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CONCENTRATIONS OF ELEMENTS IN DYING THYMOCYTES FROM THE THYMUS GLAND OF DIABETIC RATS

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Abstract

Atrophy of the thymus gland occurs in rats made diabetic by a single injection of the drug streptozotocin. Histological studies show the presence of thymocytes with pyknotic nuclei in thymus tissue taken from diabetic animals. Analysis of the elemental content of the pyknotic cells was carried out on freeze dried frozen sections of thymus tissue using the technique of X-ray microanalysis. There was no loss of elements from cells which had undergone the early morphological changes characteristic of pyknosis and which showed the condensed chromatin of pyknotic nuclei. However as the cells shrank and lost the clear distinction between nucleus and cytoplasm there was a loss of elements, particularly Mg and K from the cells. The changes in distribution of elements in the dying thymocytes suggest a method of cell death which does not involve disruption of the plasma membrane.

Key words: Thymocytes, rats, diabetes, cell death, cryosectioning, X-ray microanalysis.

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Introduction

Atrophy of the rat thymus gland occurs after the onset of both drug induced and genetically determined diabetes mellitus (Tabata et al. 1984; Chatamra et al. 1983; 1985; Warley et al. 1987). This atrophy is due to loss of thymocytes from the gland. The loss of thymocytes probably occurs by a combination of several processes: by migration of mature thymocytes from the gland; by a decrease in the number of cells undergoing division; and by death of thymocytes within the gland. In the present paper the morphological changes that occur in degenerating thymocytes are described. In addition the procedure of X-ray microanalysis is also used to identify the changes in the concentrations of elements which occur as the cells die.

Materials and Methods

The material used in the present work was identical to that used in a previous study (Warley, 1988). The findings from control thymocytes are those reported in Warley (1988) and are pooled analyses of thymocytes from the cortical region of the glands of four untreated animals. The findings from pyknotic thymocytes are the results of analyses of thymus glands of six diabetic animals. Diabetes was induced in male rats 250-300 g body weight by a single intraperitoneal injection of the drug streptozotocin (55 mg/kg body weight). Untreated rats of the same weight range were used as control animals. The animals were anaesthetised with sodium pentabarbitone and the thymus glands were dissected out rapidly before death. Small pieces (1 mm approx.) were quench frozen by plunging into stirred Freon 22 cooled in liquid nitrogen and stored under liquid nitrogen until sectioning.

Sections, 300 nm thick, were cut at -65°C to -70°C on a Slee cryoultramicrotome. The sections were transferred onto Formvar or Pioloform coated grids and freeze dried for at least 1h in the nitrogen atmosphere of the cryostat. After drying the sections were put into a Petri dish, surrounded by molecular sieve, placed in a precooled desiccator in the cryostat, and allowed to warm to room temperature overnight. The grids containing sections, still surrounded by mole-

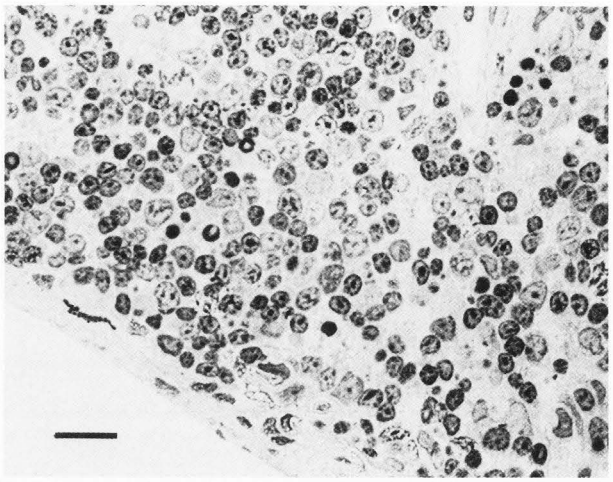

Fig 1. Light micrograph of freeze substituted frozen tissue taken from an animal 4 days after the onset of diabetes. Cells with darkly staining pyknotic nuclei can be seen in the gland. Bar = 15 μm.

