

# A distinct role for apoptosis in the changes in lymphopoiesis and myelopoiesis created by deficiencies in zinc<sup>1</sup>

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**ABSTRACT** Reduced numbers of lymphocytes in the peripheral immune system appeared to be a significant cause of the loss in host defense capacity in humans and animals that are zinc deficient (ZD). A series of studies verified that ZD substantially reduced the lymphocyte compartment of both the marrow and thymus in young adult mice, with large losses noted among the pre-B and pre-T cells. Suboptimal nutriture along with chronic production of glucocorticoids generated during ZD had accelerated apoptosis among these precursor lymphocytes two- to threefold. Thus, the primary cause of the lymphopenia created by ZD was reduced production of lymphocytes and heightened cell death among precursor cells. The data will also show that myelopoiesis in the marrow was protected and enhanced numbers of myeloid progenitor cells were found in S and G<sub>2</sub>/M. Thus, as zinc became limiting the second line of defense appeared to be down-regulated via reduction of lymphopoiesis while cells of the myeloid lineage were protected to maintain the first line of defense that provides innate immunity. This may represent an important adaptation of the immune system to suboptimal nutriture that deserves further exploration.—Fraker, P. J., King, L. E. A distinct role for apoptosis in the changes in lymphopoiesis and myelopoiesis created by deficiencies in zinc. *FASEB J.* 15, 2572–2578 (2001)

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SEVERAL LABORATORIES have shown that suboptimal intake of dietary zinc rapidly impairs cell and antibody-mediated responses in rodents, primates, and humans (1–3). These deleterious effects extended to the developing immune system of young rodents and primates. Clinicians had noted that infections were more numerous and the duration of the infection more prevalent in both children and adults that were significantly or partially zinc deficient (ZD) (2, 4). Using mice, significant insights into the link between zinc status, immune status, and mortality were obtained. Moderately zinc-deficient mice were challenged with a dose of the parasite *Trypanosoma cruzi* that was normally subacute for well-fed mice. It resulted in a 20-fold increase blood

levels of the parasite and 80% mortality among the ZD mice vs. no mortalities in the zinc adequate (ZA) controls (5). In children, diarrhea also accompanies suboptimal intake of zinc, which further compromises their health and heightens mortality (6). Attempts to vaccinate such children can fail to generate protective immunity in cases where malnutrition is extensive. The effects of moderate but chronic deficiencies in zinc in the elderly are less clear, but there is evidence that suboptimal intake of zinc exacerbates the already declining potency of the aging immune system (7).

Although deficiencies in dietary zinc clearly represent a significant worldwide human dietary problem, it should be remembered that deficiencies in zinc status accompany many diseases where inadequate nutriture becomes a problem as the disease advances, e.g., AIDS, cancer, GI disorders, renal disorders, and sickle cell anemia (1, 2, 8, 9). However, protein calorie malnutrition (PCM) is also a component of these diseases and is an even more prevalent human dietary deficiency (10). Several investigators have noted that subjects with PCM often have low serum zinc concentrations (11). Moreover, inanition or reduced intake of protein and calories accompany ZD (8, 12). These relationships are of interest since PCM and ZD generate common immunological changes (Table 1). In addition, when PCM and ZD develop rapidly, they both activate the hypothalamus-pituitary-adrenal cortical axis, causing chronic production of glucocorticoids (Gc) (10, 13, 14). Though the significance of this observation was initially dismissed by many, it is now clear that the endogenous Gc triggered by ZD and PCM plays a key role in the changes in immune integrity, as we will discuss. Indeed, we will provide new evidence that Gc-induced apoptosis plays a key role in the lymphopenia and thymic atrophy that accompanies both ZD and PCM. Collectively, the data indicate that certain forms of malnutrition can alter neuroendocrine function and gene expression.

