6-29-1987

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X-RAY MICROANALYSIS OF CELLS IN SUSPENSION AND THE APPLICATION OF THIS
TECHNIQUE TO THE STUDY OF THE THYMUS GLAND.

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Abstract

Although X-ray microanalysis has become a well established technique in the study of tissues there are still relatively few papers reporting results from cells in culture. Our early investigations on freshly isolated murine thymocytes reported higher than expected concentrations of Na and Cl in these cells but subsequent studies have shown that these high concentrations are artefactual. Rat thymocytes that have been isolated and incubated for a short time to allow them to recover from the isolation procedure have lower concentrations of Na and Cl. Sections prepared from pellets of cells which have been concentrated by centrifugation in a microhaematocrit centrifuge have concentrations of Na and Cl which are within the range that has been described for thymocytes in tissues. This method of concentration can be applied successfully to the study of white cells from the peripheral blood. In diabetic animals pyknotic cells occur in the thymus. These cells are characterised by low concentrations of K but no rise in the concentration of Na. Study of thymocytes isolated from a diabetic animal also show pyknotic cells low in K confirming these observations.

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Introduction

Despite the widespread use of cell culture lines in the study of cell physiology, and the numerous papers suggesting that the inorganic cations have a role in the regulation of cell function, there have been relatively few papers in which X-ray microanalysis has been used to study cells in tissue culture. Indeed, recently Somlyo and Somlyo (1985) were led to conclude that X-ray microanalysis of cultured cells has not yet been fully exploited. This is perhaps surprising in view of the fact that cells in culture offer advantages over whole tissues both from the point of view of experimental systems and as specimens for X-ray microanalysis (Wroblewski et al., 1983; Wroblewski and Roomans, 1984; Zierold et al., 1984). In addition to these technical advantages for the study of cells in culture X-ray microanalysis is a technique which can be used to compare cells in culture with their tissue counterparts, a field about which little is known.

Cells in suspension which have been studied by X-ray microanalysis are mainly components of the blood, e.g., red cells (Lechene et al., 1976; Kirk et al., 1978a,b; Lee and Kirk, 1982; Kirk et al., 1983; Jones et al., 1986), platelets (Costa et al., 1977; Yarom, 1983; Boekestein et al., 1985) and lymphocytes (Hook et al., 1986a,b). Other cells which have been studied include macrophages (Bell et al., 1979; Masters et al., 1979; DeVries et al., 1983; Smith et al., 1985) spermatazoa (e.g., Chandler & Battersby, 1976), yeasts (Roomans & Seveus, 1976; Zierold, 1982) and bacteria (e.g., Sigee et al., 1985; Chang et al., 1986). Early studies on cells of the blood were often on fixed cells (e.g., Skaer et al., 1974; Yarom et al., 1976) but such studies preclude investigation of the diffusible elements. A number of studies on cells of the blood have been carried out on whole cells. The cells can be mounted on E.M. grids and air dried, or smeared or sprayed onto supports. In any of these methods of preparation the fact that components of the medium are still present after drying needs to be taken into account when evaluating the spectra. The presence of medium may not interfere if the elements of interest within the cell are not contained in the medium (e.g., the study of Ca and P in the dense bodies of platelets,

Costa et al., 1977; Yarom, 1983; Boekestein et al., 1985). However such methods are unsuitable for the study of intracellular concentrations of elements such as Na and Cl which are usually components of the medium.

The thymus gland is the major site in the body in which T lymphocytes undergo development and differentiation (Miller, 1961; Jordan and Robinson, 1981). Previous studies (Chatamra et al., 1983; 1985) show that after the onset of diabetes the gland atrophies rapidly and many thymocytes become pyknotic. I wished to understand why this atrophy occurs and proposed to use studies on isolated thymocytes as part of this investigation. Many aspects of thymocyte function are commonly studied in vitro due to the ease with which thymocytes can be prepared in suspension. The use of cryofixation and X-ray microanalysis to study both normal thymocytes and thymocytes from diabetic animals in vivo and in vitro should give an insight into the mechanism of development of pyknosis. Increases in the intracellular concentration of Na are thought to occur when thymocytes are stimulated to begin dividing (Averdunk, 1976; Felber and Brand, 1983) and presumably the natural cycle of division ceases when pyknosis occurs. Therefore in order to undertake the studies on pyknotic cells it was necessary to prepare the isolated thymocytes for microanalysis using conditions which did not disturb the distribution of Na, and so studies on whole cells were ruled out.

