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Programmed cell death (apoptosis) in lymphoid and myeloid cell lines during zinc deficiency

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SUMMARY

Three human cell lines of lymphoid (Molt-3 and Raji) or myeloid (HL-60) origin were maintained *in vitro* under zinc-sufficient or zinc-deficient conditions. Under these conditions, cell proliferation, viability and mode of death (apoptotic or necrotic) were assessed. All three cell types decreased their proliferative capacity and viability under conditions of zinc deficiency. Cell death in the HL-60 and Raji cultures occurred primarily via apoptosis, while most cells in zinc-deficient Molt-3 cultures died via necrosis. Apoptosis in zinc-deficient cultures of HL-60 and Raji cells was characterized by a slow decline in culture viability as cells with condensed and fragmented nuclear DNA appeared. These morphological changes were accompanied by an increase in cell buoyant density, which allowed separation of viable apoptotic cells from their non-apoptotic counterparts by means of percoll step-density gradients. Necrosis in zinc-deficient Molt-3 cultures was characterized by rapid loss of cell culture viability as these cells underwent direct lysis. Intact necrotic cells were easily identified by the flocculated state of their chromatin as well as the decreased basophilia of their cytoplasm. Analysis of DNA from apoptotic HL-60 and Raji cells revealed that internucleosomal DNA degradation, indicative of endogenous endonuclease activation, had occurred, whereas the nuclear DNA of necrotic Molt-3 cells remained relatively unfragmented. The different modes of cell death evoked may reflect the relative sensitivities of cells of these lineages to zinc levels *in vivo*.

Keywords Zinc deficiency apoptosis necrosis

INTRODUCTION

Clinical and experimental observations have highlighted the importance of zinc status in maintaining immunological integrity (Fraker, Haas & Luecke, 1977; Fernandes *et al.*, 1979; Chandra, 1980; Cossack, 1989; Moulder & Steward, 1989). Zinc deficiency affects both cell-mediated and antibody-mediated adaptive immune responses (Malave & Benaim, 1984; Mathe *et al.*, 1985), natural killer (NK) cell function, and macrophage phagocytosis and killing (Winchurch, 1988). An increased susceptibility to a variety of diseases, including those of an infectious aetiology, has also been reported (Oleske *et al.*, 1979). The principle cell type thought to be down-regulated by zinc deficiency, at least in animal models, is the helper subpopulation of T lymphocytes. Other T cell types and B cells show relative resistance to such effects (Fraker *et al.*, 1978; Zanzonico, Fernandes & Good, 1981). Secretion of interleukins-1, -2 and -4 has been seen to be compromised and down-regulation of IL-2 receptor expression has been reported (Dowd, Kelleher & Guillou, 1986). Such observations are supported by the finding of thymic involution (Fraker *et al.*, 1977; Fernandes *et al.*, 1979; Chandra, 1980) and decreased thymic DNA content (Ku, Ullrey & Miller, 1970) in zinc-deficient animals. Zinc deficiency in humans may result from inadequate dietary intake, dietary factors which limit bio-availability, defective zinc transport across brush borders and competitive interactions between zinc and other elements (Hambridge, 1981).

The potential role of zinc deficiency in promoting cell death has not yet been elucidated. Cell death can occur as a consequence of pathological processes, or may be a more normal physiological event (Kerr, Wyllie & Currie, 1972; Wyllie, Kerr & Currie, 1980). These two distinct modes of cell death are known as necrosis and apoptosis (programmed cell death), respectively, and have been shown to be morphologically and biochemically distinct (Wyllie *et al.*, 1980; Wyllie, Duvall & Blow, 1981). A particularly unique characteristic of apoptosis is the early activation of an endogenous endonuclease which effects internucleosomal DNA cleavage, demonstrable as a 'ladder pattern' when apoptotic cell DNA is separated on agarose gels (Wyllie, 1980; Duke, Chervenak & Cohen, 1983; Cohen & Duke, 1984; Yamada & Ohyama, 1988; Cotter &

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Martin, 1989). In rodent thymocytes this endonuclease has been shown to require magnesium and calcium for its activation and to be strongly inhibited by zinc ions (Duke *et al.*, 1983; Cohen & Duke, 1984; Sellins & Cohens, 1987).

Here, we investigated the effects of zinc deficiency and repletion on cell proliferation, viability and mode of cell death in two human cell lines of lymphoblastoid origin (Molt-3 and Raji, derived from T and B cells, respectively) and one of myeloid origin (HL-60).