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TABLE 1. Changes in immune parameters created by zinc deficiency and protein/calorie malnutrition among humans and animals

Zinc deficiency	Protein/calorie malnutrition
<ul style="list-style-type: none"> <li>• Lymphopenia</li> <li>• Thymic atrophy</li> <li>• Reduced cell-mediated responses</li> <li>• Reduced antibody mediated responses</li> <li>• Increased rate of infection</li> <li>• Prolonged duration of infection</li> <li>• Chronic elevation of glucocorticoids</li> <li>• Suboptimal intake of calories</li> <li>• Reduced lymphopoiesis</li> <li>• Heightened apoptosis among lymphoid progenitor cells</li> </ul>	<ul style="list-style-type: none"> <li>• Lymphopenia</li> <li>• Thymic atrophy</li> <li>• Reduced cell-mediated responses</li> <li>• Reduced antibody mediated responses</li> <li>• Increased rate of infection</li> <li>• Prolonged duration of infection</li> <li>• Chronic elevation of glucocorticoids</li> <li>• Low serum zinc</li> <li>• Reduced lymphopoiesis?<sup>a</sup></li> <li>• Heightened apoptosis among lymphoid progenitor cells?<sup>a</sup></li> </ul>

<sup>a</sup> Remains to be answered for protein calorie malnutrition.

## QUESTIONS TO BE ADDRESSED

The result of two decades of studies in our lab and others of the zinc-deficient adult mouse raised a number of key questions. Why do deficiencies in zinc and protein calories so quickly disassemble the immune system? Why do these nutritional deficiencies cause the endogenous production of glucocorticoids at chronically elevated concentrations? Do steroids play a role in the lymphopenia and thymic atrophy that are the hallmarks of ZD and PCM? Since glucocorticoids and nutrient deprivation are well known as *in vitro* inducers of apoptosis in thymocytes, does heightened cell death play a role in the loss of young developing precursor cells of the immune system when the diet is suboptimal? Are the changes observed in immune status during the course of zinc deficiency just a chaotic dismantling of the immune system or are there some positive adaptive changes made in immune defense that are designed to provide some form of fail-safe immune defense as zinc deficiency progresses?

## CHANGES IN LYMPHOPOIESIS CREATED BY ZINC DEFICIENCY

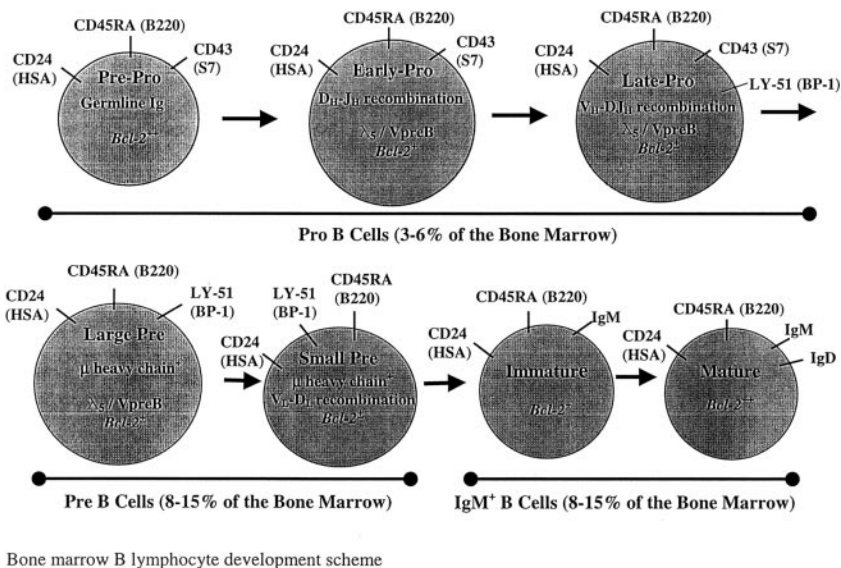
As discussed, clinicians noted several decades ago that lymphopenia and thymic atrophy accompanied PCM and ZD in children and adults (1, 2, 10). From our studies, it was evident that reduced numbers of lymphocytes rather than impaired function of surviving lymphocytes played a key role in loss of antibody and cell-mediated defense (15). Thus, it seemed clear that ZD probably impaired lymphopoiesis or the production of lymphocytes. Production of all cells of the immune system of the adult mammal takes place in the bone marrow, with final maturation of the T cells occurring in the thymus. Examination of the effect of suboptimal nutrition on the marrow was long in coming, no doubt because of the difficulties in studying this complex and highly heterogeneous tissue. Phenotypic markers to identify the progenitor cells of the various cell lineages that develop in the marrow have provided the tools

needed to answer many of the questions raised above by using multiparameter flow cytometry (FACS) (16, 17).

