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CHARACTERIZATION OF APOPTOSIS IN THYMOCYTES ISOLATED FROM DEXAMETHASONE-TREATED RATS

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Abstract—The induction of apoptosis by glucocorticoids in isolated thymocytes has been studied extensively. However, it is not known whether or not the same changes occur after *in vivo* glucocorticoid treatment. In order to investigate this, we have studied the changes occurring in thymocytes isolated from rats, from 2–24 hr after a dose of dexamethasone (1 mg/kg), which caused 50% thymic atrophy. Thymocytes were separated into four fractions by isopycnic Percoll gradients. A loss of cells occurred within 2–8 hr, primarily in only one of the two major fractions of normal thymocytes. This loss of normal thymocytes coincided with the appearance of small dense cells with characteristic features of apoptosis including condensed chromatin, increased DNA fragmentation, internucleosomal DNA cleavage and a "hypodiploid" peak on flow cytometric analysis. Striking differences occurred in the cellular composition of the different Percoll fractions with time. Initially (up to 4 hr), the pattern of changes occurring *in vivo* resembled those found *in vitro*. However, at later times, the complex fate of apoptotic cells *in vivo*, such as phagocytosis, are not observed in the *in vitro* studies.

Apoptotic cell death involves the controlled intrinsic disposal of cellular components without cell rupture; it plays a major role in cell turnover and many aspects of pathology [1–4]. This process is involved in both the elimination of autoreactive or unselected immature thymocytes that accompanies T lymphocyte maturation and also in normal physiological regression of the gland [5–8]. Glucocorticoid hormones are thought to induce thymic atrophy *in vivo* through enhancement of apoptosis [9–12]. The morphological and biochemical characteristics of thymocyte apoptosis have been primarily established using cultures of isolated immature rodent thymocytes treated *in vitro* with various glucocorticoids, particularly dexamethasone and methylprednisolone [13–16]. Such studies have shown that, within a few hours, a high proportion of the cells undergo a sharp increase in buoyant density concomitant with a decrease in size. These changes have facilitated separation of cells possessing the characteristic condensed nuclear chromatin associated with apoptosis from apparently normal thymocytes by isopycnic centrifugation on Percoll gradients [14, 17, 18]. Cells in this fraction also demonstrate the widely accepted biochemical characteristic of apoptosis, the internucleosomal fragmentation of DNA, most commonly detected by agarose gel electrophoresis of the post-nuclear supernatant fractions from cell lysates [13, 15, 19, 20]. More recent *in vitro* investigations have identified changes in the DNA binding characteristics of intercalating nuclear dyes in apoptotic cells, which can be demonstrated as a "hypodiploid" fraction by flow cytometry [18, 21, 22]. However, to date only limited studies on dexamethasone-induced thymic apoptosis *in vivo* have been reported, which have examined changes either in DNA fragmentation or in cluster designation (CD†) antigen subset populations [23–25]. The present study was initiated to determine if the cellular changes produced in immature rat thymocytes, following *in vivo* administration of dexamethasone, were similar to those identified previously following treatment of the cells *in vitro*.

MATERIALS AND METHODS

Chemicals and reagents

Dexamethasone, ethidium bromide, Percoll, minimum essential medium Eagle (MEM), RNAse A and proteinase K were purchased from the Sigma Chemical Co. (Poole, U.K.). Ficoll 70, 2206-105 Agarose and density marker beads were obtained from Pharmacia Biosystem (Milton Keynes, U.K.). Foetal calf serum and RMPI-1640 were from Gibco (Paisley, U.K.). Other chemicals were from British Drug Houses (Poole, U.K.).

Animals and treatment

Male Fischer 344 rats (4–5 weeks), bred at the MRC Toxicology Unit, Carshalton, Surrey, were allowed food and water *ad lib*. Dexamethasone was dissolved in absolute ethanol, then diluted with normal saline. The final ethanol concentration was not more than 5%. Dexamethasone (0.1–5 mg/kg) was injected intraperitoneally and animals killed from 2 to 24 hr after treatment. Control rats were dosed with solvent alone.

Thymocyte preparation

Thymuses were removed at the indicated times and diced in a McIlwain tissue chopper (Mickle Labs, Gomshall, U.K.). The tissue mince was scraped into cold (4°) carbogen-gassed Krebs–

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† Abbreviations: CD, cluster designation; MEM, minimum essential medium Eagle.

