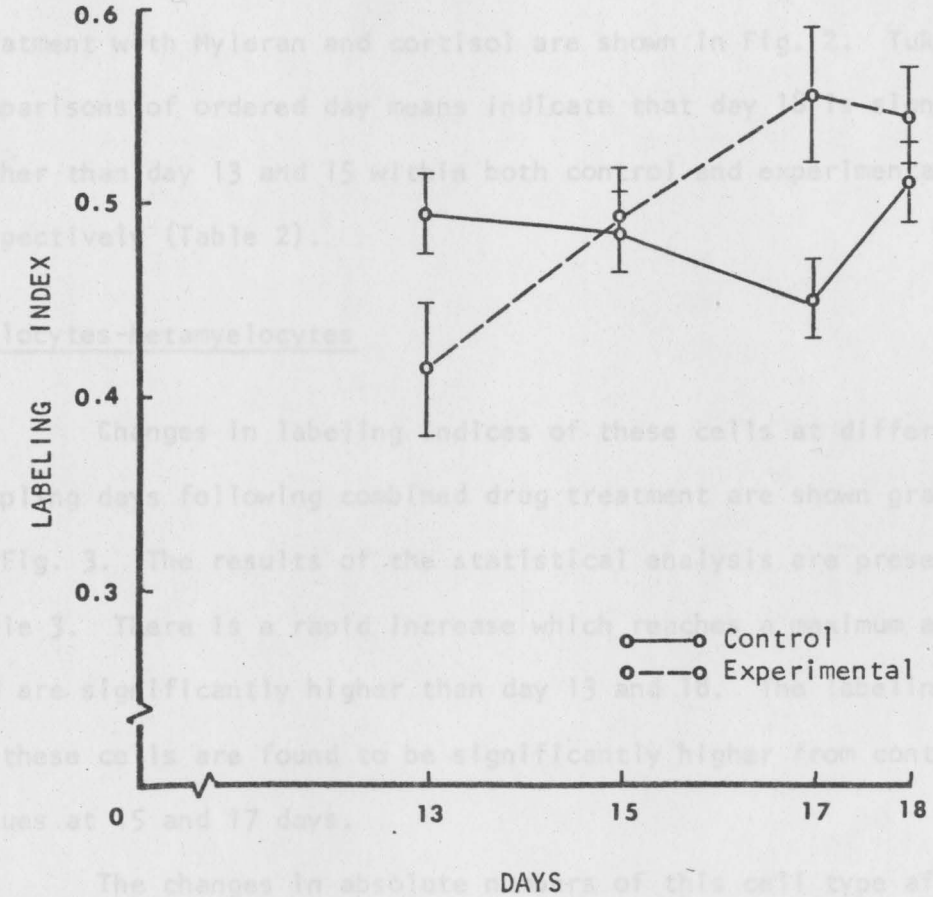

Fig. 1. Graph showing mean (±S.E.) changes in labeling index of myeloblasts - promyelocytes after treatment with Myleran and cortisol.

Tukey (a) comparisons of ordered means for experimental animals at different days sampled indicate that for the labeling index of these cells at day 15, 17, and 18 are significantly higher than day 13 (Table 1).

The changes in absolute numbers of this cell type after treatment with Myleran and cortisol are shown in Fig. 2. Tukey (a) comparisons of ordered day means indicate that day 18 is significantly higher than day 13 and 15 within both control and experimental animals respectively (Table 2).

Myelocytes-Metamyelocytes

Changes in labeling indices of these cells at different sampling days following combined drug treatment are shown graphically in Fig. 3. The results of the statistical analysis are presented in Table 3. There is a rapid increase which reaches a maximum at day 15 and are significantly higher than day 13 and 18. The labeling index of these cells are found to be significantly higher from control values at 15 and 17 days.

The changes in absolute numbers of this cell type after treatment with Myleran and cortisol are shown in Fig. 4. Comparisons between experimental and control animals indicate that a significant difference exists at day 13 only. Within the experimental group day 18 is significantly higher than all other days sampled except 17. This is also true for control animals (Table 4).

TABLE 1

LABELING INDEX OF MYELOBLASTS-PROMYELOCYTES

Analysis of variance

Source of variance	ss	df	ms	F
Drug condition	.00303	1	.00303	.92
Days sampled	.02692	3	.00897	2.75
Drug x Days sampled	.04404	3	.01468	4.50 *
Error	.10431	32	.00326	
Total	.17830	39		

* $p < .05$

Tukey (a) comparisons of ordered means *

**	E 13	C 17	C 15	E 15	C 13	C 18	E 18	E 17
Mean	0.4146	0.4488	0.4836	0.4928	0.4950	0.5088	0.5448	0.5536

* Any two means not underlined by a common line are significantly differently at the 0.05 level.

** E = Experimental, C = Control animals, number denotes days after Myleran treatment.

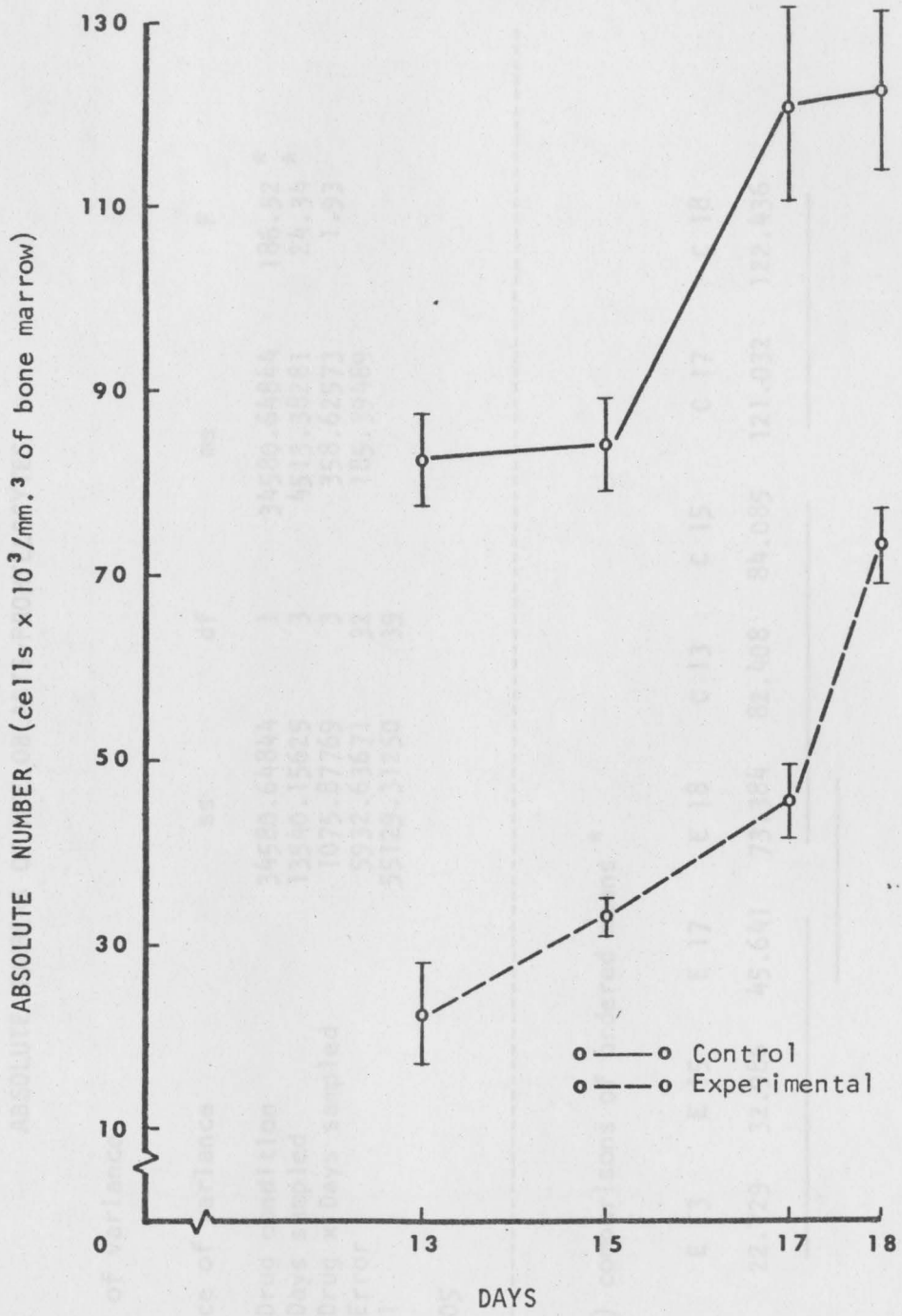

Fig.2 Graph showing mean (± S.E.) changes in absolute number of myeloblasts - promyelocytes after treatment with Myleran and cortisol.