A typical 30 day ZD dietary study used young adult mice. Restricted fed (RZA) controls were included because initiation or protein calorie deficiencies accompany ZD as it advances (12). There were clear differences in the coats of the deficient mice, some exhibiting the *Acrodermatitis* on their tails and ears that is characteristic of ZD (1, 8). These differences along with variances in thymic weights and other immune parameters allowed us to divide the ZD mice into moderately zinc-deficient (MZD) and severely zinc-deficient (SZD) categories. This represented evidence of the tight correlation between zinc status and immune status and suggests that analysis of immune status may be a better indicator of zinc status than is the zinc content of sera, hair, etc. (1).

In adult mammals, the entire developmental sequence for the production of mature B cells bearing immunoglobulin M (IgM) and IgD takes place in the marrow beginning with the earliest B cell progenitor, the pre-pro-B cell that bears B200 (see Fig. 1). The array of phenotypic markers shown there provides a system for identifying changes in distribution, cell cycle status, maturation, etc., within the B cell compartment by using FACS. Note that the late pro- and pre-B cells must rearrange their Ig genes to produce a viable antibody molecule, a process fraught with an 80–90% mistake rate (18). Because of this flawed process, it is not surprising that pre-B cells are vulnerable to spontaneous apoptotic death (18). Indeed, they (along with late pro-B cells) have low levels of the anti-apoptotic proto-oncogene Bcl-2 and, thus, are subject to cell death (19).

Being mindful of this phenotypic marker scheme, multicolor FACS was performed using the marker proteins presented in Fig. 1 to ascertain how ZD might affect lymphopoiesis in the marrow of the young adult mouse (20, 21). The results are summarized in Fig. 2. Note the significant losses in overall proportion of cells of the B lineage within the marrow of MZD and SZD mice (total B220<sup>+</sup>). This represents an absolute loss since the number of nucleated cells in the marrow did not change during 30 days of ZD (20, 21). A 50–70%



**Figure 1.** Phenotypic marker array for cells of the B lineage in the marrow of young adult mice. Stage of rearrangement of Ig genes is outlined as is the distribution of the anti-apoptotic proto-oncogene *bcl-2*.

loss of pre-B cells was noted depending on the degree of deficiency. Substantial losses were noted in the immature IgM-bearing B cells, but mature IgD-bearing B cells survived reasonably well in MZD mice. Surprisingly, some of the earliest of B cells, the pro-B cells, also survived. To summarize, the very early and mature cells of the B lineage described in Fig. 1 survived ZD, with heavy losses noted in the middle of the developmental scheme.

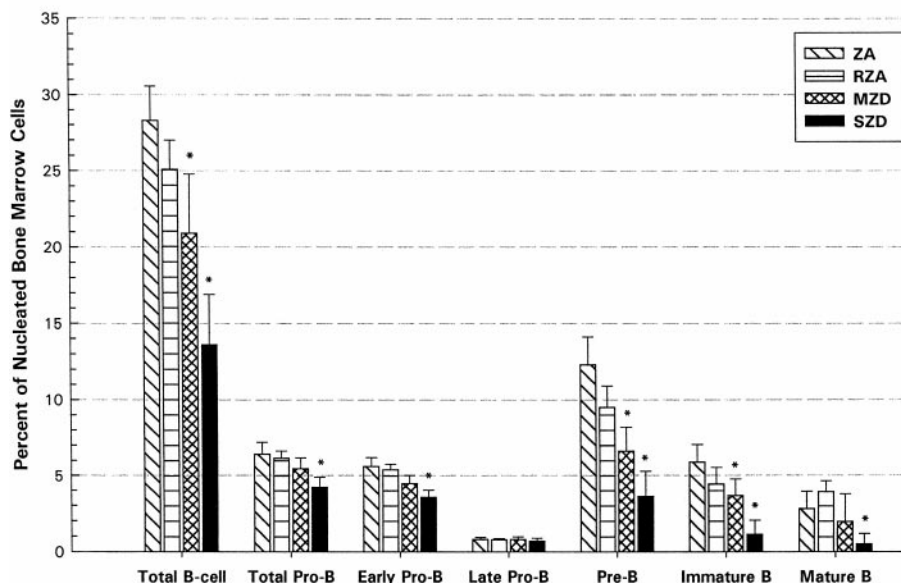
As far as we can determine, the above studies were the first series of studies to show that a dietary deficiency—namely, ZD—greatly reduced the B cell compartment of the marrow, eliminating to a large degree those cells with modest expression of *bcl-2* (19). Clearly, ZD altered lymphopoiesis in a way that must lead to lymphopenia. Since the survival of B cells during ZD tended to correlate with higher expression of the anti-apoptotic protein *bcl-2*, it seemed highly