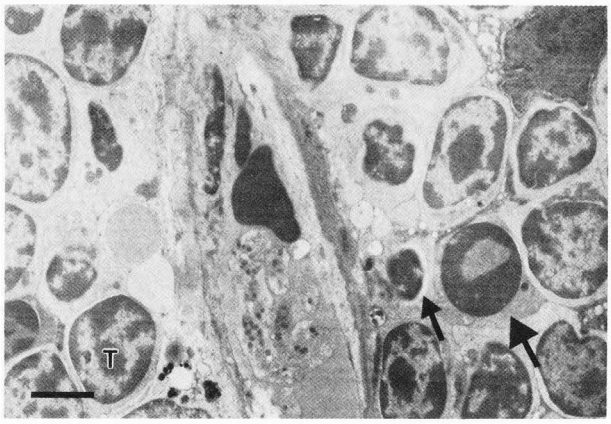

Fig 2. Electron micrograph of thymus from the cortical region of an animal 24 h after the onset of diabetes. A cell showing the early changes of pyknosis can be seen (large arrow) as well as thymocytes with normal morphology (T). The small arrow denotes a late pyknotic cell which is included within the cytoplasm of a macrophage. Bar = 2.5 μm

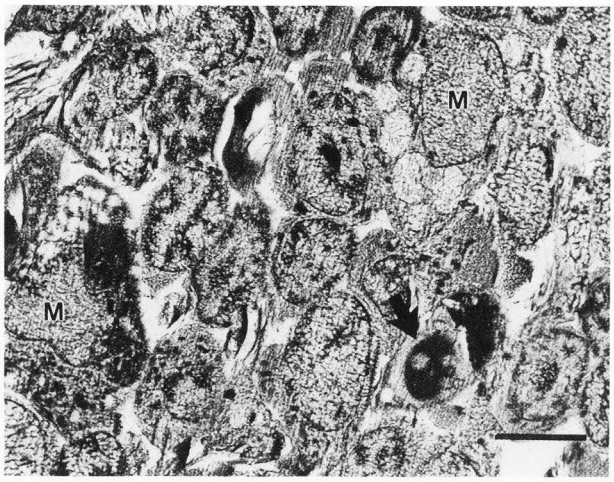

Fig 3. Freeze dried frozen section of cortical region of thymus gland taken from an animal 4 days after the onset of diabetes. A cell showing the condensed chromatin typical of early pyknotic cells can be seen (arrow). Macrophages can also be identified (M), one contains a pyknotic cell. Bar = 3 μm.

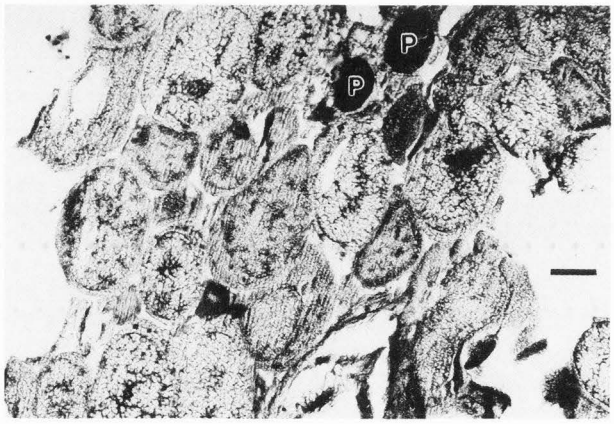

Fig 4. Freeze dried frozen section taken from thymus tissue of an animal 4 days after the onset of diabetes. Late pyknotic cells appear shrunken (P). Bar = 2 μm.

-cular sieve, were transferred to a carbon coater and coated with carbon before analysis.