TABLE 2

ABSOLUTE NUMBERS OF MYELOBLASTS-PROMYELOCYTES

Analysis of variance

Source of variance	ss	df	ms	F
Drug condition	34580.64844	1	34580.64844	186.52 *
Days sampled	13540.15625	3	4513.38281	24.34 *
Drug x Days sampled	1075.87769	3	358.62573	1.93
Error	5932.63671	32	185.39489	
Total	55129.31250	39		

* $p < .05$

Tukey (a) comparisons of ordered means *

**	E 13	E 15	E 17	E 18	C 13	C 15	C 17	C 18
Mean	22.729	32.986	45.641	73.384	82.408	84.085	121.032	122.436

* Any two means not underlined by a common line are significantly different at the 0.05 level.

** E = Experimental, C = Control animals, number denotes days after Myleran treatment.

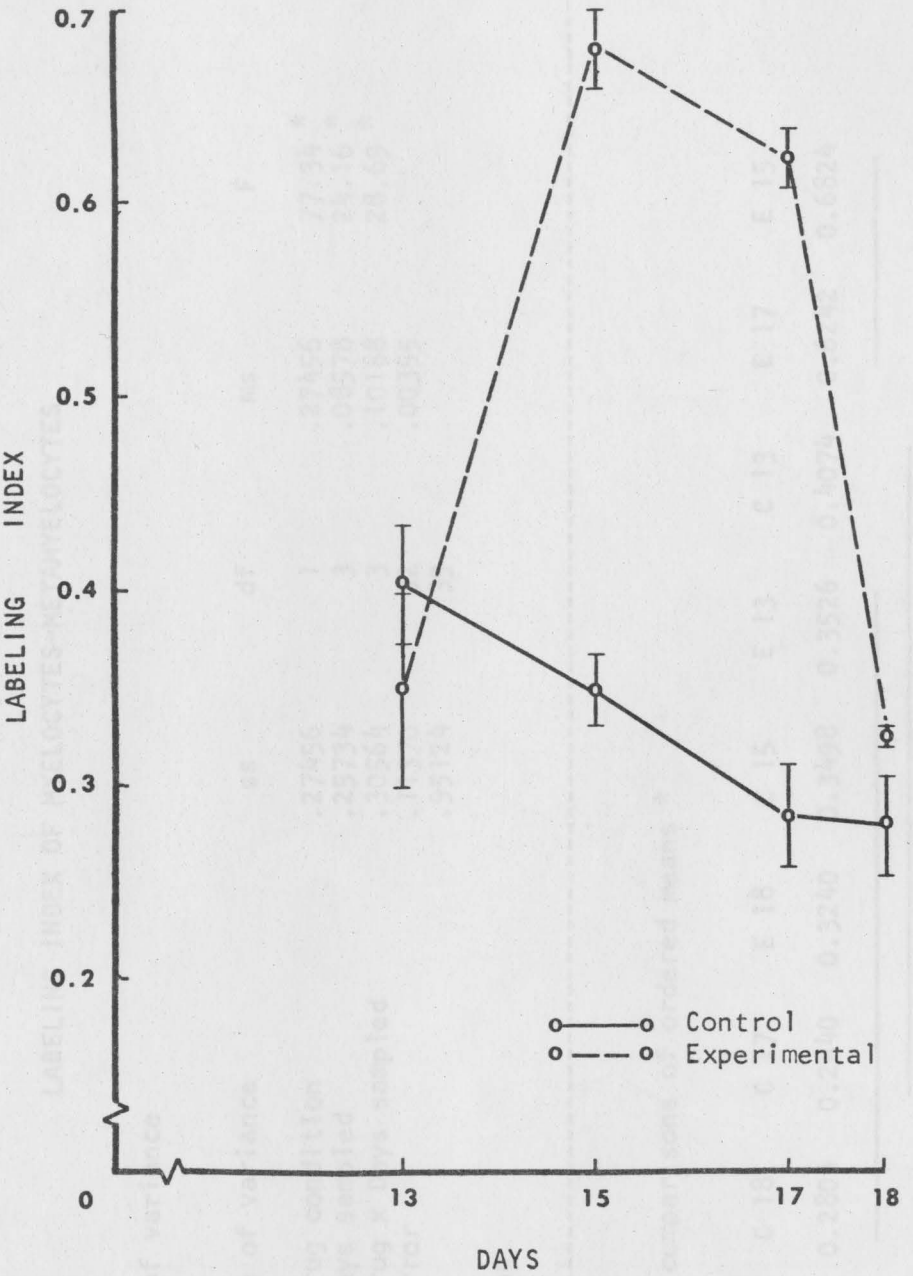

Fig.3 . Graph showing mean (±S.E.) changes in labeling index of myelocytes - metamyelocytes after treatment with Myleran and cortisol.

TABLE 3

LABELING INDEX OF MYELOCYTES-METAMYELOCYTES

Analysis of variance

Source of variance	ss	df	ms	F
Drug condition	.27456	1	.27456	77.34 *
Days sampled	.25734	3	.08578	24.16 *
Drug x Days sampled	.30564	3	.10188	28.69 *
Error	.11370	32	.00355	
Total	.95124	39		

* $p < .05$

Tukey (a) comparisons of ordered means *

**	C 18	C 17	E 18	C 15	E 13	C 13	E 17	E 15
Mean	0.2804	0.2840	0.3240	0.3498	0.3526	0.4074	0.6242	0.6824

* Any two means not underlined by a common line are significantly different at the 0.05 level.