Henseleit buffer (pH 7.4, 2.5 mL/thymus), filtered first through a tea strainer then nylon gauze. The resulting cells were suspended in ice-cold RMPI-1640 containing 10% foetal calf serum prior to subsequent processing or analysis. Cell viability was determined by Trypan blue exclusion.

Fractionation of thymocytes on Percoll

Subfractions of thymocytes were separated on a discontinuous (60:70:80:100%) Percoll gradient as described previously [14]. Cells were removed from each interface with a pasteur pipette, diluted to 5 mL with normal MEM–25 mM Hepes (pH 7.4), centrifuged at 200 *g* for 5 min, washed with 1 mL MEM–Hepes and then recentrifuged before final resuspension in 1 mL buffer. The buoyant density of the cells was determined by a comparison with density marker beads and agreed closely with values reported previously [14], i.e. 1.063, 1.075, 1.099 and 1.119 g/mL for top (F1), 60–70% (F2), 70–80% (F3) and 80–100% (F4), respectively.

Assessment of thymocyte morphology

Electron microscopy. Cell pellets of up to 1 mm thickness were prepared by centrifugation of 1×10^6 cells in 1.5 mL Eppendorf tubes at 17,000 *g* for 2 min. These pellets, together with diced samples (1 mm^3) of thymic cortex, were fixed overnight at 4° with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.3. After rinsing in the buffer, samples were post-fixed for 1 hr with 1% osmium tetroxide in 0.1 M cacodylate buffer containing 0.04 M potassium ferrocyanide. All samples were then stained for 30 min with 2% aqueous uranyl acetate prior to dehydration through an ethanol series and embedding in Araldite. Semi-thin (1 μm) sections were stained with toluidine blue and used to select areas for ultramicrotomy. Ultrathin sections were stained with lead citrate and examined in a Jeol 100-CX electron microscope.

Coulter counter analysis. Cells (approximately 1×10^6) were suspended in 20 mL Isoton II (Coulter) and counted with a model ZM Coulter Counter plus 256 Channelyser using settings of lower and upper threshold 2.486 and 9.046 μm, respectively, and an attenuation of 2. The instrument was calibrated with size marker beads.

Methods of DNA analysis

Flow cytometry. Cells (2×10^6) were vigorously mixed with 70% ethanol (2 mL), previously cooled to −20°, and the mixtures were maintained at 4° for at least 30 min, then centrifuged at 400 *g* for 5 min and washed in phosphate-buffered saline before resuspension in 2 mL filtered (0.22 μm) DNA fluorochrome stain (containing 0.1% sodium citrate, 0.005% ethidium bromide and 0.1% Triton X-100). RNAse A was added to a final concentration of 0.25 mg/mL and the mixtures were maintained at room temperature for 1 hr prior to flow cytometric analysis of a minimum of 10^4 cell nuclei with an Ortho System 50H Cytofluorograf model 2150 using argon laser excitation at 488 nm and emission measurement at above 630 nm. The data was displayed by the instrument as a cell cycle phase histogram depicting G_0/G_1, S and G_2/M regions and in some cases with an additional sub-G_0/G_1 (hypodiploid) peak (see Fig. 5). The hypodiploid peak, as reported by others [21, 22], has been identified as a characteristic of apoptotic cells. The percentage values of the regions were calculated using the Ortho program DNADISC.

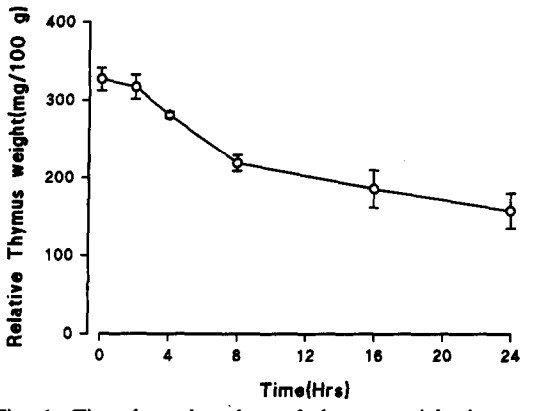

Fig. 1. Time-dependent loss of thymus weight in rats treated with dexamethasone (1 mg/kg).

