

Kinetics of Mobilization of Neutrophils and Their Marrow Pool in Protein-Calorie Deficiency

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Migration of marrow neutrophils under basal conditions and their mobilization, following subcutaneous implantation of cover slips, were investigated in groups of protein-deficient rats, using $^3\text{HTdR}$ with sequential autoradiography of the peripheral blood smears. Animals fed a protein-rich diet served as controls. The pattern of appearance of labeled neutrophils in the blood was identical in the two groups under basal conditions. However, a higher percentage of labeled neutrophils appeared earlier in the blood following cover slip implantation in the deficient rats as compared to controls. The inflammatory exudate on the cover slips was low throughout the period of observation in deficient animals, with a delay in the appearance of monocytes. A pool of mature neutrophils resides in the bone marrow. It is proposed that in PCM there is atrophy of all neutrophil compartments, including that of the marrow pool, associated with

a proportionate reduction in the efflux of cells from one compartment to another. This proportionate reduction in efflux would explain the normal kinetics of migration of neutrophils under basal conditions in the deficient rats in spite of a reduction in the marrow pool. On the other hand, in cover slip-implanted deficient rats, appearance of larger numbers of labeled neutrophils in the blood is attributed to a reduction in size of the marrow pool. This hypothesis is substantiated by the experiments in protein-deficient monkeys in which estimation of the marrow pool of neutrophils revealed a marked reduction in the deficient animals. It is further proposed that a diminution of the marrow pool of neutrophils and retarded mobilization of cells at the site of inflammation are important mechanisms responsible for the increased susceptibility of the malnourished host to infections.

PROTEIN-CALORIE MALNUTRITION (PCM) in both man and animals increases the susceptibility of the host to infections.^{1,2} The mechanism of the increased susceptibility is not fully understood. Spreading inflammation associated with a poor neutrophilic response is frequently observed in PCM.^{3,4} Studies on the kinetics of granulopoiesis, in both man and laboratory animals, have established the existence of a large bone marrow pool of mature neutrophils that is several times greater than their circulating pool.^{5,6} The poor neutrophil response in malnutrition may be a consequence of a reduction in the size of the marrow pool and/or mobilization of these cells. In this study, these aspects have been investigated in rats and rhesus monkeys.

MATERIALS AND METHODS

Experiment in Rats

Four to 6-wk-old male albino rats from our inbred colony of AS₂ strain (originally obtained from Otago, Dunedin, New Zealand) were used. They were divided into two dietary groups, each

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consisting of 22 animals. The animals in the low-protein group (also referred to as deficient) were fed a diet containing 3% protein (casein) for a period of 5–6 wk. The control (high-protein) animals were fed a protein-rich diet containing 16% casein. Each animal in the control group was pair-fed with the corresponding deficient animal. The animals were housed individually in cages of perforated galvanized iron sheet, and water was provided ad libitum. Except for the protein content, the two diets were identical and isocaloric. The composition of the diets and vitamin and mineral mixtures has been described earlier.⁷

At the end of the dietary regimen, the following experiments were carried out.

Experiment I. Four animals from each group received a pulse of ³H-thymidine* (³HTdR), 1 μ Ci/g body weight intraperitoneally. Total and differential leukocyte counts were performed on blood samples drawn at 12, 24, 36, 48, 72, 96, and 120 hr following administration of the isotope. Peripheral smears were subjected to autoradiography.

Experiment II. As in experiment I, six animals from each group received a pulse of ³HTdR. Twenty-four hours later, four sterile round (22 mm diameter) glass cover slips were implanted subcutaneously in the back of each animal under light ether anesthesia. Blood samples were collected at 24, 30, 36, 48, 72, and 96 hr following administration of ³HTdR. One of the cover slips was also withdrawn at each of the later four intervals. The blood smears and the cover slips were subjected to autoradiography.

Experiment III. As described later, the percentage of labeled neutrophils in the peripheral blood was higher in the deficient rats than in the controls following cover slip implantation. In order to ascertain that these differences were not due to labeling of a larger group of precursor cells, a feature observed in certain tissues in protein-calorie deficiency,^{8–10} animals in this experiment were given four doses of ³HTdR at 6-hr intervals. A total of 1 μ Ci/g body weight of the isotope was administered in four equal doses. This procedure achieved a "continuous" labeling for 24 hr and eliminated differences that could be attributed to disproportionate labeling of the precursors in the single pulse experiment. Twenty-four hours after the first dose of ³HTdR, a sample of blood was collected and four sterile cover slips were introduced. The cover slips were removed at 30, 36, 48, and 96 hr following administration of the first pulse of ³HTdR. Blood samples were collected simultaneously. In order to obviate the possible effects of repeated surgical manipulations, all cover slips in any one particular animal were removed at the same time. At each time interval, at least three animals from each group were used.