Cells in suspension can be prepared for X-ray microanalysis by centrifugation to concentrate the cells, followed by freezing and sectioning of the pellet. This technique has been used successfully for the study of erythrocytes (Shuman et al., 1976; Andrews et al., 1983; Kirk et al., 1983). The cells can be prepared in the presence of the medium in which they are suspended and, because the material is studied in section, the medium should not interfere with subsequent analyses. This is the approach that was adopted in our studies on isolated thymocytes. However early studies (Kendall et al., 1985) had shown an excess of Na and Cl in the isolated cells and it was necessary to understand the source of this increase before meaningful comparisons could be made between thymocytes in vivo and in vitro.

Outline of the present work

In this review paper results highlighting some of the problems encountered in X-ray microanalytical studies on freshly isolated thymocytes and peripheral white cells will be summarised. These problems will be discussed with reference to those encountered by other authors studying either isolated cell suspensions or cells in culture grown as monolayers. Detailed discussion of general methods for specimen preparation and problems encountered in quantitation for X-ray microanalysis will not be attempted as these are covered in reviews from the laboratory of Hall and Gupta (e.g., Hall, 1979; Gupta and Hall, 1982; Hall and Gupta, 1983; Hall and Gupta, 1986). A detailed review

of methods used for the study of single and cultured cells has been published (Wroblewski and Roomans, 1984). Comparisons between tissue thymocytes and isolated thymocytes and preliminary results from studies on thymocytes of diabetic animals will be presented as examples of the use of X-ray microanalysis for comparative studies between cells in vitro and their tissue counterparts. I will also discuss some of the recent studies in which X-ray microanalysis has been used to investigate cells in suspension.

Materials and Methods

Thymus glands from male CSE Wistar rats were used as a source of thymocytes. The rats were killed under chloroform or sodium pentabarbitone anaesthesia. Small pieces of thymus gland were excised rapidly and frozen onto silver stubs by plunging into Freon 22 cooled in liquid nitrogen and stored under liquid nitrogen. The methods used for preparing suspensions of thymocytes and their preparation for freezing in the presence of different media have been outlined previously (Kendall et al., 1985; Warley et al., 1986; Warley, 1986b). To allow recovery from the isolation step the cells were resuspended at 1×10^6/ml and incubated at 37°C for 1h. In experiments where the cells were concentrated further, the isolated cells were harvested by centrifugation and the pellet was resuspended in 0.1 ml medium. The cells were taken up into a haematocrit centrifuge tube which was sealed before centrifugation at 10,000g for 90s. The portion of the tube containing the cells was scored and snapped off, the tube was inverted and the cell pellet extruded onto silver stubs and frozen as for the tissues.

Diabetes was induced by a single intra-peritoneal injection of the drug streptozotocin (55mg/kg body weight in citrate buffer). Glucose levels in blood and urine were monitored as a matter of routine. Full details of the methods used for handling the diabetic animals are given in Warley et al. (1987).

Peripheral blood lymphocytes were prepared by standard isolation procedures. The animals were bled out through the heart, the blood was diluted 1:1 with culture medium and 5ml of diluted blood was layered onto 10 ml lymphocyte separation medium (Flow Labs., Irvine, Scotland). The preparation was centrifuged (400g, 30min), cells which collected at the barrier were aspirated, resuspended in culture medium, washed twice and the pellet frozen either immediately, or after concentration in a microhaematocrit centrifuge.

Cryosections and X-ray microanalysis

Frozen samples were stored in liquid nitrogen before sectioning at -65°C to -70°C in a Slee cryoultramicrotome. Sections (approximately 300nm thick) were cut, collected onto Formvar or pioliform coated Ni grids and allowed to freeze dry for 1h in the nitrogen atmosphere of the cryostat. The sections were placed in a Petri dish, which was then placed in a second larger Petri dish containing molecular sieve. The dishes were placed in a desiccator and allowed to warm to room temperature overnight. The Petri dishes

containing the sections were transferred to a carbon coater and coated before analysis. Analysis was performed in an AEI EMMA-4 electron microscope fitted with a Link systems 860 series 2 EDS detection system. Analysis was carried out for 100s at 60kV accelerating voltage and 4nA beam current measured using a Faraday cage. A circular probe diameter 0.5μm was used for the analysis of nuclei from thymocytes. In the study of thymocytes from diabetic animals the probe was extended to cover the area of the section of a cell. A gaseous nitrogen cooled anticontamination device surrounding the specimen was used to reduce contamination. Spectra were processed and quantitative information obtained by use of the Quantem/FLS program; this program is described in Gupta and Hall (1982). Full details of the analytical and quantitative procedures are given in Kendall et al. (1985).