MATERIALS AND METHODS

Cell culture

HL-60 cells (Collins, Gallo & Gallagher, 1977), Molt-3 (Minowada, Ohnuma & Moore, 1972) and Raji (Epstein & Barr, 1964) were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (FCS). Cells were maintained at 37°C in a 5% CO_2/95% air incubator and were passaged every 2 days. In all experiments cells were used during the exponential phase of growth and were seeded at an initial concentration of 5×10^5 cells/ml. The zinc content of the growth media was varied to yield zinc-deficient or zinc-repleted conditions as described below.

Preparation of zinc-deficient medium

The zinc content of the culture media was decreased by overnight dialysis at 4°C of FCS against 0·1 M pyridine-2,6-dicarboxylic acid (DPA) in 0·01 M Tris–HCl, pH 7·0, prepared according to the method of Hunt, Rhee & Storm (1977). To ensure removal of DPA from dialysed FCS, the serum was further dialysed for 12 h at 4°C against three changes of 0·1 M Tris–HCl buffer, pH 7·5. Atomic absorption spectroscopy was then used to determine the concentrations of Zn, Cu, Fe, Ca and Mg in all media. Zinc repletion of media was achieved by the addition of $ZnCl_2$ to a final concentration of 50 μM, close to the level of zinc in the undialysed control.

Cell viability was determined using both the trypan blue exclusion test and acridine orange/ethidium bromide staining. Cell morphology was evaluated on Rapi-Diff II stained (Paramount Reagents, Merseyside, UK) cytocentrifuge cell preparations.

Isolation of apoptotic cells

Apoptotic cells were purified to near homogeneity (>90%) using percoll step density gradients. Briefly, gradients were constructed by sequential layering of iso-osmotic percoll solutions of densities 1·08 g/ml (1 ml), 1·075 g/ml (2 ml) and 1·07 g/ml (2 ml) into a conical-bottomed centrifuge tube. Cells ($>10^7$) were washed twice in PBS, resuspended in 2 ml of 1·06 g/ml percoll solution and were then layered on top of the gradient. The separation was carried out by centrifugation of the gradient in a swing-out rotor at 400 *g* for 30 min. Apoptotic cells pelleted to the bottom of the centrifuge tube leaving the remaining cell population at the interface between the 1·07 g/ml and 1·075 g/ml layers. Apoptotic cells in the pellets were 90–95% trypan blue negative.

Isolation of DNA

DNA was isolated from apoptotic and non-apoptic cells using a procedure slightly modified from one previously described (Martin *et al.*, 1990). Briefly, cells were washed twice with PBS and spun at 200 *g* for 5 min at room temperature. Cell pellets were resuspended at 2×10^7 cells/ml in cell lysis buffer (10 mM EDTA, 50 mM Tris, pH 8, containing 0·5% (w/v) *N*-lauroylsarcosine and 0·5 mg/ml proteinase K) and incubated for 1 h in a 50°C water-bath. RNAse A (previously heat-treated to remove contaminating deoxyribonuclease activity) was then added to a concentration of 0·25 mg/ml and incubation at 50°C was continued for 1 h. The crude DNA preparations were then extracted (twice) with phenol, buffered with 0·1 M Tris–HCl (pH 7·4) to remove proteinaceous material, followed by two chloroform/isoamyl alcohol (24:1) extractions for removal of residual traces of phenol. DNA preparations were then brought to 2·5 volumes by the addition of 10 mM Tris–HCl, pH 8, 1 mM EDTA (TE buffer), and were centrifuged at 13 000 *g* to separate intact from fragmented chromatin. The supernatants contained fragmented DNA. DNA in both pellet and supernatant was precipitated overnight in two volumes of ethanol at −70°C and were recovered by centrifugation at 13 000 *g* for 10 min, air-dried for 5 min at room temperature, resuspended in TE buffer and stored at 4°C. DNA concentration was calculated by determining the optical density (OD) at 260 nm.

Electrophoresis of DNA

Electrophoresis of the fragmented DNA contained in the 13 000 *g* supernatants was carried out in 1% agarose gels. Prior to electrophoresis, loading buffer (10 mM EDTA, 0·25% (w/v) bromophenol blue and 50% (v/v) glycerol) was added to each sample in a 1:5 ratio. The samples were then heated to 65°C for 10 min in a water-bath and were then plunged into ice. Approximately 10 μg of DNA was loaded into each well and electrophoresis was carried out at 6 V per cm of gel in TBE buffer (2 mM EDTA, pH 8, 89 mM Tris, 89 mM boric acid). An *Hin*dIII digest of λ-DNA was applied to each gel to provide size markers of 23·5, 9·6, 6·6, 4·3, 2·2, 2·1 and 0·5 kbp, respectively. Following electrophoresis, DNA was visualized by soaking the gel in TBE containing 1 μg/ml ethidium bromide and destaining briefly in this buffer.

RESULTS

Cell proliferation and viability in zinc-deficient medium

Cell culture media were successfully rendered zinc deficient, with only minor changes in the levels of other divalent metals, as measured by atomic absorption spectrophotometry (Table 1).