probable that losses of pre-B cells, etc., were due to heightened apoptosis created by ZD and/or the accompanying production of glucocorticoids (19). Earlier work from our lab had shown that the concentrations of glucocorticoids generated in ZD mice were indeed sufficient to induce substantial apoptosis in pre-B cells both in vivo and in vitro (22, 23). Gc-induced cell death in thymocytes represents one of the best-characterized cell death pathways (24).

## THE ROLE OF APOPTOSIS IN LYMPHOPENIA

It seemed likely that many changes associated with nutritional deficiencies, e.g., altered development, reduced growth, wasting, poor reproductive capacity, etc., would be exacerbated by accelerated apoptosis among

### Bone Marrow B-Cell Population Composition

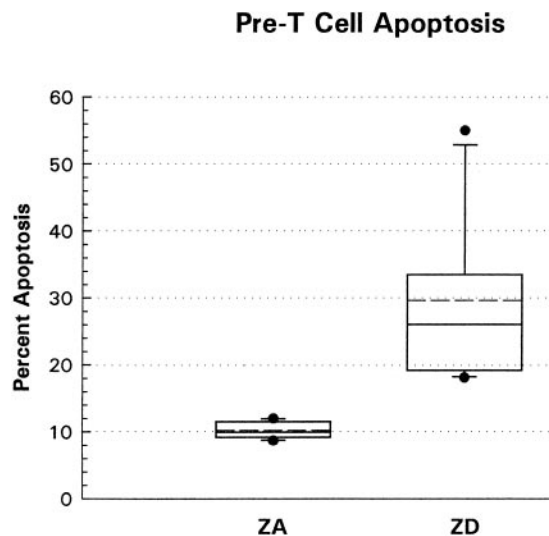


**Figure 2.** Effect of ZD on the phenotypic distribution of cells of the B lineage after a 30 day dietary study using the markers shown here to define the effect of zinc deficiency on the earliest progenitor B cells through mature IgM<sup>+</sup>IgD<sup>+</sup>-bearing B cells. This is representative of three experiments, where  $n = 6$  to 9 mice for each dietary treatment group, including zinc adequate (ZA) fed and restricted fed mice (RZA) that serve as a control for the inanition that accompanies the deficiency: marginally zinc-deficient (MZD) and severely zinc-deficient (SZD) mice. Data shown are mean  $\pm$  SD, where \* denotes data significantly different from the ZA group at  $P < 0.05$  or greater.

young developing cells and tissues. It also seemed highly probable that losses in the B cell compartment in the marrow of ZD mice was driven by accelerated apoptosis. However, this would be exceedingly difficult to prove *in vivo*, since phagocytic cells rapidly remove apoptotic cells at early stages of cell death due to the many 'eat me' markers that appear on the membrane of an apoptotic cell (25). In addition, some of the subsets of B cells represent only a small percent of the marrow, which increases the difficulty in studying them, especially since ZD further reduces their numbers. Thus, we decided to use the thymus for this study because it consists of 85% pre-T cells that are vulnerable to apoptosis (24). We also decided to harvest thymuses part way through a ZD study when the thymuses would be only partially atrophied, so that substantial degrees of apoptosis should still be occurring. To avoid the work of zealous macrophages, we removed the thymuses from both ZD and adequately fed (i.e., ZA) mice, processed them into single-cell suspensions, and cultured them for 8 h away from macrophages. It was our hope that at the time of harvest, more death signals would have been sent to thymocytes in the ZD mice than to thymocytes in the adequately fed mice, where only spontaneous cell death should be occurring (~5–12%) (18, 22).