Results

X-ray microanalysis of freshly isolated cells from normal animals

Initial studies on mouse thymocytes (Kendall et al., 1985; Warley et al., 1986) showed that the freshly isolated cells contained high concentrations of Na and Cl (778 and 937 mmoles/kg dry weight, respectively) when compared to their tissue counterpart (Na 152, Cl 247 mmoles/kg dry weight). The isolated cells also contained significantly lower concentrations of K. There was a high correlation between the values for Na and Cl in the isolated cells (correlation coefficient 0.84) whereas no such correlation was found in the tissue cells (correlation coefficient 0.15). The high values for Na and Cl coupled with a strong correlation between the two elements suggested that the medium, which consists largely of these elements, was the source of the raised values. High values of Na and Cl were also found in both peripheral blood lymphocytes and thymocytes which had been isolated by centrifugation on lymphocyte separation medium (Table 1). These cells also contained detectable quantities of iodine (Table 1, I_2 expressed as peak area/background) presumably originating from the lymphocyte separation medium which consists of Ficoll and sodium diatrizoate, an iodine containing compound. These observations led to a systematic study to attempt to understand the source of the excess Na and Cl. All subsequent studies were carried out on thymocytes from the rat. Preliminary results were published in Warley et al. (1986), the full study in Warley (1986b). Thymocytes were isolated in the presence of different media to establish whether the composition of the medium affected the intracellular elemental concentrations. These studies on thymocytes (Table 2, Warley et al., 1986; Warley, 1986b) showed that in the freshly isolated cells the concentrations of Na and Cl were not as high as those found previously in mouse thymocytes, but the concentrations of these elements were still higher than values recorded for thymocytes in tissues. There also appeared to be some variability between cells isolated in the presence of different media. The

effect of incubating the isolated cells was then studied. After incubation there was a decrease in the concentration of Na and Cl in the cells. Again, when different media were used for suspending the cells, some variability in the concentration of Na and Cl was found (Table 3).

Studies on isolated macrophages (Masters et al., 1979; Bell et al., 1979) had shown that centrifugation at higher forces (10,000g) than are normally used to concentrate cells from tissue culture (500g) yielded cell pellets which, when frozen and sectioned, showed excellent morphology. This technique was applied to both isolated incubated thymocytes and white cells from peripheral blood to investigate the effect of the procedure on their elemental composition. The results are shown in Table 4. Thymocytes which had been

Table 1. Concentrations of Na,Cl and K and presence of I_2 rat peripheral blood white cells and thymocytes isolated on lymphocyte separation medium (mmol/kg dry weight ± SE, I_2 (A/B).

	n	Na	Cl	K	I_2
PBWC*	5	606±87	635±75	433±43	0.13
Thy	17	571±75	747±81	604±63	0.29

Table 2. Concentrations of Na, Cl and K in nuclei of freshly isolated rat thymocytes and tissue thymocytes. Isolated cells were prepared for freezing in the presence of Hanks (H) Serum (S) or Dextran (D 20% in Hanks, mmol/kg dry weight ± SE). Data from Warley (1986a,b).

|	n	Na	Cl	K
H	69	323±24	487±28	661±23
S	73	218±13	294±13	632±24
D	69	175±14	331±20	561±30
Tissues	99	91±8	220±12	676±19

Table 3. Concentrations of Na, Cl and K in nuclei of rat thymocytes after incubation at 37°C for 1h. Cells prepared for freezing in the presence of Hanks (H) serum (S) or Dextran (D 20% in Hanks, mmol/kg dry weight ± SE). Data from Warley (1986b).

	n	Na	Cl	K
H	19	161±29	132±16	668±47
S	19	195±23	242±18	504±33
D	21	110±13	109±13	562±15

Table 4. Concentrations of Na, Cl and K in nuclei of rat thymocytes and in peripheral blood white cells after concentration in a microhaematocrit centrifuge. (mmol/kg dry weight ± SE, I_2 A/B).Thymocytes prepared in the presence of Hanks(H) or serum (S). Data from Warley (1986b).