Analysis was carried out using an AEI EMMA 4 electron microscope fitted with a Link Analytical 860 series 2 energy dispersive detection system. Spectra were collected for 100s live time, the area of analysis was adjusted to fit within the area of the section of a cell. The accelerating voltage was 60 kV and the beam current, measured with a Faraday cage, was 4 nA. Spectra were processed and quantitative information derived using the Quantem program supplied by Link Analytical. The system was calibrated by reference to standards composed of known quantities of salts made up in gelatin. Details of the standardisation procedures are described in Warley et al. (1983) and Kendall et al. (1985). Statistical analyses were carried out where appropriate.

After frozen sectioning the remainders of the blocks were freeze substituted in 2% Osmium tetroxide in 100% acetone, containing molecular sieve, using the method described in Ornberg and Reese (1981). After warming to room temperature the tissue was embedded in Epon and sections 1 μm thick were cut and stained with toluidene blue for examination under the light microscope.

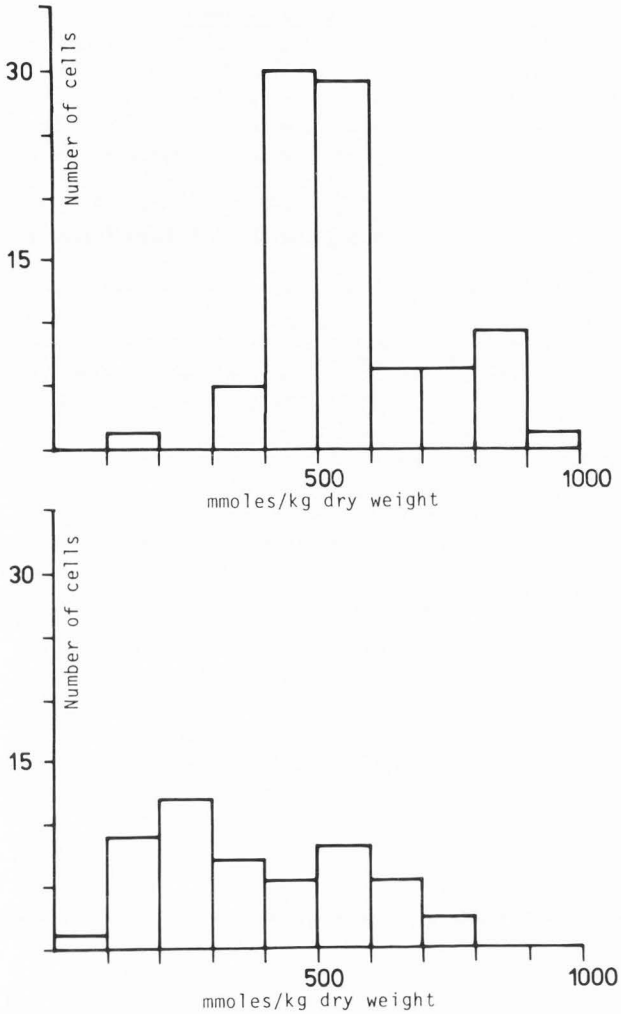

Fig. 5. Distribution of concentrations of K in cortical thymocytes from control rats (top) and in pyknotic thymocytes from diabetic rats (bottom).