To avoid the effects of diurnal variations, all experimental procedures were carried out at fixed hours in the morning in both groups.

Laboratory Techniques

Blood samples were diluted in duplicate. Total leukocyte counts were made on each dilution in duplicate, using a Neubauer chamber. The count on each sample thus represented the average of four determinations. The total blood neutrophil counts were estimated on the basis of the total and differential leukocyte counts.

Autoradiography was performed by the "dip-coating" method on methanol-fixed peripheral smears and cover slips, using Kodak NTB₃ nuclear track emulsion. The smears and the cover slips were kept in contact with the emulsion for 8 wk. The autoradiographs were developed, using Kodak D-19b developer and acid fixer at 16°C, and stained with Giemsa at pH 6.8. At least 300 consecutive cells were counted for estimation of the differential leukocyte count and the labeling index on the blood smears.

The pattern of migration of neutrophils and monocytes (macrophages) onto the cover slips was assessed by counting a minimum of 300 cells from three different areas at least 20 high-power fields apart. A single pulse of ³HTdR labels only a small cohort of cells. In this system, the rate of appearance of labeled cells on the cover slips may be modified by the division and subsequent migration of unlabeled cells which were in the G₁ phase at the time of administration of ³HTdR. For this reason, no attempt was made to measure the labeling indices of the cells on the cover slip. The intensity of cellular infiltration was arbitrarily graded from + to + + + + +. The cells were also examined for morphological alterations.

*Specific activity 6.4 Ci/mM obtained from the Bhabha Atomic Research Centre, Trombay, Bombay, India.

Table 1. Monkey Diets

Constituents	High Protein (g)	Low Protein (g)
Lactogen	11.25	1.4
Lactose	—	6.0
Sucrose	—	5.5
Milk fat	—	1.7*
Water to make	100 ml	100 ml
Calorie contents/100 ml	52.0	52.0
Protein contents/100 ml (g)	2.40	0.300

Lactogen: (ingredients are shown in grams) milk fat, 19; milk protein, 21.6; sucrose, 20; lactose, 30.8; salt, 5.6; moisture, 3.0; vitamin A, 1500 I.U., vitamin D, 400 I.U., and 6 mg organic iron/100 g of powder.

*Extraneous milk fat was added only to give the flavor. Being lighter, it used to float on the surface. Not more than 10% of it was consumed by the animal. It was therefore not taken in account for purposes of calculations of calories.

Each low-protein animal received every day 1 ml of vitamin B complex liquid (Lederplex; Cyanamid India, Ltd.) and twice a week additional quantities of 400 I.U. vitamin A, 100 I.U. vitamin D, and 5 mg of ferrous sulfate.

Each 5 ml of Lederplex contains: thiamine hydrochloride, 2 mg; riboflavin, 2 mg; niacinamide, 10 mg; pyridoxine hydrochloride, 0.2 mg; panthenol, 2.0 mg; choline dihydrogen citrate, 30 mg; inositol, 10 mg; soluble liver fraction, 305 mg (on dry basis); cyanocobalamin, 5 μ g.

Experiment in Monkeys

A total of eight rhesus monkeys, obtained from a local dealer, were used. They were housed separately in cast-iron cages, and water was provided ad libitum. Four animals weighing between 3 and 7 kg were fed a stock diet made of whole wheat chapatis, groundnut, roasted Bengal gram, and seasonal fruits. The remaining four monkeys were brought to the laboratory when they were 1–2 mo old. Two of them were bottle-fed with a whole milk diet (Lactogen, Nestle's Products, India). The animals adapted well to this diet and showed good growth. The other two were fed 1:8 diluted milk made isocaloric by addition of suitable quantities of sucrose, lactose, and milk fat. These animals also received supplements of both water and fat-soluble vitamins. The composition of the two milk diets is given in Table 1. The animals were kept on the diet for a period of 10 wk. The animals on the stock diet and the two fed the full-milk diet served as the controls.