	n	Na	Cl	K	I_2
H	59	54±7	140±6	602±21	
S	68	104±6	170±7	602±20	
PBWC*	17	24±9	176±14	548±38	0.007

* Peripheral White Blood Cells
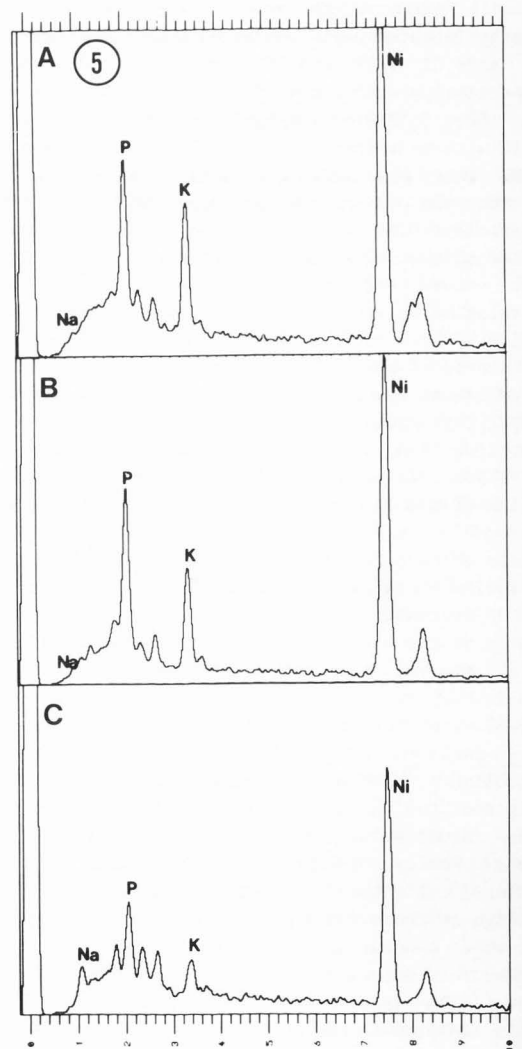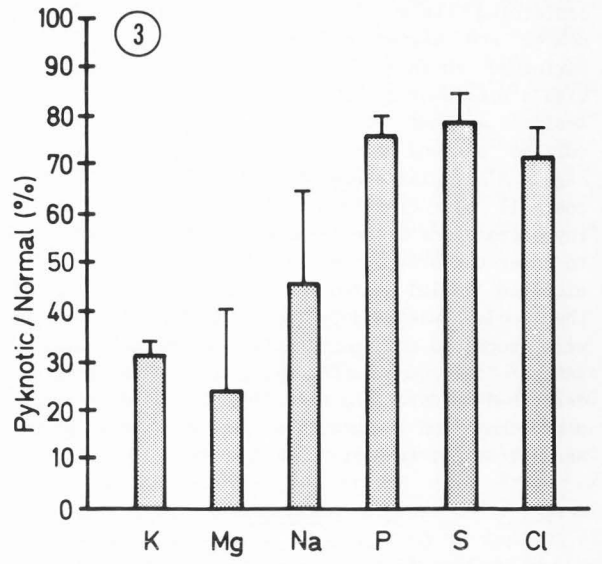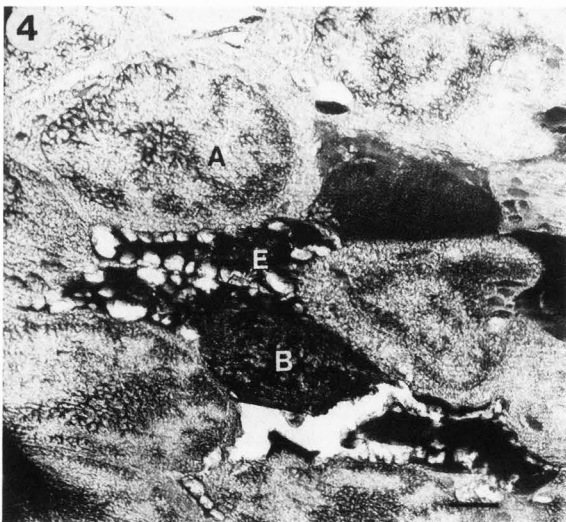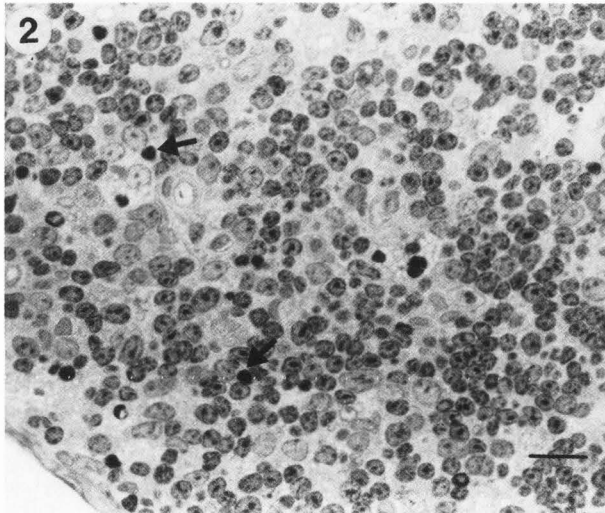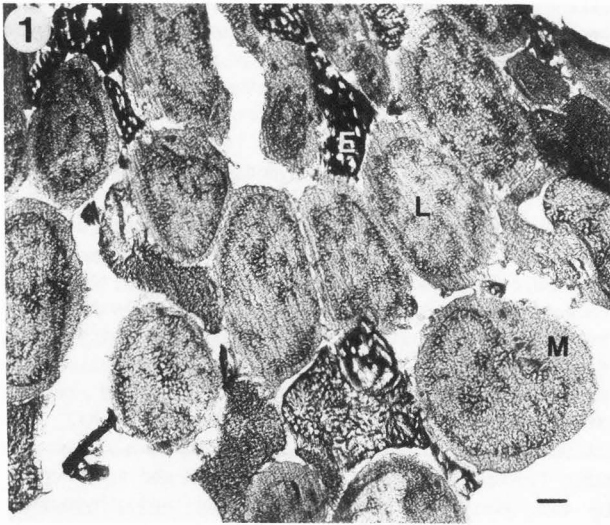
Fig 1. Freeze-dried frozen section cut from a pellet of peripheral white blood cells prepared by concentration in a microhaematocrit centrifuge. Different cell types can be identified L=lymphocyte M=monocyte E=erythrocyte. Bar=1μm.