** E = Experimental, C = Control animals, number denotes days after Myleran treatment.

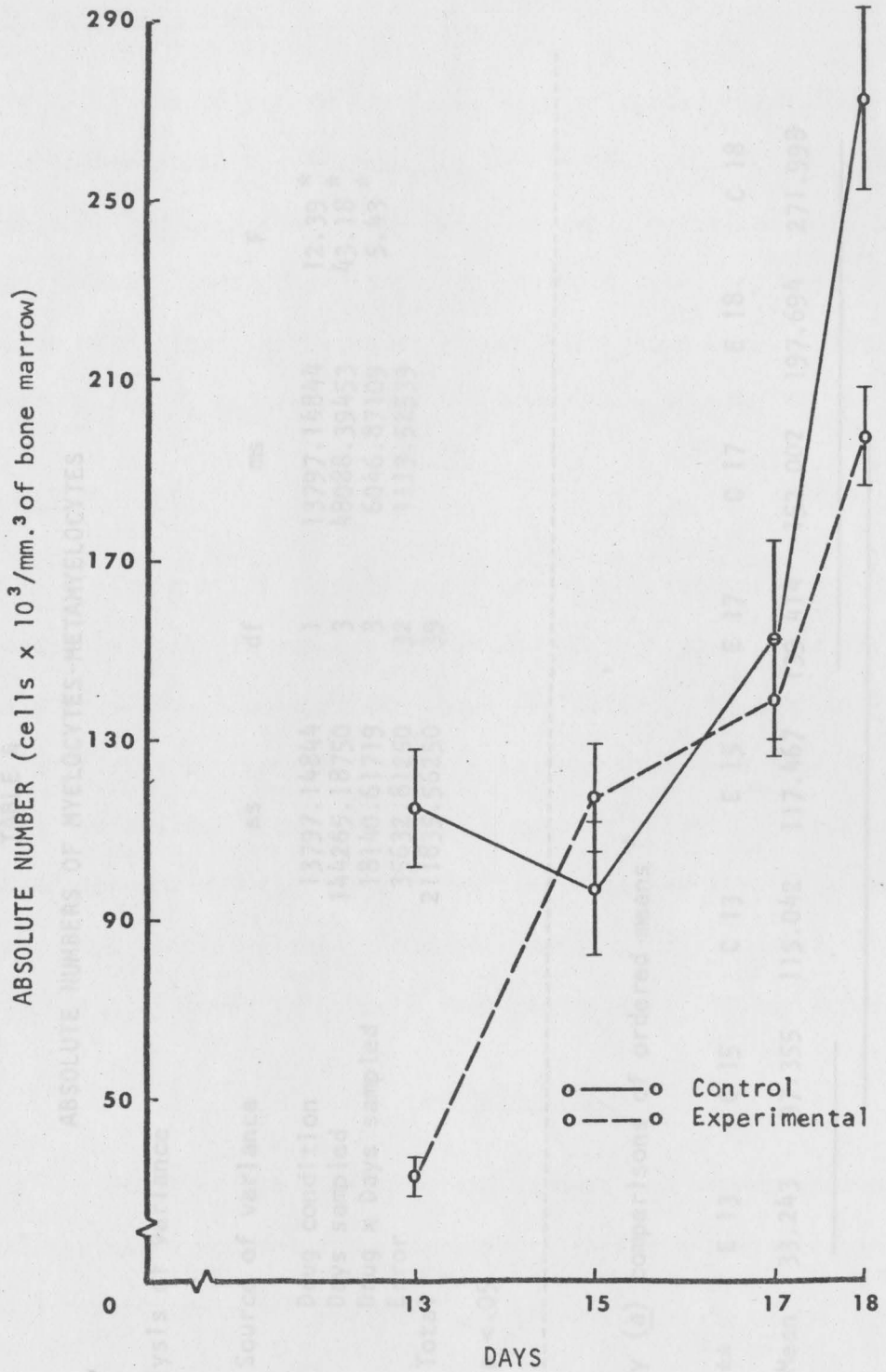

Fig. 4 Graph showing mean (± S.E.) changes in absolute number of myelocytes - metamyelocytes after treatment with Myleran and cortisol.

TABLE 4

ABSOLUTE NUMBERS OF MYELOCYTES-METAMYELOCYTES

Analysis of variance

Source of variance	ss	df	ms	F
Drug condition	13797.14844	1	13797.14844	12.39 *
Days sampled	144265.18750	3	48088.39453	43.18 *
Drug x Days sampled	18140.61719	3	6046.87109	5.43 *
Error	35632.81250	32	1113.52539	
Total	211835.56250	39		

* $p < .05$

Tukey (a) comparisons of ordered means *

**	E 13	C 15	C 13	E 15	E 17	C 17	E 18	C 18
Mean	33.243	97.355	115.042	117.467	139.414	152.002	197.694	271.998

* Any two means not underlined by a common line are significantly different at the 0.05 level.

** E = Experimental, C = Control animals, number denotes days after Myleran treatment.

Non-segmented Rings

These cells did not label with tritiated thymidine. Changes in absolute numbers are presented in Fig. 5. Statistical comparisons (Table 5) indicate that these cells in experimental animals are significantly lower than in control animals at all days sampled. There are no significant differences within each group except that in controls day 13 is higher than all other days sampled.

Segmented Rings

These cells also do not label with tritiated thymidine. Changes in absolute numbers are presented in Fig. 6. Statistical analysis of these changes are shown in Table 6. In controls there is a significant increase in these cells from day 13 to day 17. After this time a significant decrease in numbers occurs. Tukey (a) comparisons of ordered means do not indicate any significant differences among days sampled within the experimental group; however, they are significantly lower in numbers than controls at all day sampled.

Lymphoid Series

Means (± S.E.) of absolute numbers and percentages of this cell series in control and experimental rats are presented in Table 2 and 3 respectively (APPENDIX).

Lymphocytes

Changes in labeling index of these cells after treatment with Myleran and cortisol are presented graphically in Fig. 7. Table 7 presents the statistical results. The labeling index of experimental

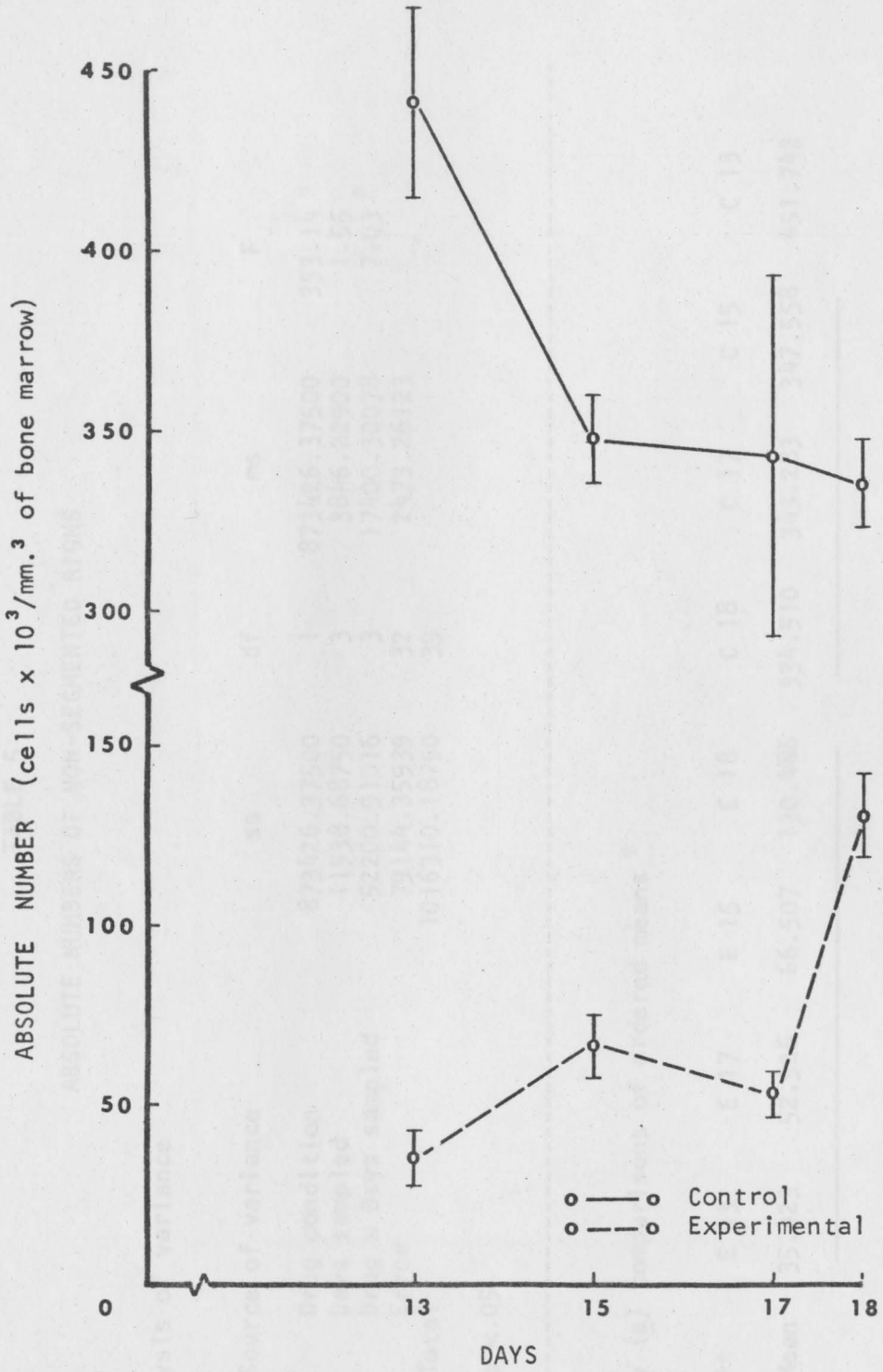

Fig.5 Graph showing mean (± S.E.) changes in absolute number of non-segmented rigns after treatment with Myleran and cortisol.