Estimation of the Marrow Pool in Monkeys

The bone marrow pool was measured using the technique of Bishop et al.¹¹ After overnight fasting, the animals were lightly anesthetized with Nembutal (30 mg/kg body weight). One hour later, a blood sample was drawn from the dorsal vein, and through the same needle hydrocortisone (10 mg/kg body weight) was administered. This dose was selected on the basis of a pilot study. Total and differential leukocyte counts were performed in blood samples drawn before and after 2, 3, and 5 hr of administration of the steroid. The counting procedures were the same as described above for the rats. The highest reading between 3 and 5 hr was used to assess the marrow pool.

RESULTS

General Features

Growth retardation was the dominant feature both in rats and monkeys fed the deficient diets. The deficient animals did not show any appreciable weight gain throughout the period of observation. On the other hand, the controls grew well (Fig. 1). Except for the failure of weight gain, the deficient animals were otherwise healthy, active, and free of infection.

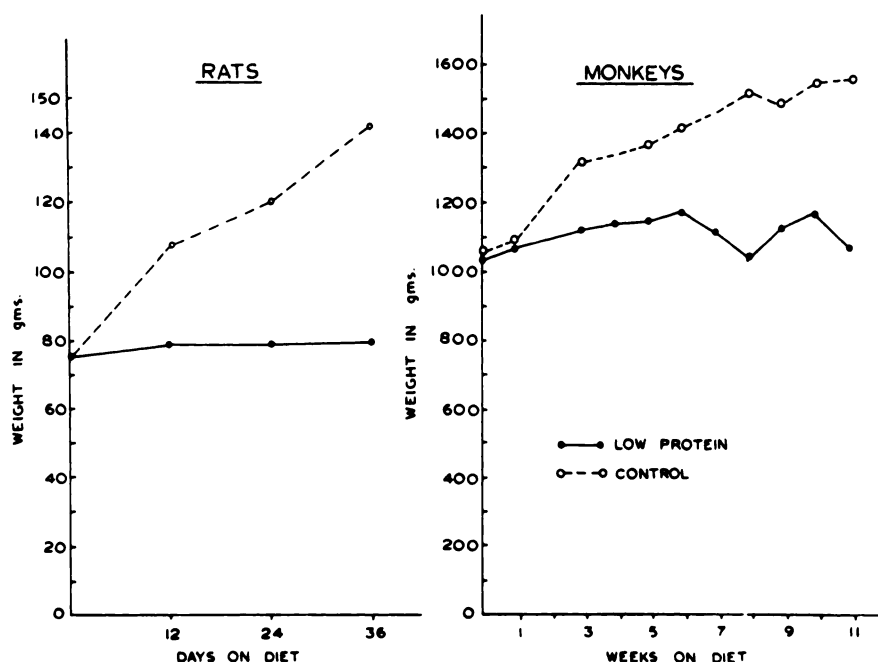


Fig. 1. Body weights of rats and monkeys on diet.

Kinetics of Leukocyte Migration in Rats

Blood leukocyte counts, both total and differential, with and without implantation of cover slips, are depicted in Table 2. The mean total blood neutrophil count in the normal rats, under basal conditions, was 3380/ μ l. Protein deficiency induced a statistically significant reduction in total neutrophil counts. Implantation of cover slips resulted in neutrophilia of similar magnitude at 6 hr in both groups. By 12 and 24 hr it returned to the original level in the control and deficient animals, respectively.

Under basal conditions, labeled neutrophils appeared in the peripheral blood

Table 2. Blood Total Neutrophil* Counts Under Basal Conditions and After Implantation of Cover Slips in Rats

Group		Basal (Without Cover Slips)	Hours After Cover Slip Implantation		
			6	12	24
Low protein	M	2130 (13)	4760 (8)	3700	2040
	SD	± 1199	± 896	± 531	± 1070
			$p < 0.01$		
High protein	M	3380 (13)	5090 (7)	3970	1180
	SD	± 1215	± 1202	± 570	± 996
			$p < 0.01$		
p		< 0.02	NS	NS	NS

*Expressed as number of neutrophils/ μ l of blood.

Figures in parentheses denote the number of animals studied.

M, mean; SD, standard deviation.