Culturing of HL-60, Molt-3 and Raji cells under zinc-deficient conditions resulted in an almost complete loss of proliferative capacity, which was most pronounced in Molt-3 cultures, within a single cell cycle. Repletion of zinc to 50 μM in parallel cultures maintained proliferative capacity to within 90–95% of the control levels in Molt-3 cultures, and to a lesser but significant degree in HL-60 and Raji cultures (Fig. 1).

Viabilities of the three cell lines under zinc-deficient conditions closely paralled the effect of this ion on cell proliferation.

Table 1. Culture medium trace metal concentrations (parts/million)

	Zn	Cu	Ca	Fe	Mg
Control	0·47	0·06	23·9	0·46	11·8
Zinc deficient	0·09	0·04	18·1	0·36	8·8

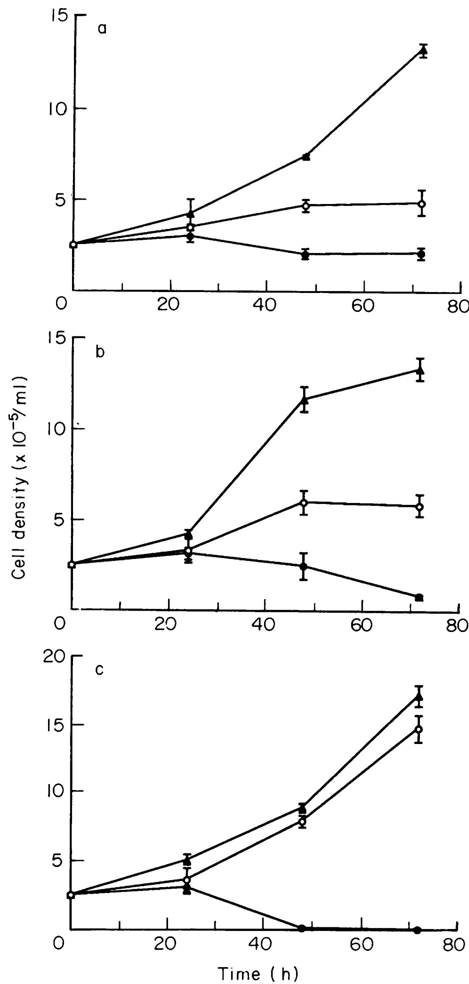

Fig. 1. Proliferative response of (a) HL-60; (b) Raji; and (c) Molt-3 cells maintained *in vitro* under conditions of zinc deficiency (●), zinc repletion to 50 μM (○), and control (▲). Cells were seeded at an initial cell concentration of 5×10^5/ml into RPMI 1640 medium containing 10% FCS, which was zinc depleted and repleted as described in Materials and Methods. Each data-point represents the mean (±s.e.m.) of quadruplicate determinations, from a representative experiment.

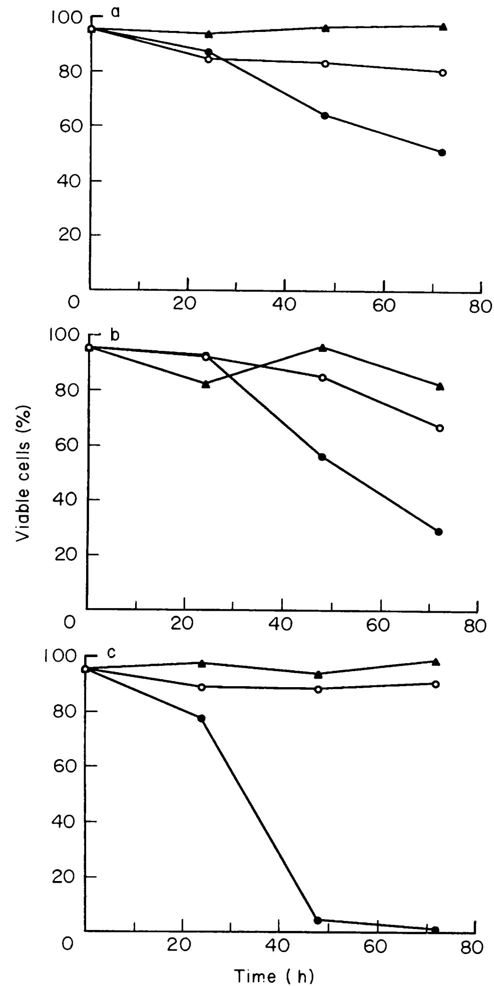

Fig. 2. Viability, as assessed by trypan blue exclusion of (a) HL-60; (b) Raji; and (c) Molt-3 cells maintained *in vitro* under conditions of zinc deficiency (●), zinc repletion to 50 μM (○), and control (▲). Cells were seeded at an initial cell concentration of 5×10^5/ml into RPMI 1640 medium containing 10% FCS, which was zinc depleted and repleted as described in Materials and Methods. Each data-point represents the mean of quadruplicate determinations from a representative experiment. All values deviated from the mean by no more than 5%.