TABLE 5

ABSOLUTE NUMBERS OF NON-SEGMENTED RIGNS

Analysis of variance

Source of variance	ss	df	ms	F
Drug condition	873426.37500	1	873426.37500	353.14 *
Days sampled	11538.68750	3	3846.22900	1.55
Drug x Days sampled	52200.91016	3	17400.30078	7.03 *
Error	79144.35939	32	2473.26123	
Total	1016310.18750	39		

* $p < .05$

Tukey (a) comparisons of ordered means *

**	E 13	E 17	E 15	E 18	C 18	C 17	C 15	C 13
Mean	35.423	52.526	66.507	130.486	334.510	343.283	347.558	451.742

* Any two means not underlined by a common line are significantly different at the 0.05 level.

** E = Experimental, C = Control animals, number denotes days after Myleran treatment.

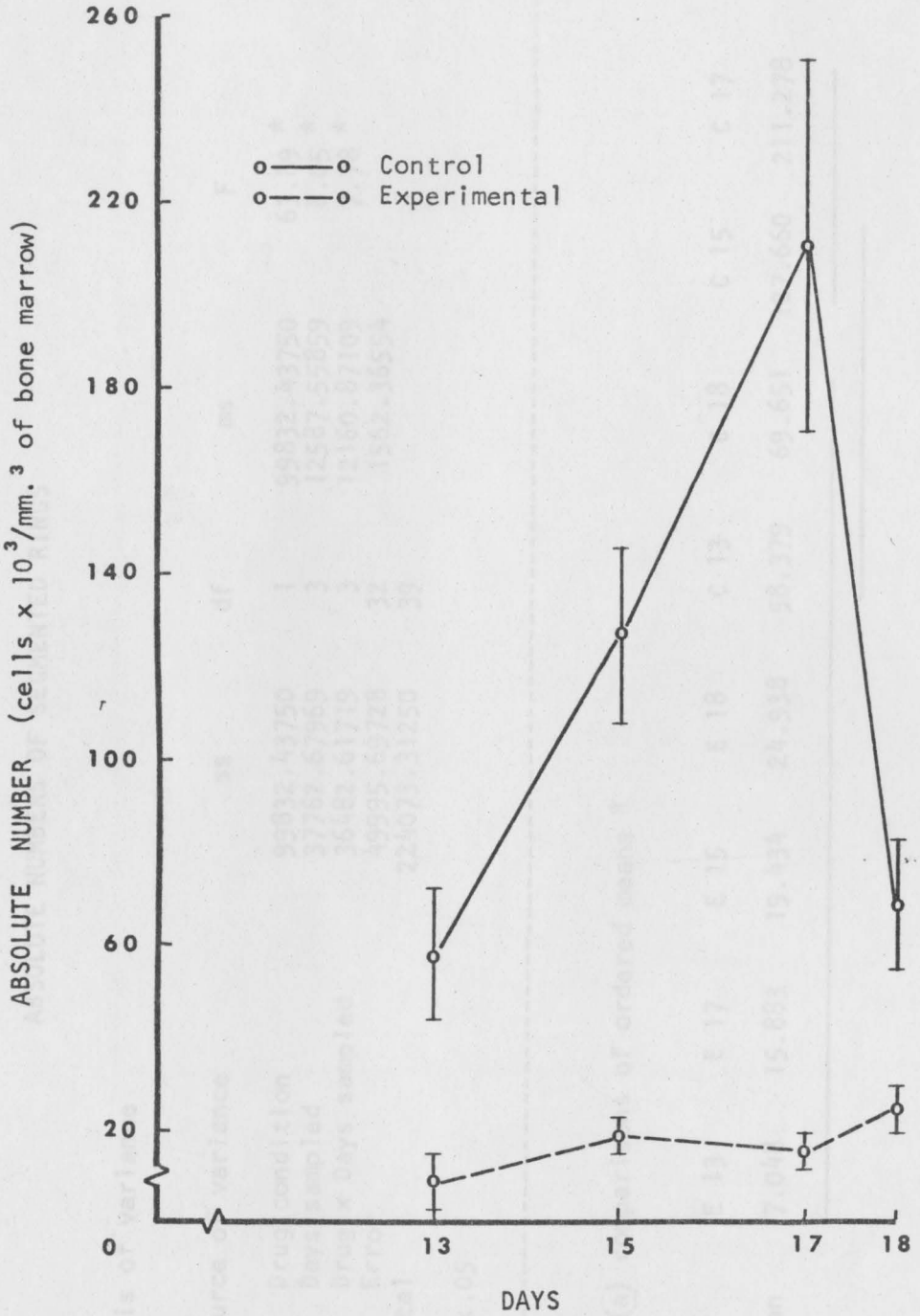

Fig. 6 Graph showing mean (± S.E.) changes in absolute number of segmented rings after treatment with Myleran and cortisol.

TABLE 6

ABSOLUTE NUMBERS OF SEGMENTED RINGS

Analysis of variance

Source of variance	ss	df	ms	F
Drug condition	99832.43750	1	99832.43750	63.89 *
Days sampled	37762.67969	3	12587.55859	8.05 *
Drug x Days sampled	36482.61719	3	12160.87109	7.78 *
Error	49995.69728	32	1562.36554	
Total	224073.31250	39		

* $p < .05$

Tukey (a) comparisons of ordered means *

**	E 13	E 17	E 15	E 18	C 13	C 18	C 15	C 17
Mean	7.048	15.883	19.434	24.938	58.379	69.651	127.660	211.278

* Any two means not underlined by a common line are significantly different at the 0.05 level.

** E = Experimental, C = Control animals, number denotes days after Myleran treatment.

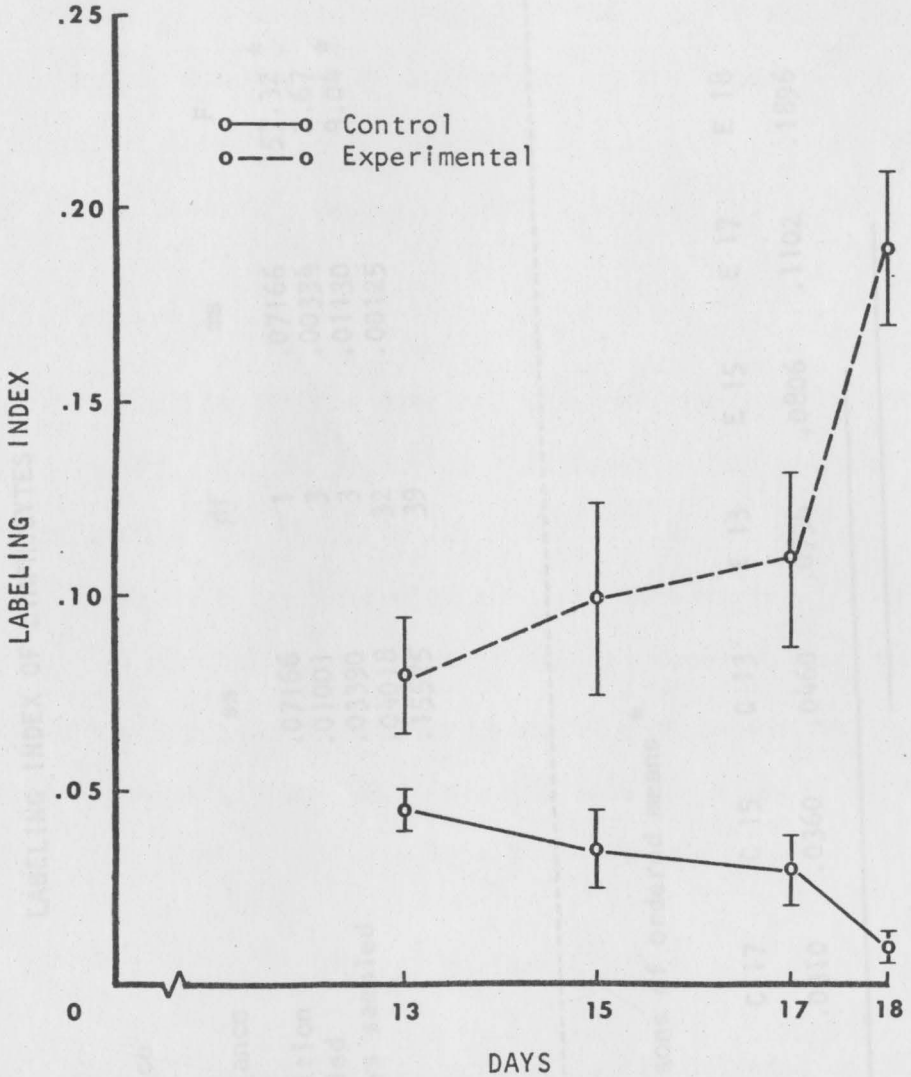

Fig. 7 Graph showing mean (±S.E.) changes in labeling index of lymphocytes after treatment with Myleran and cortisol.