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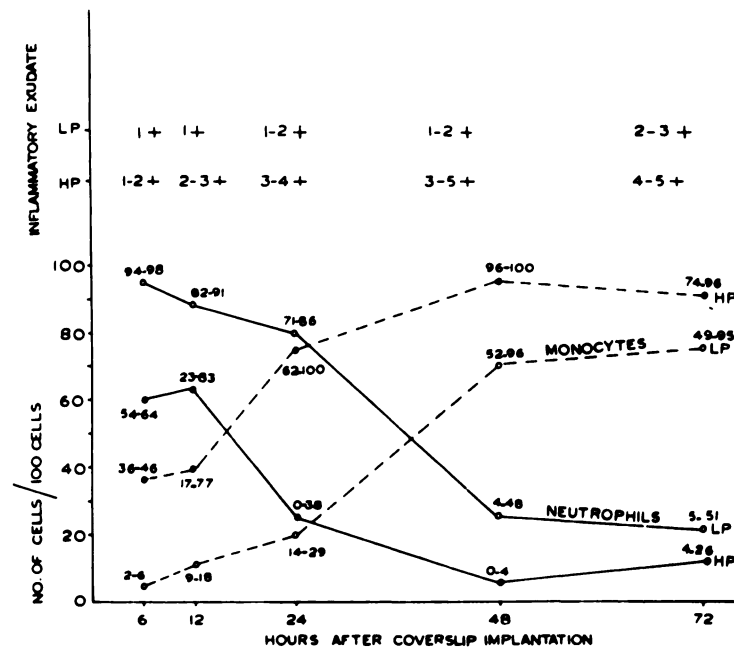


Fig. 3. Pattern of appearance of inflammatory cells on the cover slips. Intensity of inflammatory exudate graded from 1+ to 5+ is indicated at the top of the figure. The figures at each point denote ranges. LP, low protein; HP, high protein; straight line, neutrophils; dashed line, monocytes. There were at least three animals for each time interval in each group.

trarily graded as grade + to +++++, was low throughout in the deficient as compared to the control rats (Fig. 3, top portion).

An interesting pattern was observed in the relative proportions of neutrophils and monocytes. In the deficient animals, the cellular exudate contained, on the average, 92% and 80% neutrophils, respectively, at 6 and 24 hr after cover slip implantation. On the other hand, in the controls, the exudate contained a high percentage of monocytes even at 6 hr, and by 24 hr they constituted 75% of the cells.

There was not only a quantitative reduction in monocytes, but even their transformation to macrophages was qualitatively inferior in the deficient rats as compared to the controls. In the latter, the cells had prominent vesicular nuclei and abundant basophilic cytoplasm; a few giant cells were also observed at 96 hr. In the deficient animals, however, the macrophages were poorly developed and smaller in size with scanty and less basophilic cytoplasm, and no giant cells were seen.

Monkeys. As depicted in Table 4, no differences were observed in the two control groups. Their results are therefore discussed together. The basal mean total blood neutrophil count in the control animals was $9156/\mu\text{l}$, and in the deficient animals was $8204/\mu\text{l}$. Administration of cortisone resulted in a marked increase in the blood neutrophils in all animals. In the controls, on the average 19,494 neutrophils/ μl of blood were mobilized from the marrow pool which

Table 4. Marrow Reserve of Neutrophils in Protein-deficient and Control Monkeys

Animal No.	Diet	Total Leukocytes/ μ l		Total Neutrophils/ μ l		Neutrophils Mobilized From the Marrow/ μ l of Blood
		Basal	After Cortisone	Basal	After Cortisone	
1	SD	11,400	29,225	8,775	27,875	19,097
2	SD	17,350	37,150	11,104	32,792	21,688
3	SD	18,575	35,800	10,959	31,862	20,903
4	SD	15,750	31,150	9,078	27,412	18,334
5	HP	14,755	37,650	9,586	32,003	22,417
6	HP	8,883	20,450	5,415	20,041	14,526
Mean	SD & HP	14,452	31,904	9,153	28,664	19,494
7	LP	9,600	15,700	6,528	14,444	7,916
8	LP	12,050	17,050	9,881	15,516	5,635
Mean	LP	10,825	16,375	8,204	14,980	6,775

SD, stock diet; HP, high protein (synthetic full milk diet); LP, low protein.

was markedly reduced in the deficient animals; in these, it averaged only 6775/ μ l (Table 4).

DISCUSSION

Under basal conditions, the pattern of migration of neutrophils in the deficient and control animals was essentially similar. Mobilization of neutrophils was accelerated in both groups following implantation of cover slips. Moreover, the percentage of labeled neutrophils in the blood was higher at 12 and 24 hr in the deficient as compared to the control animals.

These observations could be explained on the basis of migration of a larger number of unlabeled neutrophils out of the circulation in response to the inflammation induced by the cover slips in deficient animals. However, poorer cellular exudate on the cover slips in the deficient rats throughout the observation period did not support this contention.