Fragmentation analysis. Cells (approximately 20×10^6) were allowed to lyse in ice-cold buffer containing 10 mM Tris, 20 mM EDTA, 0.5% Triton X-100, pH 8.0, for 20 min prior to centrifugation at 13,000 *g* for 30 min. Both pellet (intact chromatin) and supernatant (DNA fragments) fractions were assayed for DNA content using the diphenylamine reagent method [26] and the level of DNA fragmentation was expressed as a percentage value [13].

Agarose gel electrophoresis. DNA fragmentation was assessed in whole cells (1×10^6) by gel electrophoresis [27] using 1.8% agarose (Agarose 10) in 89 mM Tris, 89 mM boric acid and 2.5 mM EDTA (pH 8.0).

RESULTS

Dexamethasone-induced thymic atrophy

In preliminary studies, dexamethasone (0.1–5 mg/kg) caused a dose-dependent thymic atrophy. Based on these studies, a dose of 1 mg/kg was chosen, which caused a 50% loss in thymic weight after 24 hr (Fig. 1). Thymocytes, isolated 8 hr after treatment, were 75–80% viable.

Dexamethasone-induced changes in cell density and size

Percoll fractionation of freshly isolated thymocytes from untreated rats established that about one third of the cells remained at the top of the gradient (F1) with two thirds residing at the 60–70% Percoll interface (F2). Only 1% were located at the 70–80% interface (F3) and <1% at the 80–100% interface (F4). The cells in both F1 and F2 exhibited normal cell morphology. In contrast, cells derived from F4 illustrated the characteristics usually associated with apoptosis. Treatment of rats with dexamethasone

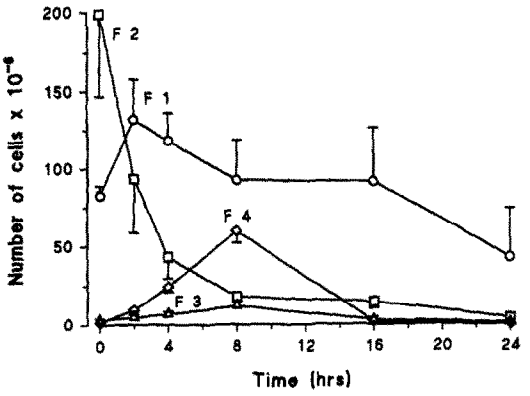

Fig. 2. Time-dependent changes in thymocyte subfractions isolated from rats dosed with dexamethasone. Thymocytes were isolated at 0, 2, 4, 8, 16 and 24 hr after treatment with dexamethasone (1 mg/kg) and separated by Percoll gradient centrifugation into fractions (F1–F4) with modal densities of 1.063 (—○—), 1.075 (—□—), 1.087 (—△—) and 1.119 (—◇—) g/mL, respectively.

resulted in very marked changes in the distribution of cells between different Percoll fractions (Fig. 2). The most striking change was the rapid fall of cells in F2 observed during the first 8 hr, which was accompanied by an increase in the number of high density cells in F4 (Fig. 2). Over the same period, a small increase was also observed in the number of cells of intermediate density (F3). After 8 hr, the number of cells recovered from the thymus declined markedly (Fig. 2). In contrast to the marked loss of cells from F2, very few cells were lost from F1 until after 16 hr (Fig. 2).

Size analysis of thymocytes, isolated from untreated rats, showed that most of the cells had a modal diameter of 5.83 μm. Treatment with dexamethasone resulted in the appearance of a second population of cells with a modal diameter of 4.89 μm. The number of these smaller cells increased markedly up to 8 hr (Fig. 3) and, from 8–24 hr, these smaller cells were the most numerous population of cells recovered from the thymus (Fig. 3). Examination of Percoll-separated cell fractions showed that the small diameter cells were located exclusively in F4 during the first 4 hr (Table 1). However, by 8 hr the smaller cells were present in all Percoll fractions, and at all subsequent times their proportion increased most notably in F3.