Both HL-60 and Raji cell cultures had substantial losses in cell viability within 48 h in zinc-deficient medium while repletion of zinc to 50 μM in parallel cultures maintained cell viabilities to within 10% of control levels (Fig. 2 a, b). The effect of zinc deficiency in Molt-3 cultures was more dramatic, with an almost total loss of cell viability within 48 h. Parallel cultures revealed that re-addition of 50 μm of zinc could replenish cell viability almost completely (Fig. 2c). Thus, Molt-3 cells were the most sensitive to fluctuation of zinc levels, while the HL-60 and Raji cell lines exhibited a more measured, dose-dependent response.

Mode of cell death in zinc-deficient medium

The different responses of the HL-60 and Raji cell lines and the Molt-3 cell line to zinc deficiency suggested that different modes of cell death were being triggered. With this in mind, cytospun preparations of HL-60, Raji and Molt-3 cells were examined for cells exhibiting apoptotic or necrotic morphological features. Previous studies (Kerr *et al.*, 1972; Wyllie *et al.*, 1980), including studies carried out in this laboratory (Martin *et al.*, 1990), have described the morphological characteristics of apoptosis and necrosis. The most striking feature of an apoptotic cell is its compact nuclear chromatin along with fragmentation of the nucleus into many discrete masses dispersed throughout the cell cytoplasm. Necrotic cells, on the other hand, do not undergo nuclear fragmentation or condensation; instead, there is marked cell and nuclear swelling with flocculation of the chromatin and a decreased cytoplasmic basophilia. These cells rapidly burst open and disgorge their contents, while apoptotic cells maintain membrane integrity for many hours even after separation of the cell into many apoptotic bodies (Wyllie *et al.*, 1981; and our own unpublished observations).

Analysis of the morphological features of HL-60, Raji and Molt-3 cells under zinc deficient conditions revealed significant increases in the proportion of cells with the morphological features of apoptosis in all three cell lines after 24 h of culture (Fig. 3). These increases were particularly marked in Raji and

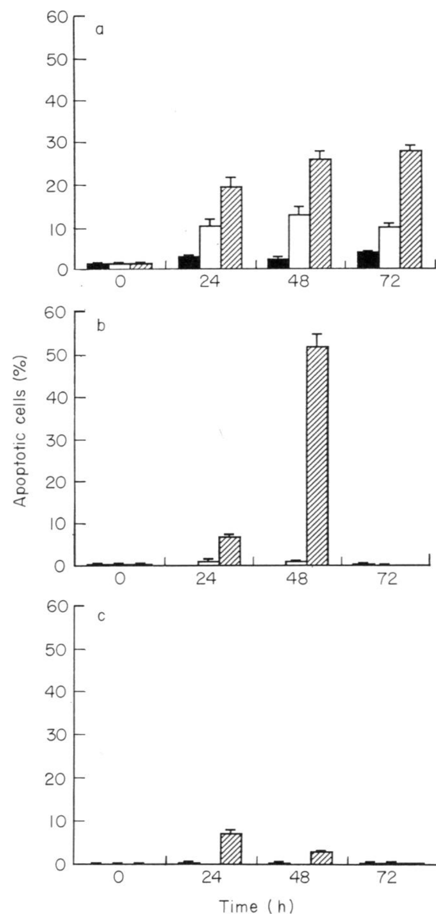

Fig. 3. Time course of appearance of cells with the morphological characteristics of apoptosis in cultures of (a) HL-60; (b) Raji; and (c) Molt-3 cells maintained *in vitro* under conditions of zinc deficiency (■), zinc repletion to 50 μM (□), and control (■). Morphological evaluation of cells at the indicated time-points was performed on stained cytocentrifuge preparations (see Materials and Methods). Results are expressed as the mean ($\pm$s.e.m.) percentages of the total number of cells counted (>500 per slide) from five randomly selected fields.