TABLE 7

LABELING INDEX OF LYMPHOCYTES

Analysis of variance

Source of variance	ss	df	ms	F
Drug condition	.07166	1	.07166	57.32 *
Days sampled	.01001	3	.00334	2.67
Drug x Days sampled	.03390	3	.01130	9.04 *
Error	.04018	32	.00125	
Total	.15575	39		

* $p < .05$

Tukey (a) comparisons of ordered means *

**	C 18	C 17	C 15	C 13	E 13	E 15	E 17	E 18
Mean	.0084	.0310	.0360	.0460	.0796	.0806	.1102	.1896

* Any two means not underlined by a common line are significantly different at the 0.05 level.

** E = Experimental, C = Control animals, number denotes days after Myleran treatment.

animals are significantly higher than controls at day 17 and 18. At day 18 the labeling index is significantly higher than all other days sampled within experimental animals.

Changes in absolute numbers of these cells are presented in Fig. 8. Absolute numbers of lymphocytes in the controls are significantly higher than in experimentals at all days sampled. There are no significant differences within each group (Table 8).

Transitional Cells

The results in the labeling index of these cells are presented in Fig. 9. No significant differences between any two means are found (Table 9).

There is a depression of absolute numbers at all days sampled (Fig. 10). Tukey (a) comparisons of ordered means do not indicate any significant differences among days sampled within both experimental and control animals. However, experimentals are significantly lower in number than control animals at all days sampled (Table 10).

Total Nucleated Cells

Changes in absolute numbers of total nucleated cells are presented graphically in Fig. 11.

Analysis of variance of absolute numbers of these cells indicates that days sampled, drug condition, and the interaction of these two effects are statistically significant. Comparisons of ordered mean cell counts of control and experimental animals at different days sampled show that these cells are significantly lower

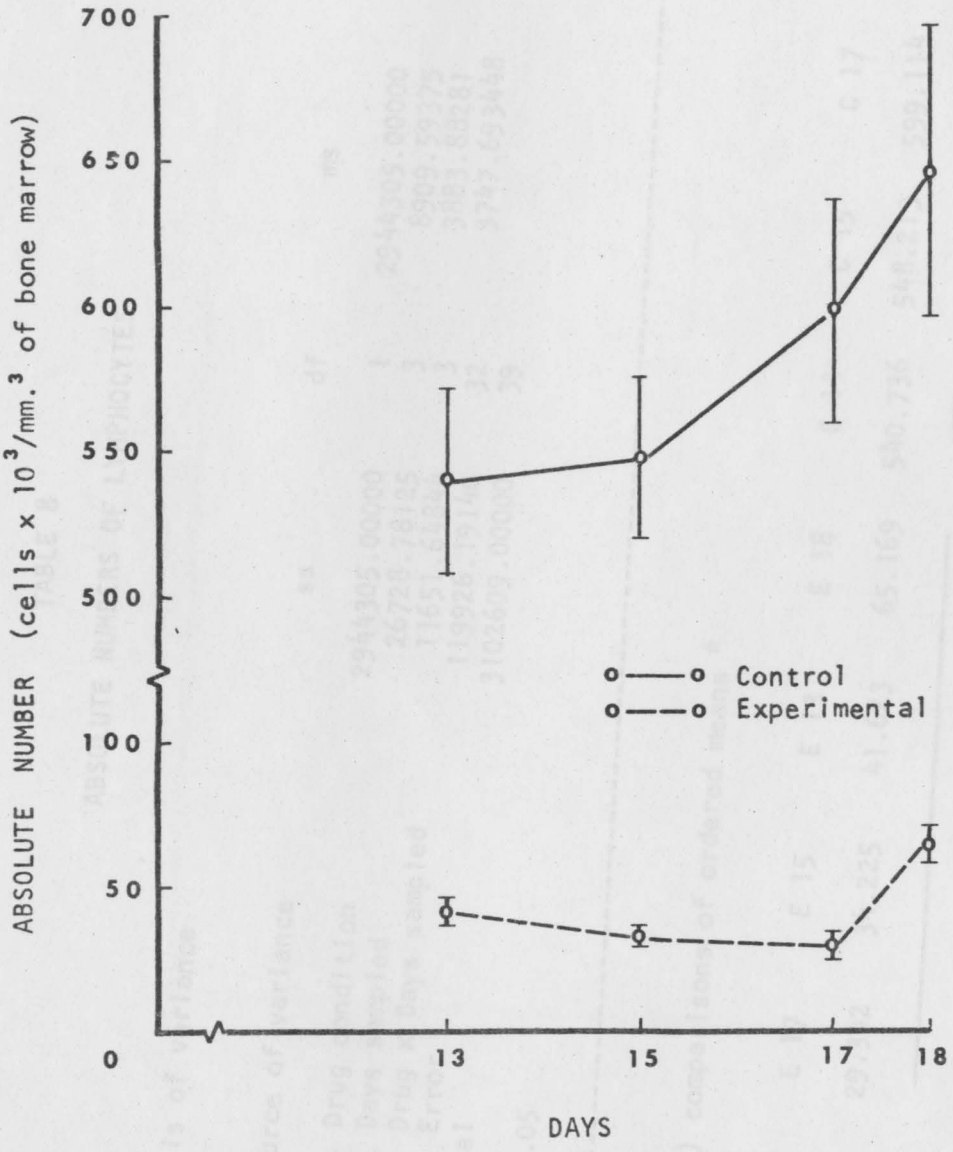

Fig. 8 Graph showing mean (± S.E.) changes in absolute number of lymphocytes after treatment with Myleran and cortisol.

TABLE 8

ABSOLUTE NUMBERS OF LYMPHOCYTES

Analysis of variance

Source of variance	ss	df	ms	F
Drug condition	2944305.00000	1	2944305.00000	785.63 *
Days sampled	26728.78125	3	8909.59375	2.37
Drug x Days sampled	11651.64844	3	3883.88281	1.04
Error	119926.19140	32	3747.693448	
Total	3102609.00000	39		

* $p < .05$

Tukey (a) comparisons of ordered means *

**	E 17	E 15	E 13	E 18	C 13	C 15	C 17	C 18
Mean	29.342	34.225	41.623	65.169	540.736	548.213	599.114	646.755

* Any two means not underlined by a common line are significantly different at the 0.05 level.