Fig 2. Light micrograph of thymic tissue from a 4 day diabetic rat showing the presence of pyknotic cells (arrows). Bar=15μm

Fig 3. Concentration of different elements in pyknotic thymocytes in thymus tissue from diabetic rats. The concentrations are expressed as percentage of the value found in thymocytes from a control animal. Bars represent standard error.

Fig 4. Electron micrograph of cells isolated from a 4 day diabetic rat showing A) normal thymocytes and B) pyknotic thymocytes. E=erythrocyte. Bar=1μm.

Fig 5. Spectra of thymocytes isolated from a 4 day diabetic rat. A) normal looking thymocyte B) pyknotic thymocyte C) cell "ghost." Spectra A and B are from cells A and B in Fig 4.

Fig 6. P/K in thymocytes from a normal rat (a) in normal looking cells from a diabetic rat (b) in pyknotic cells in vitro (c) and pyknotic cells in vivo (d).
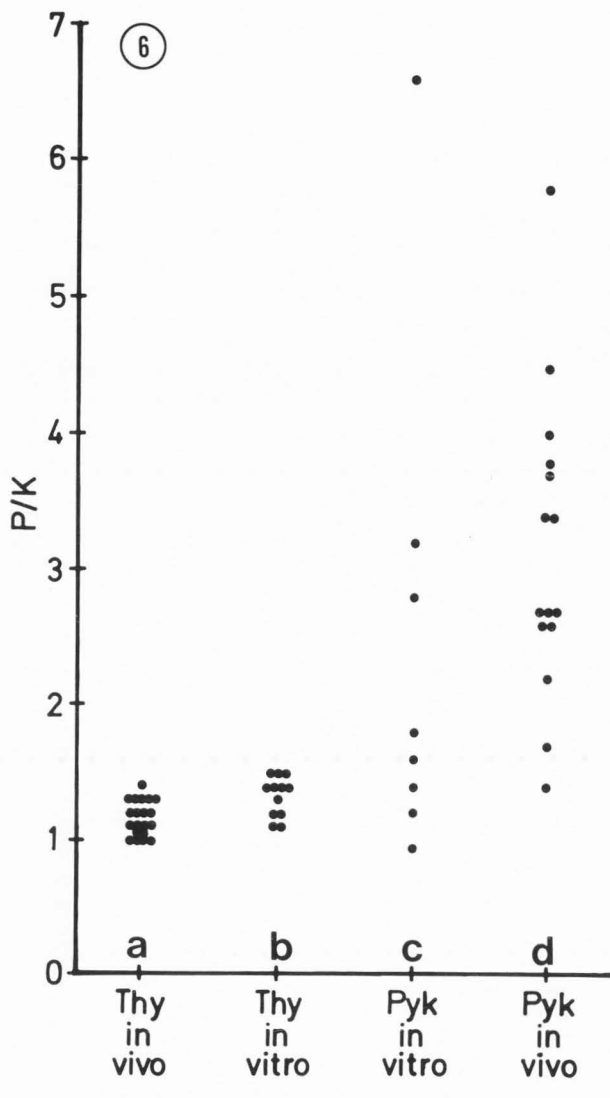
prepared in this way showed the lowest concentrations of Na and Cl. Low concentrations of Na and Cl were also found in the white cells. After the higher speed centrifugation there was no detectable I_2 peak in the spectra from these cells (Table 4). This procedure gave sections in which the morphological detail was well preserved, an electron micrograph of peripheral blood white cells prepared in this way is shown in Fig. 1. When thymocytes were concentrated by centrifugation at higher g force there were consistent differences in the concentrations of Na between cells prepared in the presence of Hanks' solution and those prepared in the presence of serum (see Table 4). This difference could be due to differential centrifugation occurring in the presence of serum, which is denser than Hanks, the result being a partial separation of different cell populations.