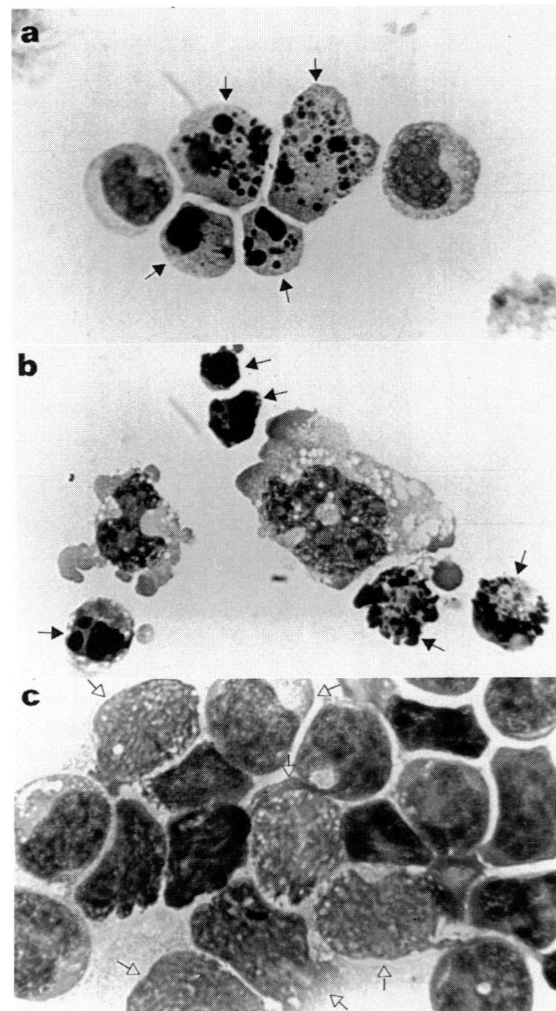

Fig. 4. Morphological appearance of (a) HL-60; (b) Raji; and (c) Molt-3 cells, cultured for 48 h under zinc-deficient conditions and illustrating apoptotic (solid arrows) and necrotic (blank arrows) cells. Note the condensed and fragmented state of the nuclear chromatin within apoptotic cells while necrotic cells display nuclear chromatin flocculation and plasma membrane disruption. Magnification ×2200.

HL-60 cell cultures, while only small increases over control levels were found in Molt-3 cultures. The proportion of apoptotic cells in the Raji and HL-60 cultures continued to rise over the next 48 h, although in the Raji cell cultures estimation of the levels of apoptosis beyond 48 h was difficult due to cell debris. In Molt-3 cultures only a small increase in the levels of apoptosis was observed (Fig. 3c), while most cells (>90%) exhibited the morphological characteristics of necrosis. Although necrotic cells, due to their membrane fragility, ruptured easily upon cytocentrifugation, intact necrotic cells could be identified by their swollen appearance as well as the flocculated state of their chromatin and decreased basophilia of their cytoplasm (Fig. 3c). These cells were invariably trypan blue positive. Figure 4 illustrates the morphological features of apoptotic cells from zinc-deficient cultures of HL-60 and Raji cells (a and b, respectively); necrotic cells from zinc-deficient Molt-3 cell cultures are illustrated in Fig. 4c.

Parallel cultures growing under zinc-repleted conditions demonstrated that these morphological effects were substantially reduced particularly in Molt-3 and Raji cell cultures (Fig. 3). It is important to emphasize that cultures of zinc-deficient Molt-3 cells did not exhibit any increases in the levels of apoptotic cells observed during the critical period between 24 h and 48 h of zinc deficiency when these cells underwent massive losses in cell viability. Thus the necrosis which was observed was not likely to be the result of secondary necrosis of apoptotic Molt-3 cells. Neither was it likely that these cells were undergoing a morphologically aberrant form of apoptosis, since it has recently been shown that Molt-3 cells adopt the classical features of apoptosis *en masse*, soon after short periods of u.v. irradiation, in common with several other human cell lines (Martin & Cotter, 1991). Apoptosis in Molt-3 cells was characterized by condensation of chromatin, progressive nuclear fragmentation, separation of cells into apoptotic bodies, and was accompanied by DNA cleavage into internucleosomal fragments. Note that while a number of cells in Fig. 4c illustrate the features of necrosis, cells with the normal Molt-3 morphology are also present, while apoptotic cells are completely absent. Interestingly, irradiation of Molt-3 cells for extended periods provoked features identical to those induced in the

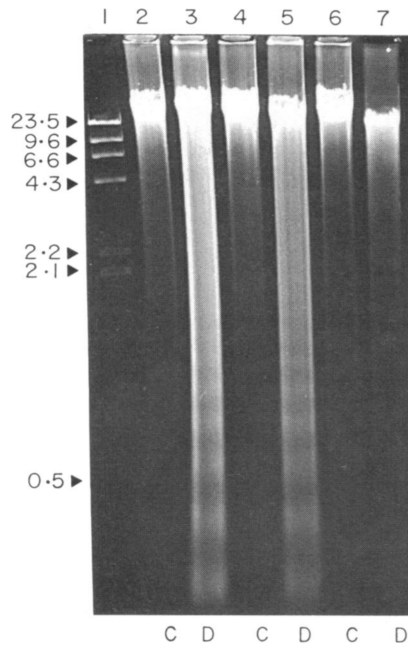

Fig. 5. Electrophoresis in 1% agarose of DNA extracted from the 13 000 *g* supernatants of HL-60 (lanes 2–3); Raji (lanes 4–5); and Molt-3 (lanes 6–7) cells maintained under control (C), or zinc-deficient (D) conditions. DNA was extracted and electrophoresed as described in Materials and Methods and was detected by u.v. fluorescence after ethidium bromide staining. An *Hin*dIII digest of λ-DNA was included to provide molecular size markers, as indicated (lane 1).

majority of cells by zinc deficiency, and which were characteristic of necrosis. Several other human cell lines were also found to behave in this manner (Martin & Cotter, 1991).