** E = Experimental, C = Control animals, number denotes days after Myleran treatment.

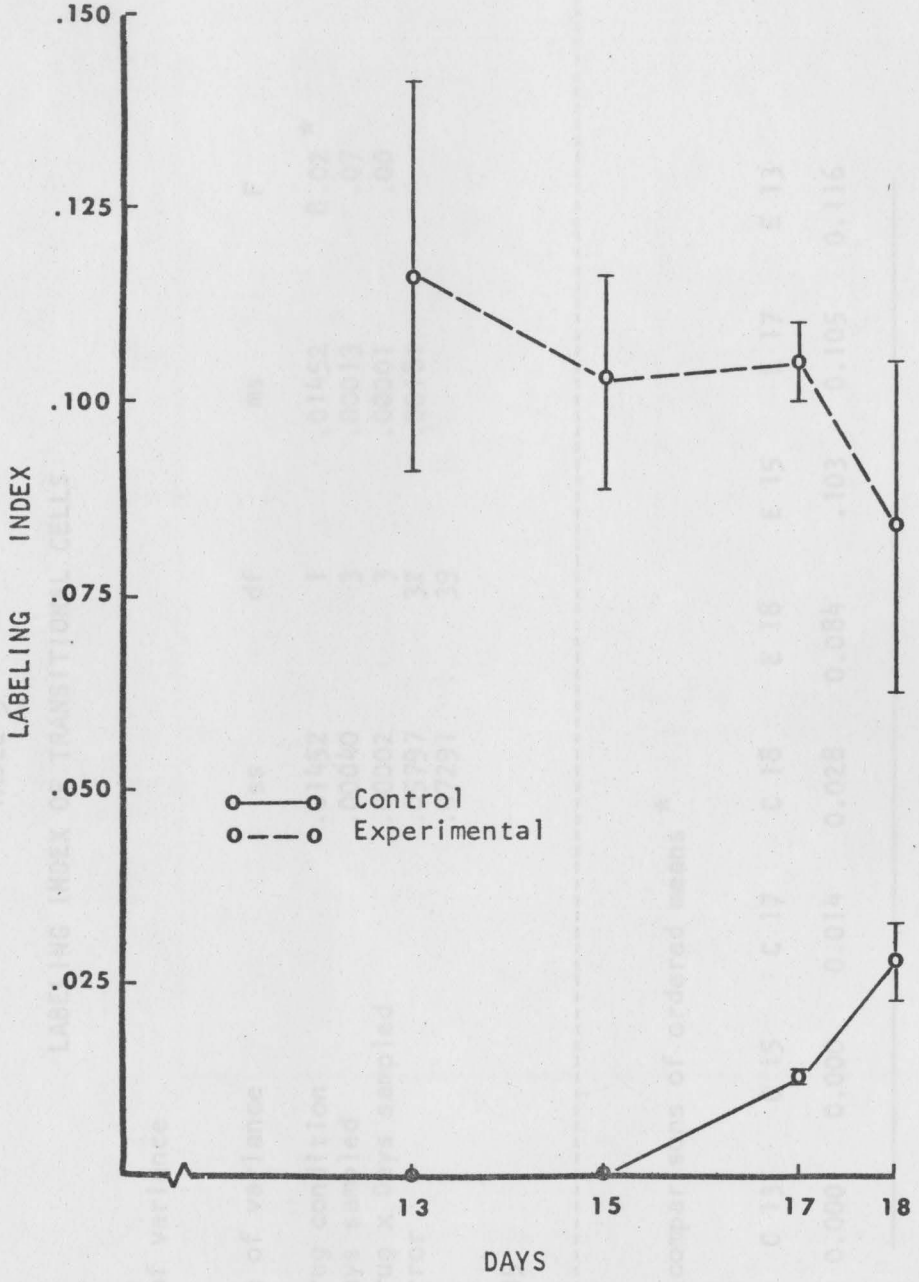

Fig. 9 . Graph showing mean (±S.E.) changes in labeling index of transitional cells after treatment with Myleran and cortisol.

TABLE 9

LABELING INDEX OF TRANSITIONAL CELLS

Analysis of variance

Source of variance	ss	df	ms	F
Drug condition	.01452	1	.01452	8.02 *
Days sampled	.00040	3	.00013	.07
Drug x Days sampled	.00002	3	.00001	.00
Error	.05797	32	.00181	
Total	.07291	39		

* $p < .05$

Tukey (a) comparisons of ordered means *

**	C 13	C 15	C 17	C 18	E 18	E 15	E 17	E 13
Mean	0.000	0.000	0.014	0.028	0.084	.103	0.105	0.116

* No significant differences between any two means are found.

** E = Experimental, C = Control animals, number denotes days after Myleran treatment.

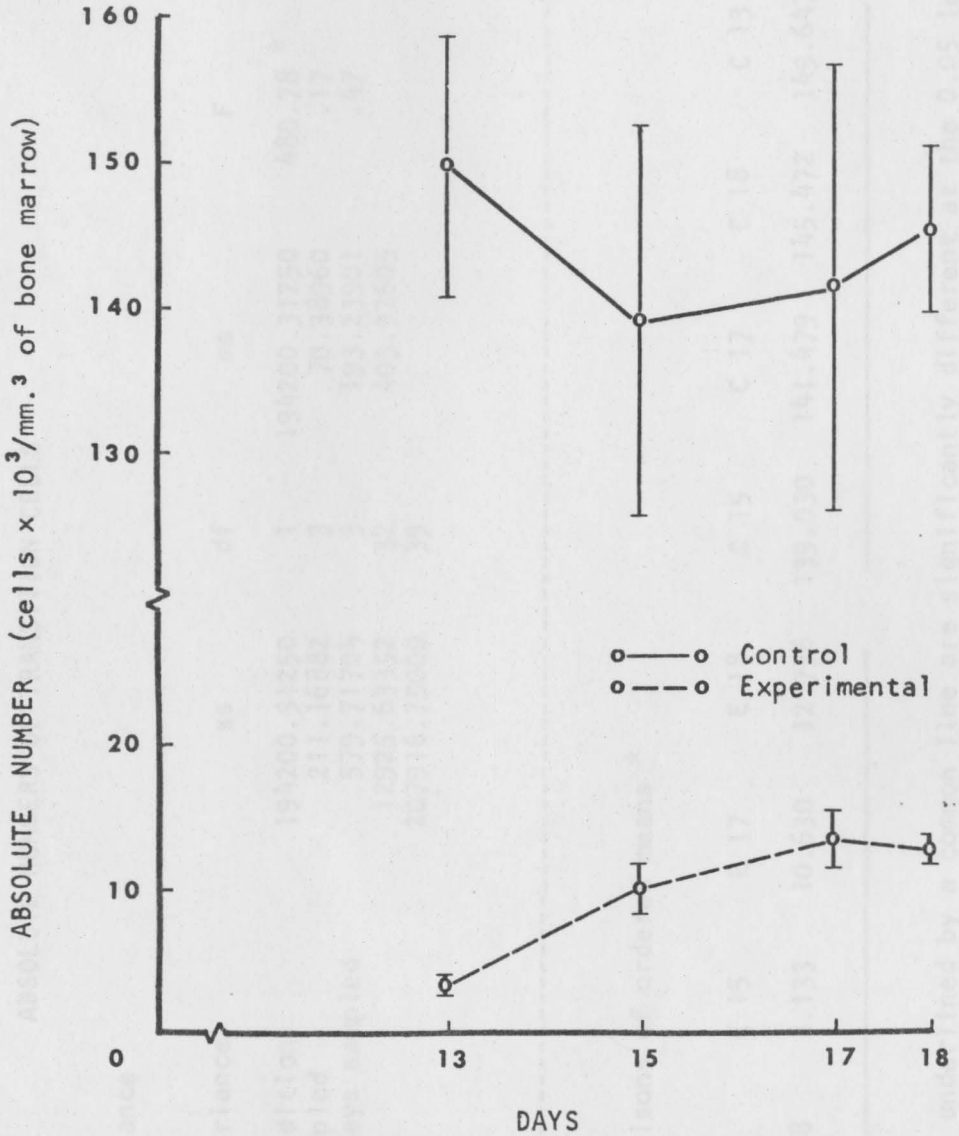

Fig. 10 Graph showing mean (± S.E.) changes in absolute number of transitional cells after treatment with Myleran and cortisol.

TABLE 10

ABSOLUTE NUMBERS OF TRANSITION CELLS

Analysis of variance

Source of variance	ss	df	ms	F
Drug condition	194200.31250	1	194200.31250	480.78 *
Days sampled	211.16882	3	70.38960	.17
Drug x Days sampled	579.71704	3	193.23901	.47
Error	12925.63352	32	403.92605	
Total	207916.75000	39		

* $p < .05$

Tukey (a) comparisons of ordered means *

**	E 13	E 15	E 17	E 18	C 15	C 17	C 18	C 13
Mean	2.668	8.133	10.630	12.736	139.030	141.479	145.472	149.647

* Any two means not underlined by a common line are significantly different at the 0.05 level.