Dexamethasone-induced changes in the DNA profile

Little or no DNA laddering was observed in thymocytes at 2 hr, but a gradual time-dependent increase in internucleosomal DNA cleavage was observed which reached a maximum 8 hr after dexamethasone administration (Fig. 4b). DNA fragmentation analyses also showed a maximum at 8 hr (Table 2). Flow cytometric analyses showed that very small numbers of hypodiploid cells were present at 2 hr but by 8 hr this population had enlarged markedly (Table 2 and Fig. 5). These results were confirmed by the detection of DNA ladders in

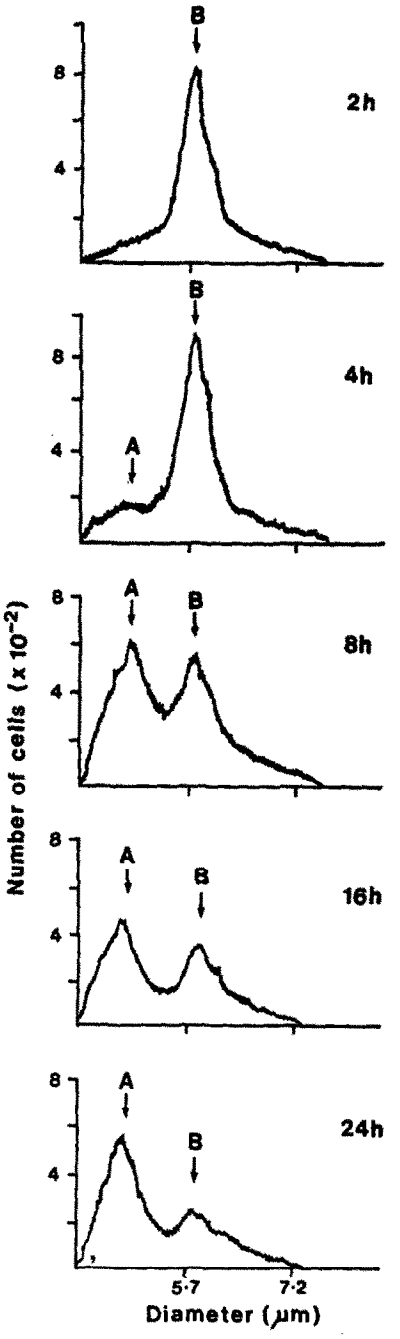

Fig. 3. Dexamethasone-induced increases in a population of small cells *in vivo*. Thymocytes were isolated from 2–24 hr after treatment with dexamethasone (1 mg/kg). Peaks A and B indicate cells estimated by Coulter counter analysis to have a mean diameter of 4.89 and 5.83 μm, respectively.

Percoll fractions F3 and F4, at both 2 and 4 hr (Fig. 4c and d), and also a hypodiploid peak (Table 2). However, at 8 hr there was an increase in both the hypodiploid peak and DNA laddering in F2 (Table 2 and Fig. 4e) and by 16 hr hypodiploid cells and DNA laddering were found in all fractions (F1–F4)

Table 1. Time-dependent induction of small diameter thymocytes by dexamethasone

Time (hr)	% small diameter cells in Unfractionated thymocytes	F1	F2	F3	F4
2	0	0	0	0	100
4	11	0	0	0	100
8	62	5	8	18	100
16	54	16	29	60	100
24	72	25	42	63	79

Rats were dosed with dexamethasone (1 mg/kg) and thymocytes isolated at the times indicated. The proportion of small diameter (4.89 μm) cells present in unfractionated and Percoll fractions (F1–F4) was determined by Coulter counter analysis. The values were estimated from the peak areas obtained in the Coulter cell size analyses histograms and corresponded to peak A depicted in Fig. 3.

(Fig. 4f). Interestingly, thymocytes isolated by Percoll fractionation 8 hr after treatment with dexamethasone (0.1 mg/kg) showed DNA laddering in only F3 and F4 (Fig. 4g), whilst after a higher dose (5 mg/kg), DNA laddering was found in all four fractions (Fig. 4h). Thus, the extent of DNA fragmentation in different Percoll fractions was both time and dose dependent. At 16–24 hr, a high percentage of hypodiploid cells was found in all fractions (Table 2) and all Percoll fractions showed evidence of DNA laddering (Fig. 4f).