Another possibility is that the alterations in the cell generation cycle in PCM may be responsible for the higher percentage labeling of neutrophils in the deficient animals. PCM prolongs the S phase and unevenly affects the phases of the cell generation cycle.⁸⁻¹⁰ As the result of prolongation of the S phase, larger numbers of cells are labeled in organs of high cell renewal such as the intestine, hair follicle, and regenerating liver, in the deficient animals as compared to the control animals, given a pulse of ³HTdR.^{8-10,12} This situation may be true of myeloid cells in the present study. An inflammatory stimulus such as implantation of cover slips results in mobilization of marrow neutrophils. In the control, in this study (experiment II) the labeled neutrophils may be followed by the unlabeled ones derived from the precursors which are in the G₁ phase of the cell cycle at the time of administration of ³HTdR. On the other hand, a larger cohort of labeled precursors in the marrow may be able to provide labeled neutrophils for a longer period in the deficient animals. This finding may explain the higher percentage of labeled neutrophils observed at 48 hr in the cover slip-implanted deficient animals (Table 2) as compared to the controls. However, if

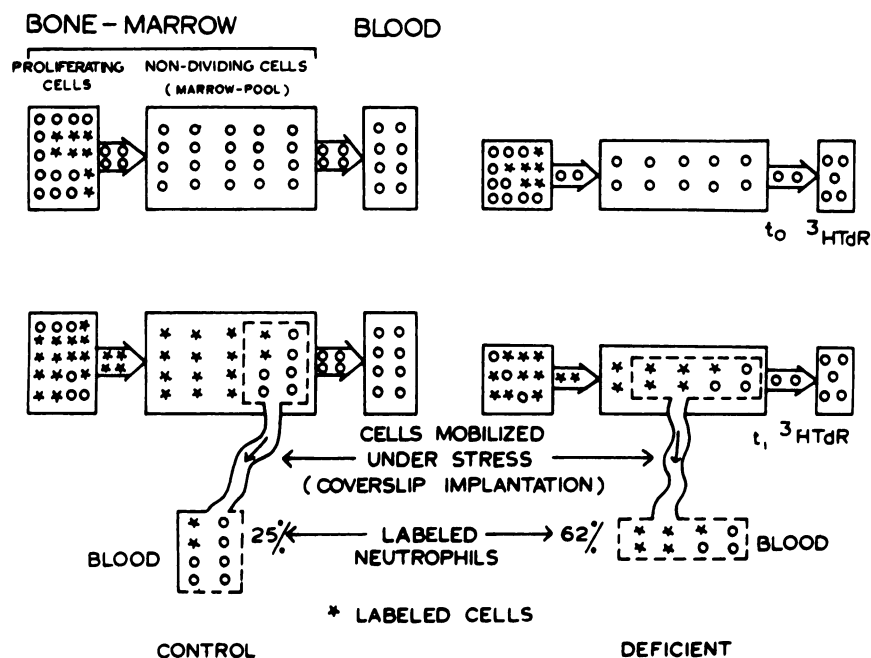


Fig. 4. Conceptual diagram to show movement of neutrophils in different compartments. There is atrophy of all compartments, associated with a proportionate decrease in the number of migrating cells, in protein deficiency. Under stress, large numbers of cells are mobilized in both groups. However, the percentage of labeled cells is higher in the deficient than that in the control.

this is true, the controls should have exhibited an earlier peak of labeled neutrophils. This, however, has not been the case in this study. Moreover, the higher percentage of labeled neutrophils observed at 48 hr in experiment III, in which a 24-hr "continuous" pulse is used to label the entire population of cells in the cell cycle, also does not support this possibility. As explained earlier, this procedure will eliminate differences due to labeling of unequal numbers of cells in the single pulse experiment (experiment II).

Several workers have reported atrophy of the marrow and neutropenia in protein-calorie deficiency.^{3,13-15} The marrow pool of neutrophils may likewise be affected. In such a situation, in order to maintain a steady state, the quantity of cells migrating from one compartment to another might be reduced in the deficient rats. As a consequence of this proportionate decrease in the efflux of cells, no differences would be observed in the pattern of appearance of labeled cells in the blood in the two groups. A slowing down of cell migration is a feature of protein deficiency in other tissues.^{8,9,12} An entirely different picture would be observed, however, when the marrow cells are mobilized under stress. As shown in Fig. 4, mobilization from a shrunken marrow pool would result in higher labeling of neutrophils in the peripheral circulation in the deficient animals. This contention is further strengthened by observations that the marrow pool of neutrophils, as measured by administration of hydrocortisone, is markedly reduced in the deficient monkeys.