** E = Experimental, C = Control animals, number denotes days after Myleran treatment.

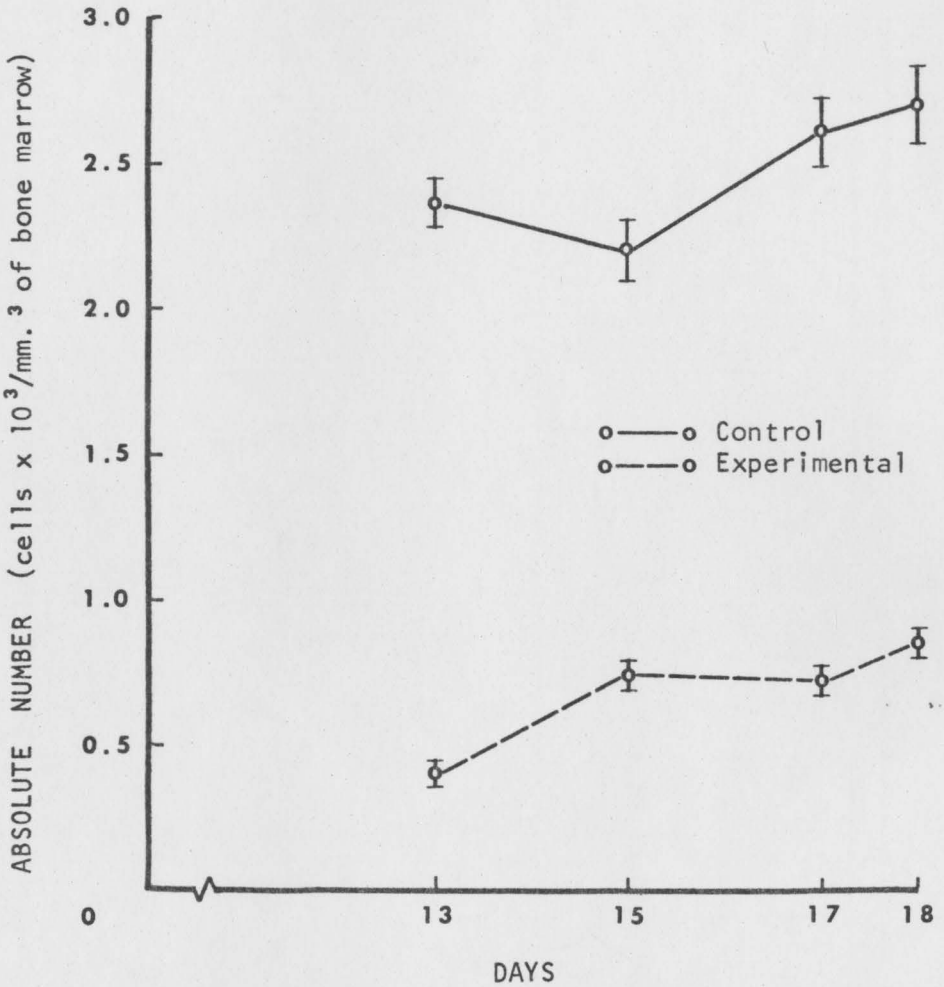

Fig. 11 Graph showing mean (± S.E.) changes in absolute number of total nucleated cells after treatment with Myleran and cortisol.

than controls at all days sampled. There is a highly significant difference between day 13 and 18 in the experimentals and between day 15 and 18 in the controls (Table 11).

TABLE 11

ABSOLUTE NUMBERS OF TOTAL NUCLEATED CELLS

Analysis of variance

Source of variance	ss	df	ms	F
Drug condition	32.10005	1	32.10005	864.90 *
Days sampled	0.96100	3	0.32033	8.63 *
Drug x Days sampled	0.35275	3	0.11758	3.17 *
Error	1.18764	32	0.03711	
Total	34.60141	39		

* $p < .05$

Tukey (a) comparisons of ordered means *

**	E 13	E 17	E 15	E 18	C 15	C 13	C 17	C 18
Mean	.403	.717	.740	.857	2.204	2.370	2.596	2.703

* Any two means not underlined by a common line are significantly different at the 0.05 level.

** E = Experimental, C = Control animals, number denotes days after Myleran treatment.

CHAPTER IV

DISCUSSION

Previous cytological work concerning the effects of Myleran and cortisol treatment on the distribution of rat bone marrow cells (Toepfer, 1969) has established that severe depression of myeloid cells results (15 per cent of control at 10 days after initiation of treatment). However, after treatment, regeneration of myeloid cells occurs. In this study, the results of the absolute numbers of the several cell types in the neutrophil production lineage confirms that a marrow depression does exist. Also, there is evidence that significant increases in the numbers of dividing neutrophil precursors (myeloblasts-promyelocytes, and myelocytes-metamyelocytes) in drug treated marrow is occurring, thus confirming that myeloid regeneration appears to be taking place. These interpretations are made difficult, however, when the numbers of dividing neutrophil precursors of control animals are considered. Here, too, significant increases in the numbers of cells are occurring.

It is well known that the antigenic state of the animal does affect the proliferative state and the cellular content of bone marrow. Thus, for instance, in guinea-pigs (Yoffey, 1966) the number of myeloblasts show a marked increase after an initial depression two days after injection with typhoid vaccine. Thirty-six hours prior to this increase there is a highly significant increase in the mitotic index of immature neutrophils. Also, of importance was the fact that mature neutrophils are almost totally discharged from the marrow two hours

after injection of typhoid vaccine. In the present study, the antigenic state on marrow cellular distribution of the control animals can not ruled out because of certain facts: (1) at 13 days the absolute numbers of the control segmented neutrophils are very low compared with day 17, (2) the absolute number of myeloblasts-promyelocytes and myelocytes-metamyelocytes increase significantly over the sampling times, and (3) the labeling index of myelocytes-metamyelocytes is significantly higher at day 13 than at day 18.

The present study was designed to investigate the mechanism by which the re-establishment of neutrophil production following drug depression occurs. This was accomplished by identifying the types of neutrophil precursors which were preparing to divide (labeling index). The mechanism appears to be an actual increase in the rate of neutrophil production. Thus, the labeling index of myeloblasts-promyelocytes increases significantly at day 17. Also, the labeling index of myelocytes-metamyelocytes are significantly higher at days 15 and 17. An increase in labeling index can be interpreted either of two ways: (1) an increase will occur if the cell cycle time decreases, thus resulting in more cells active in DNA synthesis at any given time, although, the absolute numbers of cells may not increase, (2) an increase in labeling index may reflect an actual increase in absolute numbers of dividing cells given the condition that the cell cycle times remain unaltered. Absolute numbers of both the myeloblasts-promyelocytes and myelocytes-metamyelocytes do increase significantly. Therefore, it is probable that the increases in labeling indices obtained in this study reflect increases in the absolute numbers of cells. It is conceivable, however, that cell cycle times may also change, although the experimental

design cannot show this. Also of interest is the fact that no significant increases in absolute numbers of non-segmented and segmented neutrophils in drug-treated marrows has yet occurred.

Other studies indicate that the total transit time for myeloid cell division and maturations is approximately 1½ days and the transit time for the maturing-only compartment is 1½ - 2 days duration (Bond et al., 1965). The total marrow transit time for neutrophil production (i.e., from myeloblasts to entry into the circulatory system) is about 3 - 3½ days. Based on this data it would seem in this study that the appearance of mature neutrophils (i.e., non-segmented and segmented neutrophils in bone marrow) should occur about three days after an increase in the rate of division of myeloblasts. Increased labeling index of myeloblasts-promyelocytes occur at 17 days whereas there are no differences between the experimental and control animals at 15 days. Therefore, the earliest indication of an increase in absolute numbers of mature neutrophils would be expected to appear somewhere between 18 - 20 days. Although not significant, an increase in non-segmented neutrophils seems to start at day 18. Also, the labeling index of myelocytes-metamyelocytes increases at 15 days. The input of the daughter cells resulting from divisions into the mature pool of bone marrow cells would be expected to occur. Since these cells occur at the end of the dividing-maturing compartment, the time of appearance into the mature compartment would be about 1½ days (the transit time for the maturing-only marrow compartment). This means that the earliest increase in absolute numbers of non-segmented and segmented neutrophils would be expected to occur at about 17 days. This does not occur. An explanation for this observation may be that transit time data is unavoidably highly

variable because of the complex kinetics of neutrophil production and release and the inherent experimental difficulties in the measurement of marrow transit time. In spite of these difficulties, the observation that mature neutrophils do not appear in significant numbers in the bone marrow possibly indicates that the cell cycle times of the dividing neutrophil precursors has not changed. This strengthens the idea that the significant increases in labeling indices obtained in this study reflect an increase in absolute numbers of dividing cells. It is also important to note that the significant increases in labeling indices of both the myeloblast-promyelocyte and myelocyte-metamyelocyte groups is an indication that the increase in neutrophil production does not originate with the earliest precursor cells. That is, myeloblasts are not the first cells stimulated into division, these daughter cells then giving rise to increased numbers of promyelocytes, then promyelocytes, in turn, give rise to large numbers of myelocytes, etc. The data indicates that more mature cells are, in fact, stimulated into division earlier than more immature forms.