Activation an endogenous endonuclease in HL-60 and Raji but not in Molt-3 cells

Apoptotic cells are marked by cleavage of their DNA into fragments which amount to multiples of approximately 200 bp. This inter-nucleosomal DNA cleavage is the biochemical hallmark of apoptosis, affected by an endogenous endonuclease which requires calcium and magnesium ions and is strongly inhibited by zinc ions (Duke *et al.*, 1983; Cohen & Duke, 1984; Sellins & Cohen, 1987). In order to confirm that cells from zinc-deficient cultures of HL-60 and Raji were undergoing classical apoptosis, we isolated DNA from percoll purified populations of apoptotic cells grown in zinc-deficient medium. DNA was also isolated from Molt-3 cells, in order to confirm that the DNA of these cells was not degraded in an inter-nucleosomal pattern. DNA degradation in necrotic cells is a relatively late event which occurs in conjunction with protease attack (released from ruptured lysosomal compartments) on the histones, thus exposing the whole length of the DNA to endonucleases.

Figure 5 illustrates the inter-nucleosomal pattern of DNA degradation, indicative of endogenous endonuclease activation in zinc-deficient apoptotic cells. Fragmentation is seen in both zinc-deficient HL-60 and Raji cells, while DNA from zinc-sufficient cultures remained unfragmented. DNA isolated from necrotic Molt-3 cultures, while partially fragmented, displays little inter-nucleosomal DNA degradation, and DNA from control cultures of these cells remains unfragmented.

DISCUSSION

We have shown that cell death can be induced by zinc deficiency in human cell lines of both lymphoid and myeloid origin. Of these, cells of the Molt-3 T lymphoid cell line were found to be the most sensitive to conditions of zinc deficiency. This finding is in line with the conclusions of Fraker *et al.* (1978) and Zanzonico *et al.* (1981), based on *in vivo* zinc deficiency in animal models, that diminished immune responsiveness was due primarily to impaired T lymphocyte activity. Further, these and other investigators have reported measurable reductions in many aspects of both cell and antibody mediated immunity, consistent with our observation of reduced growth rate and cell viability in Raji (B lymphoid) and HL-60 (myeloid) cultures.

In normal adult human tissues it is probable that a balance exists in many slowly and rapidly proliferating tissues between the processes of apoptosis and mitosis (Lynch, Nawaz & Gershenson, 1986). It has been suggested (Walker *et al.*, 1988) that the massive apoptosis frequently observed in lymphoid germinal centres may be a significant regulator of immune responses. Our data on the mode of cell death would suggest that zinc deficiency can induce both apoptosis and necrosis. These effects were substantially reversed by the re-addition of zinc, directly implicating this metal rather than any other divalent metal as the principal mediator of these responses. The relative frequency of each mode of cell death was found to be particular to the lineage of the cell under study.

It has previously been shown that zinc deficiency in mice causes profound thymic involution as a result of excessive thymocyte apoptosis (Fraker *et al.*, 1977; Fernandes *et al.*, 1979). In other cell populations with high cell turnover, such as the gastrointestinal epithelium, apoptosis is common (Elmes, 1977; Elmes & Gwyn Jones, 1980). Zinc added to cultures could prevent glucocorticoid or gamma-irradiation-induced apoptosis of rodent thymocytes (Cohen & Duke, 1984; Sellins & Cohen, 1987) as well as the apoptosis seen in P815 murine mastocytoma cells after exposure to cytotoxic T lymphocytes *in vitro* (Duke *et al.*, 1983). Further, the addition of micromolar concentrations of zinc, as $ZnCl_2$, also inhibits the apoptosis of HL-60 cells induced by u.v. irradiation (Martin & Cotter, 1991).