Dexamethasone-induced changes in thymocyte morphology

Changes in the DNA profile correlated with the morphological characteristics of the cells, which appeared normal at 2 hr but by 4 hr a significant number showed condensation of chromatin, a characteristic feature of apoptosis. The proportion of these apoptotic cells had increased substantially by 8 hr (Fig. 6a). At early times (up to 8 hr), typical apoptotic morphology was primarily observed in cells in F4. At 16–24 hr, the number of morphologically distinct apoptotic thymocytes had decreased considerably and the population of isolated cells in the Percoll fractions was very heterogeneous. Macrophages, most of which contained apoptotic remnants, were common primarily in F1 (Fig. 6b).

DISCUSSION

Using morphological, physical and biochemical criteria, we have shown that the cellular changes involved in dexamethasone-induced thymic atrophy *in vivo*, particularly at early times (up to 4 hr) after dosing, closely resemble those observed previously following treatment of thymocytes with glucocorticoids *in vitro* [13–16]. In common with the cultured thymocyte model, evidence of apoptosis was first seen 2–4 hr after dexamethasone administration and reached a maximum at about 8 hr. The apoptotic cells were characterized by condensed nuclear chromatin (Fig. 6) coupled with evidence, from flow

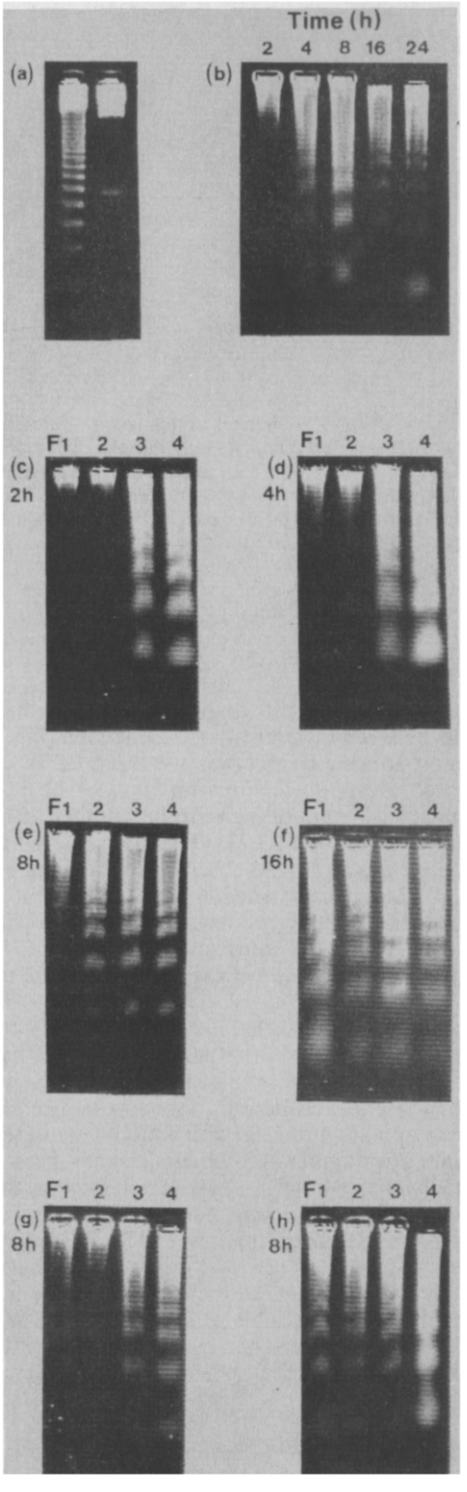

Fig. 4. Time course of DNA fragmentation in thymocytes isolated from rats treated with dexamethasone. Relative molecular mass standards showing multiples of 123 and a single 500 bp band (a). Thymocytes were isolated at the indicated times after dosing analysed by agarose gel electrophoresis either unsorted (b) or after Percoll gradient centrifugation, when fractions F1–F4 were analysed 2, 4, 8 and 16 hr (c, d, e and f, respectively) after dexamethasone (1 mg/kg) administration or 8 hr after 0.1 and 5.0 mg/kg dexamethasone (g and h, respectively).