This approach was successful in demonstrating that ZD accelerates apoptosis in pre-T and B cells. The effect of ZD on the phenotypic distribution of T cells in the thymus paralleled that of the B cell compartment of the marrow. The earliest progenitor, the pro-T cell ( $CD4^-$ ,  $CD8^-$ ), survived as did the mature helper T cell ( $CD4^+CD8^-$ ) and cytolytic T cells ( $CD4^+CD8^+$ ). The thymus weights were 34 mg for ZA mice and 12.6 mg for ZD mice. Thus, the thymuses of the ZD mice had atrophied, being one-third the normal size; their overall losses in cellularity were even more extensive: only  $1.3 \times 10^7$  nucleated cells per thymus, or ~20% of normal. The greatest loss in cellularity was among the pre-T cells or  $CD4^+CD8^+$  thymocytes, which (coincidentally) also have little *bcl-2*, being in the midst of rearranging the T cell receptor (19). They are analogous to the pre-B cells already discussed. In the ZA mice, this double-positive cell represented 83% of all cells and only 52% of the surviving cells for ZD mice. So large cell losses were also accompanied by considerable change in the phenotypic distribution of developing T cells, paralleling findings for cells of the B lineage.

Collectively, this suggested that ZD had probably heightened cell death in pre-T and B cell populations. This indeed was the case. As can be seen in **Fig. 3**, apoptotic death was heightened threefold in  $CD4^+CD8^+$  thymocytes of the ZD mice. The spontaneous death for thymocytes was ~10%, a characteristic level of death due to the ongoing demise of nonsense and anti-self clones (18). The data in **Fig. 3** are representative of three different experiments. No acceleration of cell death occurred in the mature T cell subsets that express more *bcl-2*, and very little enhanced death occurred among pro-T cells from the ZD mice (data



**Figure 3.** The degree of apoptosis found in pre-T cells ( $CD4^+CD8^+$ ) as determined by quantitating cells appearing in the hypodiploid region of the cell cycle using FACS analysis. Thymuses were removed from adequately fed (ZA) and zinc-deficient mice (ZD). The thymuses were processed into single-cell suspensions and maintained in culture for 6 h before being evaluated for number of apoptotic events in the pre-T cell population. Six to eight mice were evaluated from each dietary groups, with the distribution for individual data points shown. The data are representative of three experiments where mean  $\pm$  SD are shown.

not shown). Thus, it is clear that as ZD advances, it accelerates cell death among pre-T and probably pre-B cells. If apoptosis is accelerated threefold or more during ZD, then cell losses will over time be far greater in the deficient mice vs. adequately fed mice. It would in fact explain the thymic atrophy, high losses in numbers of precursor lymphocytes and lymphopenia observed for ZD (1–3). This accelerated apoptosis clearly is an underlying cause of lymphopenia and reduced host defense in ZD.

### WHY IS APOPTOSIS HEIGHTENED IN ZINC-DEFICIENT MICE?

Zinc is critical to the function of many enzymes and > 100 transcription factors, various hormone receptors, etc., that contain zinc fingers (26). Serum and nutrient deprivation in culture can initiate apoptosis (24). It would thus stand to reason that inadequate intake of zinc could alter lymphopoietic processes and/or enhance apoptosis among precursor cells *in vivo* as well. As discussed, we had already shown that concentrations of glucocorticoids analogous to those generated during ZD and PCM cause thymic atrophy and induce apoptosis in pre-T and pre-B cells both *in vivo* and *in vitro* (22, 23). This suggested a potential role for endogenously produced Gc in the accelerated apoptosis among precursor lymphocytes in the ZD mouse. Perhaps the most compelling evidence for the role of steroids comes from earlier experiments where adrenalectomies or

TABLE 2. Adrenalectomies and removal of production of corticosterone protected primary immune tissues in the zinc-deficient mouse

	ZA sham <sup>a</sup> (+CS)	MZD sham <sup>a</sup> (+CS)	MZD adrenalectomy (-CS)	ZA adrenalectomy (-CS)
Thymus wt (mg)	100% <sup>b</sup> (22.1)	71%	109%	124%
Early B cells (% BM)	100% <sup>c</sup> (23)	43%	134%	117%
Plasma CS (μg/dl)	100% (6.6)	413%	42%	62%
Plasma Zn (μg/dl)	100% (95.2)	72%	77%	98%