The site of action of zinc in inhibiting apoptosis is still unknown. However, the endogenous endonuclease shown to be responsible for the inter-nucleosomal pattern of DNA degradation observed during apoptosis of glucocorticoid-treated rodent thymocytes is known to be strongly inhibited by zinc ions (Duke *et al.*, 1983; Cohen & Duke, 1984; Sellins & Cohen, 1987). It has been postulated that upon receipt of the apoptosis-inducing stimulus, an exchange of zinc for calcium and magnesium within the nucleus may initiate the characteristic chromatin degradation observed. Further, since apoptosis in most systems is dependent on macromolecular synthesis in the dying cell (Wyllie *et al.*, 1984; Cohen & Duke, 1984; Cohen *et al.*, 1985; Sellins & Cohen; 1987; Yamada & Ohyama, 1988), it has also been proposed that one of the proteins synthesized may be involved in a putative zinc-transport system (Wyllie *et al.*, 1981; Duvall & Wyllie, 1986). However, evidence for such a protein is still not forthcoming.

In this study we have found that zinc deficiency induces apoptosis with endogenous endonuclease activation in two of the three cell lines studied. HL-60 cells were found to respond to zinc levels in a dose dependant fashion. The frequency of

apoptosis and the ability to proliferate and maintain viability, all depended upon the availability of zinc in the medium. Raji cells responded in a similar but slightly less dose-related manner: zinc repletion to 50 μM all but abolished apoptosis in these cultures. However, data for Molt-3 cells demonstrated that these cells are even more sensitive to zinc levels. Zinc-deficient conditions provoke a dramatic increase in cell death via necrosis, while zinc repletion totally abolished these negative effect. Molt-3 cells can be induced to undergo classical apoptosis with appropriate stimuli (Martin *et al.*, 1990; Martin & Cotter, 1990). We therefore speculate that zinc deficiency is so highly toxic to these cells that there is insufficient time for an apoptotic response to occur, and the majority of cells have no choice but to undergo necrosis. Since this study was restricted to a single cell line from each category, further work is clearly required to establish whether human T cell lines universally respond to zinc deficiency in this manner.

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REFERENCES

CHANDRA, R. K. (1980) *Immunology of Nutritional Disorders.* Edward Arnold, London.

COHEN, J.J. & DUKE, R.C. (1984) Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. *J. Immunol.* **132,** 38.

COHEN, J.J., DUKE, R.C., CHERVENAK, R., SELLINS, K.S. & OLSON, L.K. (1985) DNA fragmentation in targets of CTL: an example of programmed cell death in the immune system. *Adv. exp. Med. Biol.* **184,** 493.

COLLINS, S.J., GALLO, R.C. & GALLAGHER, R.E. (1977) Continuous growth and differentiation of human myeloid cells in suspension culture. *Nature,* **270,** 347.

COSSACK, Z.T. (1989) T-lymphocyte dysfunction in the elderly associated with zinc deficiency and subnormal nucleoside phosphorylase activity: effect of zinc supplementation. *Eur. J. Cancer clin. Oncol.* **25,** 973.

COTTER, T.G. & MARTIN, S.J. (1989) Apoptosis as a mode of cell death in cultures of differentiating human leukaemia cells. In *Cell Transformation and Radiation-Induced Cancer* (ed. by K. H. Chadwick, C. Seymour & B. Barnhart) p. 25. Adam Hilger, Bristol.

DOWD, P.S., KELLEHER, J. & GUILLOU, P.J. (1986) T-lymphocyte subsets and interleukin-2 production in zinc deficient rats. *Br. J. Nutr.* **55,** 59.

DUKE, R.C., CHERVENAK, R. & COHEN, J.J. (1983) Endogenous endonuclease-induced DNA fragmentation: an early event in cell-mediated cytolysis. *Proc. natl Acad. Sci. USA,* **80,** 6361.

DUVALL, E. & WYLLIE, A.H. (1986) Death and the cell. *Immunol. Today,* **7,** 115.

ELMES, M.E. (1977) Apoptosis in the small intestine of zinc-deficient and fasted rats. *J. Pathol.* **123,** 219.

ELMES, M.E. & GWYN JONES, J. (1980) Ultrastructural studies on Paneth cell apoptosis in zinc-deficient rats. *Cell Tissue Res.* **208,** 57.

EPSTEIN, M.A. & BARR, Y.M. (1964) Cultivation *in vitro* of human lymphocytes from Burkitt's malignant lymphoma. *Lancet,* **i,** 253.

FERNANDES, G., NAIR, M., ONDE, K., TANAKA, T., FLOYD, R. & GOOD, R.A. (1979) Impairment of cell-mediated immunity functions by dietary zinc deficiency in mice. *Proc. natl Acad. Sci. USA,* **76,** 457.

FRAKER, P.J., DE PASQUALE-JARDIEU, P., ZWICKLE, C.M. & LUECKE, R.W. (1978) Regeneration of T-helper function in zinc deficient adult mice. *Proc. natl Acad. Sci. USA,* **75,** 5660.