Table 2. Flow cytometric and DNA fragmentation analysis of thymocytes from dexamethasone-treated rats

Time (hr)	% DNA fragmentation in total thymocytes	% hypodiploid cells in				
		Total thymocytes	F1	F2	F3	F4
0	0	1.7 ± 0.4	2.0 ± 0.1	2.2 ± 0.2	ND	ND
2	7.0 ± 1.9	11.2 ± 2.2	1.5 ± 0.6	3.5 ± 1.2	48.5 ± 11.4	68.4 ± 6.7
4	15.3 ± 1.6	17.1 ± 4.4	2.1 ± 0.9	3.6 ± 0.7	44.4 ± 4.5	66.1 ± 5.5
8	36.0 ± 8.4	47.7 ± 6.5	5.3 ± 1.5	24.0 ± 1.04	63.9 ± 8.0	89.4 ± 2.3
16	24.5 ± 3.5	33.3 ± 9.1	9.0 ± 1.8	39.8 ± 7.8	ND	ND
24	29.6 ± 7.2	41.2 ± 13.7	11.8 ± 3.8	27.6 ± 8.4	ND	ND

Rats were dosed with dexamethasone (1 mg/kg) and thymocytes isolated at the times indicated, and the percentage DNA fragmentation and percentage of hypodiploid cells were determined. The remaining thymocytes were fractionated on Percoll and the percentage of hypodiploid cells in fractions F1–F4 determined using flow cytometry.

Values are means ± SE for at least four separate determinations.

ND, not determined due to insufficient cells being available for analysis.

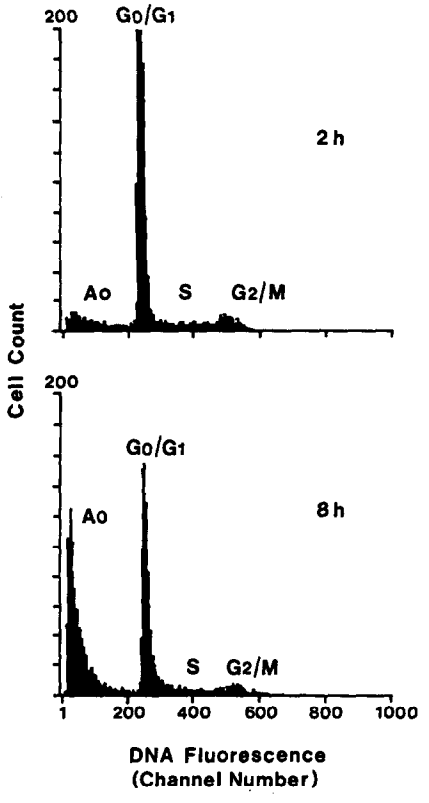

Fig. 5. Flow cytometric DNA analysis of thymocytes isolated after treatment with dexamethasone. Rats were dosed with dexamethasone (1 mg/kg) and thymocytes were isolated either 2 or 8 hr after treatment, fixed and analysed by flow cytometry. Cells in different phases of the cycle are indicated on the DNA histogram. A hypodiploid peak (A_0) indicative of apoptotic cells is also shown.

cytometry, of an altered DNA structure (Fig. 5 and Table 2), a small diameter (Fig. 3), increased buoyant density on Percoll gradients and the presence of internucleosomal DNA fragments (Fig. 4).

The observation that the apoptotic cells were derived primarily from the normal cells in Percoll F2 rather than those in F1 (Fig. 2) was of particular interest. These results suggest that cells in F2 are much more sensitive to dexamethasone-induced apoptosis than those in F1. A similar differential sensitivity of these cells has been observed *in vitro* [14] and may relate to their immunological status. Recent studies of the effects of dexamethasone on the murine thymus *in vivo* show that most thymocytes are lost from the immature double positive $CD4^+$ and $CD8^+$ and to a lesser extent from the double negative $CD4^-$ $CD8^-$ populations [23, 24]. Since in a normal thymus the elimination of autoreactive $CD4^+$ $CD8^+$ thymocytes is believed to occur as a consequence of apoptosis [7, 28], it is possible that dexamethasone simply increases the rate of cell death normally occurring in this tissue.