<sup>a</sup> Mice were sham operated, leaving behind the adrenal gland. Corticosterone (CS) was found to be elevated in these mice. <sup>b</sup> See refs 13, 14. <sup>c</sup> See ref 27.

removal of Gc from the equation provided substantial protection to the marrow and thymus, greatly reducing the effects of the deficiency (14, 27). A summary of those results is provided in **Table 2**. Adrenalectomies also protected the thymus from atrophy induced by the PCM rodents (10).

Collectively, these results suggested that the real underlying cause of heightened apoptosis among precursor cells is the endogenous, chronic production of Gc treated by ZD (13, 14, 27). This would seem to further suggest that suboptimal zinc induces the stress axis (1). Thus, the predominant cause of apoptosis among developing lymphocytes during ZD may be glucocorticoids. A series of experiments using glucocorticoid antagonists are planned in order to further define the role of these steroids in apoptotic events in the ZD mouse. Nevertheless, zinc is important to development, differentiation, and proliferation, all of which are required for lymphopoietic processes (26). Thus, it would seem there may be some role for suboptimal zinc in heightening apoptosis among precursor cells besides initiating production of glucocorticoid. Thus, it is conceivable there may be some synergy between suboptimal zinc and intensity of the death response of cells to GC.

## MYELOPOIESIS

The FACS scatter profiles from previous studies revealed an increase in the proportion of larger, somewhat more granular, cells in the marrow of mice as ZD advanced (21). A generic marker for granulocytes (Mac-1) (CD11b) suggested an increase in this population within the marrow of the ZD mice. Using a series of markers that included ER-12 (CD-31) and ER-20 (CD-

59) developed in The Netherlands, the marrow was divided into a series of compartments including granulocytes and monocytes (17). This data was verified using Gr-1 (CD-97) and Mac-1 to determine the fidelity of the granulocytic compartment and identify the monocytic compartment. Before examining these data, it is important to reiterate that nearly five different studies have suggested that ZD in the marrow does not alter the number of nucleated cells within the marrow; rather, it fostered a profound change in the composition of the marrow (20, 21). From **Table 3** it is apparent that ZD substantially increased both the number and proportion of granulocytes in the marrow by 38 to 60%. Moreover, the number and proportion of monocytes increased 75–80% depending on the degree of the deficiency. Though not shown here, the peripheral blood reflects these changes, with the proportion of lymphocytes declining and the proportion of neutrophils increasing substantially (unpublished data). Additional analyses are needed to verify these changes in the blood and to determine whether there are similar increases in the monocytic population.

## ADAPTIVE CHANGES OF THE IMMUNE SYSTEM TO SUBOPTIMAL ZINC

Why would myeloid cells such as granulocytes and monocytes not only survive ZD, but increase in numbers. Neutrophils have short half-lives, high levels of bax, and are prone to die in the peripheral tissues (25). Why, then, would their progenitors survive in the marrow? Our hypothesis is that ZD orchestrates defined changes in the immune system as it advances. These might be analogous to the metabolic changes that accompany individuals as they move from the well-fed

TABLE 3. Phagocytic cells of the myeloid series survived zinc deficiency becoming a greater proportion of the nucleated cells of the marrow

Cell type	Dietary group			
	Zinc adequate	Restricted fed	Marginally zinc deficient	Severely zinc deficient
Granulocytes (% increase)	39 ± 2.1% <sup>a</sup> (-)	39 ± 3.6% (0%) <sup>b</sup>	54 ± 8.4% (+38%) <sup>a</sup>	62 ± 5.9% (+60%) <sup>a</sup>
Monocytes (% increase)	4.8 ± 0.8% (-)	4.5 ± 0.7% (-9%) <sup>a</sup>	8.4 ± 1.4% (+75%) <sup>a</sup>	8.6 ± 2.1% (+79%) <sup>a</sup>

<sup>a</sup> Percentage (or proportion) of the nucleated cells of the marrow each cell population represents. <sup>b</sup> Relative increase in cell lineage in marrow vs. control or zinc-adequate mice.