FRAKER, P.J., HAAS, S.M. & LUECKE, R.W. (1977) Effect of zinc deficiency on the immune response of the young adult A/J mouse. *J. Nutr.* **107,** 1889.

HAMBRIDGE, K.M. (1981) Zinc deficiency in man: its origin and effects. *Philos. Trans. R. Soc. Lond. [Biol.]* **294,** 129.

HUNT, J.B., RHEE, M.J. & STORM, C.B. (1977) A rapid and convenient preparation of carbonic anhydrase. *Anal. Biochem.* **79,** 614.

KERR, J.F.R., WYLLIE, A.H. & CURRIE, A.R. (1972) Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. *Br. J. Cancer,* **26,** 239.

KU, P.K., ULLREY, D.E. & MILLER, E.R. (1970) In *Trace Element Metabolism in Animals* (ed. by C. F. Mills) p. 158. Churchill Livingstone, Edinburgh.

LYNCH, M.P., NAWAZ, S. & GERSHENSON, L.E. (1986) Evidence for soluble factors regulating cell death and cell proliferation in primary cultures of rabbit endometrial cells grown on collagen. *Proc. natl Acad. Sci. USA,* **83,** 4784.

MALAVE, I. & BENAIM, R. (1984) Modulatory effect of zinc on the proliferative response of murine spleen cells to polyclonal T-cell mitogens. *Cell Immunol.* **89,** 322.

MARTIN, S.J. & COTTER, T.G. (1991) Ultraviolet B irradiation of human leukaemia HL-60 cells in vitro induces a suicidal cell response. *Int. J. radiat. Biol.* (In press).

MARTIN, S.J., BRADLEY, J.G. & COTTER, T.G. (1990) HL-60 cells induced to differentiate towards neutrophils subsequently die via apoptosis. *Clin. exp. Immunol.* **79,** 448.

MARTIN, S.J., LENNON, S.V., BONHAM, A.M. & COTTER, T.G. (1990) Induction of apoptosis (programmed cell death) in human leukemic HL-60 cells by inhibition of RNA or protein synthesis. *J. Immunol.* **145,** 1859.

MATHE, G., BLAZSEK, I., GIL-DELGADO, M.A., CANON, C., MISSET, J.L., GAGET, M.H. & REISENSTEIN, P. (1985) The effect of zinc on normal and neoplastic T-lymphocyte proliferation. *J. Med. Oncol. Tum. Pharmacother.* **2,** 203.

MINOWADA, J., OHNUMA, T. & MOORE, G.E. (1972) Rosette-forming human lymphoid cell lines. I. Establishment and evidence for origin of thymus-derived lymphocytes. *JNCI,* **49,** 891.

MOULDER, K. & STEWARD, M.W. (1989) Experimental zinc deficiency: effects on cellular responses and the affinity of humoral antibody. *Clin. exp. Immunol.* **77,** 269.

OLESKE, J.M., WESPHAL, M.L., SHORE, S., GORDON, D., BODGEN, J.D. & NAHAMIAS, A. (1979) Zinc therapy of depressed cellular immunity in acrodermatitis enteropathica: its correction. *Am. J. Dis. Child.* **133,** 915.

SELLINS, K.S. & COHEN, J.J. (1987) Gene induction by γ-irradiation leads to DNA fragmentation in lymphocytes. *J. Immunol.* **139,** 3199.

WALKER, N.I., HARMON, B.V., GOBE, G.C. & KERR, J.F.R. (1988) Patterns of cell death. *Methods Achiev. exp. Pathol.* **13,** 18.

WINCHURCH, R.A. (1988) Activation of thymocyte responses to Interleukin-1 by zinc. *Clin. Immunol. Immunopathol.* **47,** 174.

WYLLIE, A.H. (1980) Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature,* **284,** 555.

WYLLIE, A.H., DUVALL, E. & BLOW, J.J. (1981) Intracellular mechanisms of cell death in normal and pathological tissues. In *Cell Ageing and Cell Death* (ed. by I. Davies & D. C. Sigee) p. 269. Cambridge University Press, Cambridge.

WYLLIE, A.H., KERR, J.F.R. & CURRIE, A.R. (1980) Cell death: the significance of apoptosis. *Int. Rev. Cytol.* **68,** 251.

WYLLIE, A.H., MORRIS, R.G., SMITH, A. L. & DUNLOP, D. (1984) Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. *J. Pathol.* **142,** 67.

YAMADA, T. & OHYAMA, H. (1988) Radiation-induced interphase death of rat thymocytes is internally programmed (apoptosis) *Int. J. Radiat. Biol.* **53,** 65.

ZANZONICO, P., FERNANDES, G. & GOOD, R.A. (1981) The differential sensitivity of T-cell and B-cell mitogenesis to *in vitro* zinc deficiency. *Cell. Immunol.* **60,** 203.