There is a pattern in the reaction of the body to protein-calorie deficiency.^{16,17} The organs with a high protein and cell turnover are affected earlier than those with a low turnover. The organs of high protein turnover with stable cell populations, such as the liver and pancreas, show predominantly cytoplasmic lesions.¹⁶ On the other hand, a reduction in cell number is a dominant feature in those with a high rate of cell renewal. In these organs, the cytoplasmic lesions are often minimal.¹⁶ It is, therefore, not surprising that neutrophils, which belong to the latter group of tissues, show a reduction in number but little alteration in functions.^{15,18}

Poor inflammatory response, a feature of the present study, has also been observed by others in protein-calorie deficiency.^{2,3,19,20} Both the neutrophils and the monocytes were affected. There was also a preponderance of neutrophils on the cover slips, in the deficient animals, at a time when the cellular exudate was predominantly monocytic in the controls. This difference may be due to persistent migration of neutrophils or reduced migration/proliferation of monocytes. The observation of poor cellular exudate on the cover slips throughout the study speaks against the first possibility. Ryan and Spector²¹ have demonstrated that, although local proliferation of macrophages may be observed in the late phases of certain granulomatous inflammations, macrophages on the cover slips in the first week were almost entirely derived from the blood monocytes. A diminished monocytic response, may, therefore, be a consequence of defective chemotaxis of these cells. Using a Boyden chamber, Rosen et al.¹⁵ have reported poor chemotaxis of neutrophils in kwashiorkor. Similar factors may be responsible for reduced mobilization of monocytes. Page and Good,²² using a skin window technique, have demonstrated a poor mobilization of monocytes in patients with cyclic neutropenia in the neutropenic phase, and also in rabbits made neutropenic by the administration of nitrogen mustard. This situation is also true of certain cancer patients under treatment with cytotoxic drugs that suppress myeloid elements.²³ Ward²⁴ has recently isolated from neutrophils a factor chemotactic to monocytes. On the other hand, Dale and Wolff²⁵ have not observed any alteration in the migration of monocytes in their patients with cyclic neutropenia.

Sterile foreign bodies provide only a mild stimulus for mobilization of marrow cells. Cover slip implantation therefore may not exhaust the marrow pool and may be the reason that, unlike in microbial infections,^{3,4,20} the magnitude of neutrophilia induced by cover slip implantation is not affected in protein deficiency.

The mechanism of the poor neutrophilic response to microbial infections, observed in protein-calorie malnutrition, is not properly understood. Cartwright et al.⁵ have demonstrated that bone marrow neutrophils are mobilized in response to inflammation. The poor neutrophilic response in malnutrition may be due to a reduction in marrow reserves and/or defective mobilization. The fact that neutrophils are mobilized in similar quantities in the two groups of rats implanted with cover slips suggests that their mobilization from the marrow is not defective in protein deficiency. On the other hand, the observation of a reduced marrow pool in the deficient monkeys favors the former possibility.

It is generally believed that neutropenia indicates poor resistance. However,

recent studies suggest that patients with familial neutropenia with very low circulating neutrophils do not show an increased tendency to infections. These patients have a normal bone marrow and their marrow reserves of neutrophils are also normal.^{23,26} Patients with acute leukemia are highly susceptible to infections, but the infection rate is low in those who show neutrophilia even when the initial blood granulocyte level is low.²⁷ A reduction in the marrow pool is a feature of chronic alcoholism, aplastic anemia, certain cases of leukemia, and disseminated lupus erythematosus.²⁸⁻³⁰ All of these disorders are associated with an increased risk of infection. These observations emphasize the importance of marrow reserves in host defense mechanisms. This point is also brought out in the studies of Deinard et al.²³ These authors have, in fact, proposed that cancer chemotherapy should be monitored not by the estimation of blood neutrophils but through an assessment of marrow reserves.

The precise mechanism of the increased susceptibility to infection in malnutrition is not yet fully understood. In protein-calorie malnutrition, cell-mediated immunity is depressed.³¹ The phagocytic functions of the reticuloendothelial cells,³² serum complements,³³ and the bactericidal power of the serum are likewise^{34,35} affected. The present study suggests that a reduction in the marrow reserves of neutrophils may also be an important mechanism of increased susceptibility to infection in malnutrition.

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