Following dexamethasone administration *in vivo*, the number of apoptotic cells (in F4) increased up to 8 hr and then decreased substantially (Fig. 2). This decline was caused partly by their phagocytic removal from the tissue and possibly also by their decreased generation, as a result of the relatively short half-life (2–3 hr) of dexamethasone in the thymus [10, 29]. The first suggestion was supported by the presence of macrophages, many of which contained remains of apoptotic cells (Fig. 6b), particularly at 24 hr after dosing. Macrophages recognise the cell surface changes on apoptotic cells [16]. In addition, at the later times (16 hr) all four Percoll fractions demonstrated DNA laddering (Fig. 4). This was consistent with an altered cell distribution within the gradient, which may have resulted from changes in buoyant density, following metabolic disturbances or uncontrolled re-entry of water into the apoptotic thymocytes. Similar changes have been reported in thymocytes exposed to glucocorticoids *in vitro* [3, 14] and may explain, in part, the loss of

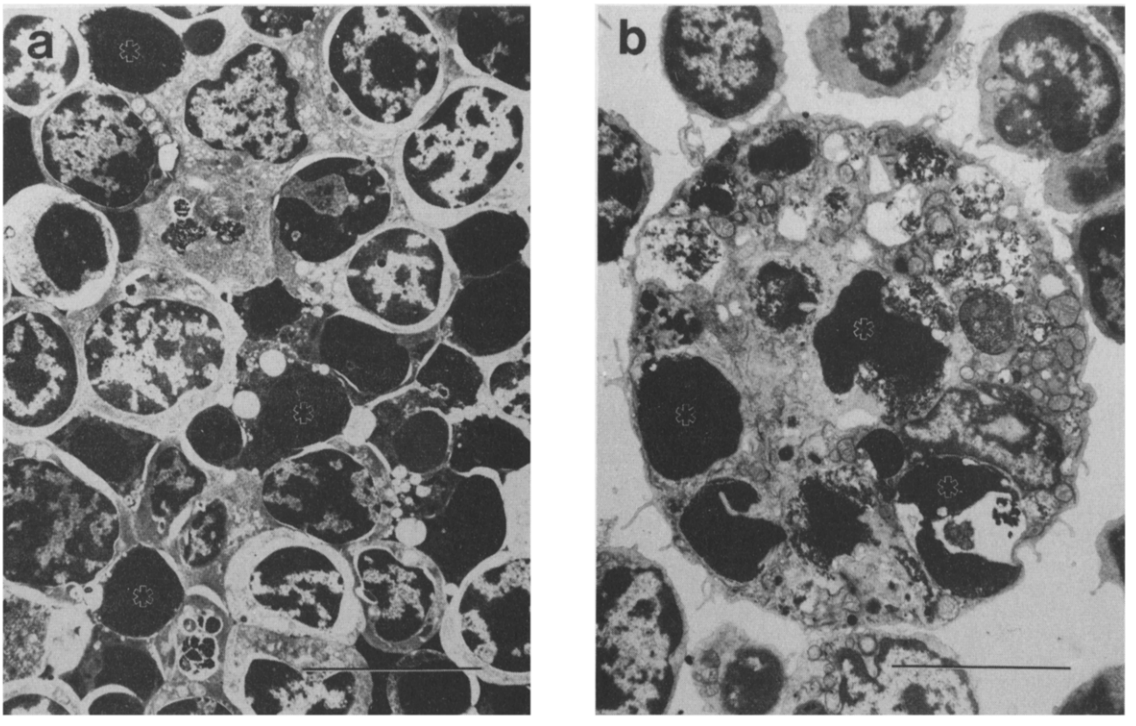

Fig. 6. Cells isolated from the thymus of rats following administration of dexamethasone (1 mg/kg). (a) Many of the cells, isolated 8 hr after dosing, show condensation of chromatin and other characteristic signs of apoptosis (asterisks). (b) One of the many macrophages present in Percoll fraction F1, prepared 24 hr after dosing, showing the partially digested remnants of phagocytosed apoptotic bodies/cells (asterisks). Bars = 5 μm.

cells from F4. Results after higher doses of dexamethasone, which resulted in a more rapid association of DNA laddering with all four Percoll fractions (Fig. 4), suggested that the level of apoptosis and the rate of subsequent events were dose dependent.

In summary, our results suggest that the widely used *in vitro* model of glucocorticoid-induced thymocyte apoptosis closely mimicks the *in vivo* situation at early times after glucocorticoid administration. However, at later times the fate of apoptotic cells *in vivo*, such as phagocytosis, is obviously not mirrored *in vitro*. Our results highlight the particular susceptibility of the F2 subpopulation of thymocytes to dexamethasone-induced apoptosis.

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