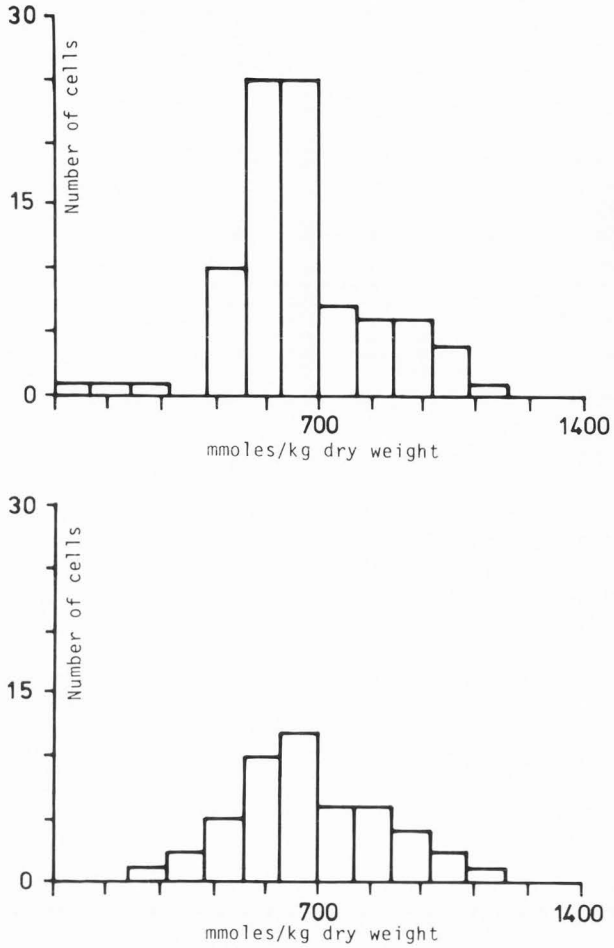

Fig. 6. Distribution of concentrations of P in cortical thymocytes from control rats (top) and in pyknotic thymocytes from diabetic rats (bottom).

Results

After the onset of drug induced diabetes there is a marked atrophy of the thymus gland, which reaches about one third of the weight of that of the control animal within 10 days. There is marked derangement of the structure of the cortical region of the gland (Warley et al. 1987). Cells with densely staining basophilic pyknotic nuclei are seen in the cortical region of the gland (Fig. 1). Pyknotic nuclei are found within 24 h of the injection with streptozotocin and were seen in all of the tissue taken from diabetic animals up to 17 days after the onset of diabetes. At the EM level the changes which take place in the nucleus of the pyknotic cells are clearly seen (Fig. 2). Initially there is condensation of the chromatin around the nuclear membrane with a loss of the typical "cartwheel" pattern of heterochromatin seen in normal thymocytes (Fig. 2). As degradation proceeds the cells shrink and some of the dying cells are engulfed by macrophages (Fig. 2, Fig. 3). The changes which take place in the dying cells can be recognised in unstained freeze dried frozen sections. In Fig. 3 a cell with condensed chromatin can be seen along with a pyknotic cell that has been engulfed by a macrophage. A pyknotic cell in which the distinction between nucleus and cytoplasm is lost is seen in Fig. 4.

Elemental concentrations in pyknotic cells compared with control thymocytes.

The elemental concentrations of cells which are undergoing degradation are compared with those of thymocytes from control animals in Table 1. All the cells whose elemental concentrations are shown in Table 1 were free in the tissues of the thymus gland, i.e. not within macrophages. When the results from the pyknotic cells are considered together, there is a decrease in the mean value of K in the pyknotic cells. The cells undergoing pyknosis can be differentiated according to morphological criteria (see Fig. 2,3,4)

Table 1. Concentrations of elements (mmole/kg dry weight) in pyknotic cells (total and divided as early and late) compared with concentrations in cortical thymocytes from control animals.
Students´ t-test.

	n	Na	Mg	P	S	Cl	K
control	87	40±4	27±3	647±20	96±7	128±6	551±6
pyknotic (total)	49	48±7	24±4	670±28	81±3	153±11	369±27
Pyknotic cells divided according to morphology							
early	19	73±14	36±5	802±35	86±6	166±21	576±22
late	30	32±6	10±4	587±31	78±4	145±11	237±17
Student´s t-test test							
early against control		**	ns	***	ns	*	ns
late against control		ns	**	ns	ns	ns	***
early against late		**	***	***	ns	ns	***

* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

and can be separated into those in the earlier stages of degradation in which nuclear chromatin has condensed but in which the nuclear membrane is still intact (Fig. 2,3) and those in later stages of degradation in which shrinkage has occurred (see Fig. 4) and in which recognisable morphology is lost.