to starved state. As zinc becomes limiting, the first line of defense that provides primary or innate immunity may be protected. To conserve nutrients, perhaps the second line of defense (the lymphocytic branch) is down-regulated. Though important to cell and antibody-mediated responses, only a small fraction of lymphocytes are ever actually engaged in antibody-mediated responses to pathogens, etc. Moreover, in the adult animal, memory cells that are much less expensive to maintain can provide defense for a variety of routine infections. The high turnover of unprimed lymphocytes along with the greater than 80% loss of pre-B and pre-T cells due to the generation of nonsense clones makes this system extremely expensive to maintain as nutrients become limiting. Moreover, past data indicate that residual lymphocytes in ZD mice retain their functions and may even be somewhat more potent than regular lymphocytes (15). All this suggests there may be more adaptations of a positive nature by the immune system to malnutrition than once thought.

With regard to apoptosis and survival of myeloid cells, it is valuable to revisit the data available for nearly two decades. It is well known from the Dexter-Witte culture systems that the addition of small amounts of cortisone to primary bone marrow cultures promotes the development of myeloid cells, gradually eliminating lymphocytic cells (28). In an odd way, ZD and perhaps PCM, where glucocorticoids are present, may reaffirm the finding by Dexter-Witte in a natural stress or real-life setting.

### INTERVENTION: THE FUTURE

The seminal cause for the loss of host defense capacity has been identified, namely, that zinc causes a reduction in lymphopoiesis or production of lymphocytes in the marrow and thymus due, at least in part, to accelerated apoptosis. Thus, it is important to begin to try to identify useful interventions. Factors such as interleukin 3 (IL-3) and IL-7 are key to the development of T and B cells (29). It is possible that the production of these factors by stromal cells in the marrow is decreased by ZD. Regardless, provision of these cytokines might help to offset the loss of precursor lymphocytes. Indeed, IL-7 alone provided pre-B cells with substantial protection against glucocorticoid-induced death (T. Laakko, unpublished results). It will also be of interest to determine whether the antagonist of glucocorticoids such as RU38486 can protect lymphopoietic processes as ZD advances (22). Given our growing understanding of the changes ZD can make in neuroendocrine function and the fact that adrenalectomies provide substantial protection to the primary immune tissues by neutralizing the role of glucocorticoids, drugs that block the synthesis of these steroids or their ability to bind to their cytosolic receptor might help stabilize the immune system when malnutrition is present. Combinations of immunotherapy, drug intervention, and zinc supplementation are likely to reduce the lymphopenia

and disrupted host defense created by inadequate zinc nutrition whether caused by diet or disease states. Because of the parallels in the effects of ZD and PCM on immune parameters (Table 1), these same interventions might also offset the effects of malnutrition and wasting associated with disease states as discussed earlier. Unfortunately, substantial numbers of children throughout the world have impaired immune development due to malnutrition. Taken together, these conditions affect large numbers of people, making the magnitude of the problem readily apparent. It is important, therefore, to continue to define how different forms of malnutrition affect various facets of the immune system. It is also time to begin to make a more rigorous effort to offset some of the outcomes by appropriate interventions.

### OVERVIEW

Deficiencies in zinc are tightly linked to the immune system, causing rapid loss in cell and antibody-mediated responses (1–3). The frequency of dietary zinc deficiency along with a variety of disease states where zinc status is suboptimal makes this a significant form of malnutrition (1, 8). The finding that suboptimal zinc status substantially reduces the proportion of pre-B and pre-T cells explains the thymic atrophy and lymphopenia known to be a substantial factor in the loss of immune defense. Heightened apoptosis in these populations caused by suboptimal zinc and associated production of glucocorticoid offers a mechanism to reduce lymphopoietic precursor cells. The perpetuation of the production of phagocytic cells and their survival in the periphery as ZD advanced suggest that changes in immune status during zinc deficiency may be a highly organized process. Identification of the causes of the lymphopenia associated with ZD and PCM opens the way for interventions that may reduce the impact of some forms of malnutrition on the immune system. It may be that those cytokines that promote T and B cell development—better nutritional management and reduction in the production of glucocorticoids—will offset the adverse changes in host defense created by ZD and other types of deficiencies. **FJ**

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