In investigating the mechanism of neutrophil replacement, it is necessary to consider the possible role of bone marrow lymphocytes as stem cells for increased neutrophil production. The present data shows that absolute numbers of marrow lymphocytes from drug-treated animals are significantly lower than controls at all days sampled. The labeling index of these cells are significantly higher than controls at days 17 and 18. This increase occurs after neutrophil production has started to increase and, therefore, does not suggest a functional relationship. Also, many hypothetical schemes of lymphocyte stem cell function involves the transformation of lymphocytes into blast cells (Rosse and

Yoffey, 1967). On the basis of *in vitro* lymphocyte culture studies with phytohemagglutinin (Elves and Wilkinson, 1962; Yoffey et al., 1965), transformation into blast cells requires at least 24 hours. In fact, maximal numbers of blast cells are present 3 days after incubation of the cultures (Yoffey et al., 1965). The time involved in transformation of lymphocytes would provide further arguments against possible stem cell function in this study. Finally, the fact that there are no significant increases in either absolute numbers or labeling index of transitional cells adds further support to the idea that lymphocytes do not function as stem cells for neutrophil production in this study.

APPENDIX

TABLE 1

LABELING INDEX OF BONE MARROW CELLS

(EXPT. = Experimental, CONT. = Control animals)

		DAY 13		DAY 15		DAY 17		DAY 18	
		CONT.	EXPT.	CONT.	EXPT.	CONT.	EXPT.	CONT.	EXPT.
Blasts-Pro myelocytes	MEAN	.495	.415	.848	.493	.449	.554	.509	.545
	S.E	±.018	±.036	±.023	±.024	±.020	±.033	±.019	±.025
Myelo-Meta myelocytes	MEAN	.407	.353	.350	.682	.284	.625	.280	.324
	S.E.	±.030	±.050	±.017	±.019	±.027	±.014	±.026	±.006
Lympho-cytes	MEAN	.046	.080	.036	.101	.031	.110	.010	.190
	S.E.	±.005	±.015	±.009	±.024	±.008	±.023	±.003	±.019
Transi-tionals	MEAN	.000	.116	.000	.103	.014	.105	.028	.084
	S.E.	.000	±.026	.000	±.014	.000	±.005	±.005	±.021

TABLE 2

MEANS (± S.E.) OF ABSOLUTE NUMBERS AND PERCENTAGES OF DIVIDING CELLS IN CONTROL RATS

		DAY 13		DAY 15		DAY 17		DAY 18	
		ABSOLUTE NUMBER	PERCENT	ABSOLUTE NUMBER	PERCENT	ABSOLUTE NUMBER	PERCENT	ABSOLUTE NUMBER	PERCENT
Blasts-Pro myelocytes	MEAN	82.408	3.5	84.085	3.8	121.032	4.7	122.436	4.5
	S.E.	±4.929	±0.3	±4.912	±0.2	±10.437	±0.4	±8.453	±0.3
Myelo-Meta myelocytes	MEAN	115.042	4.8	97.355	4.4	152.002	5.9	271.998	10.2
	S.E.	±13.538	±0.5	±15.591	±0.7	±22.097	±0.8	±20.035	±1.1
Lympho-cytes	MEAN	540.736	22.9	548.213	24.9	599.114	23.1	646.755	23.8
	S.E.	±31.647	±1.3	±27.615	±0.6	±38.751	±0.9	±50.514	±1.2
Transi-tionals	MEAN	149.646	6.3	139.030	6.4	141.439	5.5	145.473	5.5
	S.E.	±9.188	±0.2	±12.974	±0.8	±15.565	±0.6	±5.814	±0.4

TABLE 3

MEANS (± S.E.) OF ABSOLUTE NUMBERS AND PERCENTAGES OF DIVIDING CELLS IN EXPERIMENTAL RATS

		DAY 13		DAY 15		DAY 17		DAY 18	
		ABSOLUTE NUMBER	PERCENT	ABSOLUTE NUMBER	PERCENT	ABSOLUTE NUMBER	PERCENT	ABSOLUTE NUMBER	PERCENT
Blasts-Pro myelocytes	MEAN	22.729	5.3	32.986	4.6	45.641	6.4	73.384	8.8
	S.E.	±5.502	±1.0	±2.173	±0.3	±3.994	±0.4	±4.108	±0.9
Myelo-Meta myelocytes	MEAN	33.243	8.4	117.467	16.1	139.414	18.6	197.694	22.1
	S.E.	±4.670	±1.1	±12.405	±1.2	±12.814	±2.5	±11.211	±1.3
Lympho-cytes	MEAN	41.623	10.4	34.226	4.9	29.342	4.1	65.169	7.6
	S.E.	±4.848	±0.6	±3.585	±0.7	±4.954	±0.6	±7.187	±0.7
Transi-tionals	MEAN	3.332	0.9	10.166	1.4	13.287	1.8	12.736	1.5
	S.E.	±0.777	±0.2	±1.724	±0.3	±1.975	±0.3	±0.892	±0.2

TABLE 4

MEANS (± S.E.) OF ABSOLUTE NUMBERS, PERCENTAGES OF NON-DIVIDING CELLS, AND TOTAL NUCLEATED CELLS IN CONTROL RATS

		DAY 13		DAY 15		DAY 17		DAY 18	
		ABSOLUTE NUMBER	PERCENT	ABSOLUTE NUMBER	PERCENT	ABSOLUTE NUMBER	PERCENT	ABSOLUTE NUMBER	PERCENT
Non-segmented rings	MEAN	441.742	18.600	347.558	15.860	343.283	13.160	334.510	12.420
	S.E.	±26.946	±0.707	±12.047	±0.619	±50.983	±1.818	±13.090	±0.404
Segmented rings	MEAN	58.379	2.480	127.660	5.800	211.277	7.920	69.651	2.540
	S.E.	±13.823	±0.572	±19.246	±0.876	±40.620	±1.266	±14.329	±0.495
Total Nucleated cells	MEAN	2.370		2.204		2.596		2.703	
	S.E.	±0.086		±0.111		±0.116		±0.131	

TABLE 5

MEANS (± S.E.) OF ABSOLUTE NUMBERS, PERCENTAGES OF NON-DIVIDING CELLS, AND TOTAL NUCLEATED CELLS IN EXPERIMENTAL RATS

		DAY 13		DAY 15		DAY 17		DAY 18	
		ABSOLUTE NUMBER	PERCENT	ABSOLUTE NUMBER	PERCENT	ABSOLUTE NUMBER	PERCENT	ABSOLUTE NUMBER	PERCENT
Non-segmented rings	MEAN	35.421	8.460	66.507	9.120	52.526	7.360	130.485	15.280
	S.E.	±7.626	±1.172	±8.836	±0.909	±6.006	±0.826	±11.964	±1.227
Segmented rings	MEAN	8.809	2.000	19.434	2.560	15.883	2.160	24.938	2.960
	S.E.	±6.046	±1.203	±4.332	±0.453	±3.984	±0.511	±4.827	±0.564
Total Nucleated cells	MEAN	0.403		0.728		0.717		0.854	
	S.E.	±0.044		±0.049		±0.045		±0.050	

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