When cells are separated according to these morphological criteria highly significant differences in the concentrations of Na, Mg, P and K are found between the two groups (Table 1). The cells in the early stages of degradation retain concentrations of Mg, S and K similar to the levels found in control cells, but there are significant increases in Na, P and Cl in the early pyknotic cells when compared with controls. The elements Na, Mg, P and K are lost in the later stages of degradation resulting in highly significant differences in the concentrations of these elements between the early and late pyknotic cells. The results for the late cells are similar to those reported earlier for this type of cell (Warley, 1987a). The change in distribution of K in the pyknotic cells compared to control thymocytes is clearly demonstrated if the spread of observations are displayed as a histogram (Fig. 5). In thymocytes from control animals mean values of K are high, since in the majority of cells the concentration of K is greater than 500 mmoles/kg dry weight. In the pyknotic cell population the distribution of values for K changes and there is an increase in the number of cells with values of K less than 400 mmoles/kg dry weight, these are the cells which appear shrunken. When the distribution of P is analysed, in both control and pyknotic thymocytes the pattern is similar, with the majority of cells having values of P in the range 600–800 mmoles/kg dry weight (Fig. 6).

Correlation between the elements K and P.

In a previous paper (Warley, 1988) a strong correlation between the elements K and P was shown in thymocytes from control animals ($r = 0.90$) and in normal looking thymocytes from diabetic animals ($r = 0.88$). This relationship breaks down in the pyknotic cells because of the loss of K. The correlation coefficient between K and P in the early pyknotic cells is 0.30, and in the late cells 0.48; neither of these values is significant.

Discussion

It has long been recognised that many thymocytes die within the thymus gland. Histologically the dying cells are recognisable because their nuclei become pyknotic. Such pyknotic thymocytes are seen in tissue taken from apparently normal animals (Lundin and Schelin, 1965) but the exact role that pyknosis plays in balancing the production of cells by mitosis is not clear (see discussion in Bellamy, 1984, Steinmann, 1986). A number of authors even suggest that the majority of thymocytes die within the gland (see Scollay, 1983). However this view is controversial (Hinsull and Bellamy, 1981; Bellamy, 1984; Kendall, 1984a,b; Steinmann, 1986).

The thymus gland is known to be sensitive to altered levels of corticosteroid hormones. In rodents increased levels of corticosteroids cause atrophy of the thymus gland and result in the appearance of large numbers of pyknotic cells in the tissue (Ito and Hoshino, 1962; Ishidate and Metcalf, 1963; La Pushin and De Harven, 1971; Claman, 1972). Thymic atrophy with the presence of pyknotic cells also occurs as a result of other treatments (e.g. radiation, Harris, 1958). Thus alterations in the thymus gland which occur after the onset of diabetes are typical of the response of the gland to various insults.

Concentrations of elements obtained by X-ray microanalysis of freeze dried frozen sections, as in this paper, are expressed in mmoles /kg dry weight, this has serious limitations in that the more meaningful units physiologically are mmoles/l cell water. From the present results it appears that loss of K occurs from the thymocytes as they undergo shrinkage in the later stages of pyknosis. However, interpretation of the results at this stage is difficult without the knowledge of the intracellular water concentration. It is possible that when the cells shrink there is a change in cell volume without a change, or even with an increase, in intracellular K concentration. The results described in the current paper for thymocytes are similar to those reported by Forslind et al. (1985) for elemental concentrations in epidermal cells. These authors showed gradients of Na, P and K over the epidermis when the results were expressed on a dry weight basis. This gradient was partially explained by a coexisting gradient in intracellular water concentrations. Nevertheless, we believe that the changes in elemental concentrations reported in the present paper do give information about

the mechanism of cell death. The pertinent observation is that normal concentrations of Mg and K are maintained in thymocytes which show the morphological changes characteristic of the early stages of pyknosis prior to the shrinkage of the cells. Although in these early cells there is a doubling of the concentration of Na this element still remains within the range found for thymocytes from control animals (Warley, 1987b). It is noteworthy that very high concentrations of Na, Cl and Ca, typical of the extracellular space, were not found within the pyknotic cells. Such high concentrations of Na, Cl and Ca would have indicated damage to the plasma membrane (Bowen, 1984). In terms of a dry weight basis loss of elements, particularly Mg and K, only occurs later in the process of cell death and there is still no increase in the intracellular concentration of Na as would be expected if the cell membrane had been damaged.

It is interesting that there is a change in the ratios of K/P in the late pyknotic cells. In the previous paper we have shown that there is a strong relationship between the elements K and P in thymocytes from control animals, and have argued that this could represent binding of K to the condensed heterochromatin. Indeed Negendank (1982) has argued that K is bound to intracellular macromolecules in lymphocytes (the mature form of thymocytes). In this paper we show changes in the ratios of K/P in the cells which are undergoing pyknotic degradation. This raises the interesting possibility that the results could reflect loss of bound K from the degrading chromatin. Clearly an investigation of alterations in K concentrations in pyknotic thymocytes using methods in which intracellular water concentration can also be estimated (e.g. Gupta et al. 1977; Zs-Nagy et al. 1982; Zierold, 1986; von Zglinicki et al. 1987) would be merited.

It has been proposed that morphological criteria can be used to distinguish two different types of cell death, in one instance swelling of the cell and rupture of the cell membranes occurs, whereas the other is characterised by condensation of chromatin and shrinkage of the cell (Kerr, 1971; Kerr et al. 1972; Wyllie et al. 1980; Wyllie and Morris, 1982; Allan and Harmon, 1986). Shrinkage necrosis was applied initially to describe this latter type of cell death (Kerr, 1971). However the term apoptosis has been coined to describe shrinkage necrosis and to separate it from death by swelling which has been termed by these authors necrosis (Kerr et al. 1972: Wyllie et al. 1980). These latter authors consider that the development of pyknosis in thymocytes is characteristic of apoptosis. The results in the present paper support the fact that pyknosis in thymocytes occurs without lysis of the plasma membrane. These findings for dying thymocytes in vivo confirm earlier studies on thymocytes isolated from diabetic animals (Warley, 1987c). It is worth reiterating that in these isolated thymocytes there was no uptake of the vital dye Erythrosin, a finding in agreement with the observations of Claesson et al. (1972). The lack of uptake of vital dye is another indication that gross membrane damage has not occurred.

As yet the reason why an increased number of thymocytes die after the onset of diabetes is not known. It is possible that alterations in levels of hormones other than insulin occur and this could account for the atrophy. However, it is interesting that detailed biochemical studies on glucocorticoid treated thymocytes (reviewed in Claman, 1972; Munck and Crabtree, 1981) show that the first general metabolic change to be detected was lowered uptake of glucose. This change is followed by decreases in uptake of amino acids (Munck and Crabtree, 1981). Insulin also stimulates the uptake of glucose and amino acids into cells in insulin sensitive tissues (Kahn, 1985). Thus lowered uptake of glucose and amino acids into thymocytes might represent a common mechanism whereby the metabolism of the thymocyte is disturbed after treatment with glucocorticoids or the onset of diabetes.

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Discussion with Reviewers

T. von Zglinicki: We found lower K/P ratios (and also lower Mg/P ratios) in the condensed chromatin of rat liver cells as compared to the decondensed (von Zglinicki and Bimmler (1987) J. Microsc 146, 77) Taken together these results clearly demonstrate that counterions have to be bound to preserve the chromatin from collapse. Do you have any idea whether the primary event in pyknosis is nuclear , i.e. replacement of K (and other counterions , e.g. Mg) by some sort of regulatory proteins or whether it is membraneous in origin, i.e. decreased pumping activity and or opening of K channels?

Authors: Biochemical studies have shown that an early event in the development of pyknosis is the activation of an endonuclease (Wyllie et al. (1984) J. Path. 142, 67-77. The results in the present paper suggest that any membrane perturbations occur at a later stage.