Comparison between thymocytes in vivo and in vitro

Previous studies on rat tissue thymocytes have shown that levels of Na and Cl vary in different individuals (Warley, 1987a). The mean concentrations of Na and Cl in nuclei of isolated thymocytes centrifuged in the presence of Hanks (lowest value for an individual animal Na 49±8, Cl 134±10 mmoles/kg dry weight) or serum (highest value for an individual

animal Na 108±11, Cl 196±14 mmoles/kg dry weight) fall within the range of those found for tissue thymocytes (lowest value for an individual animal Na 48±10, Cl 119±6 mmoles/kg dry weight, highest value for an individual animal Na 152±22, Cl 316±17 mmoles/kg dry weight).

Studies on diabetic animals

The appearance of thymic tissue four days after the onset of diabetes showing the presence of pyknotic cells is shown at the light microscope level in Fig 2. Analysis of the pyknotic cells in freeze dried frozen sections from thymic tissue showed that these cells lost K without a concomitant rise in Na (Fig 3, Warley, 1987b). Thymocytes were isolated in medium containing Erythrosin B from an animal four days after the onset of diabetes and the pyknotic and non-pyknotic cells analysed. Micrographs of normal looking (A) and pyknotic thymocytes (B) in the isolated preparation are shown in Fig. 4; spectra from cells A and B are shown in Fig. 5. The spectrum of a cell which

appears as a "ghost" is also shown in Fig. 5. A low level of potassium was found in the pyknotic thymocytes. By comparing spectra A and B it can be seen that in the pyknotic cells the ratio of P/K changes as a result of the loss of K. A peak for I_2 was not detected indicating that there had been no uptake of vital dye. Na only becomes apparent in the spectra of the isolated cells when the cells appear as "ghosts" (spectrum C). So far an increase in Na in pyknotic thymocytes in vivo has not been detected. The change in P/K ratio in individual pyknotic cells compared to normal thymocytes is shown diagrammatically in Fig. 6.

Discussion

The preparative methods used in this work should theoretically not have led to problems with contamination from the external medium. There are several stages during the preparative procedure where artefacts could arise and it is necessary to investigate each of these to understand the cause. The high values for Na and Cl found in the isolated cells could be caused during the isolation procedure, during the preparation for freezing, or in the interval between sectioning and analysis.

Techniques for cell isolation

Cells of the blood can be isolated in the presence of buffered salt solutions such as Hanks' or tissue culture medium both of which are isotonic and should not alter intracellular concentrations of elements. Thymocytes are also easily prepared in suspension by chopping the tissue in the presence of culture medium followed by sieving to remove debris. This treatment is mild in comparison with the more severe methods of enzyme digestion often used to establish cells in culture. For instance Wroblewski et al. (1983) needed to digest auricular cartilage for 24h in the presence of collagenase to free chondrocytes, and Buja et al. (1985) isolated myocardial cells from neonatal rats by repeated digestions with pancreatin and collagenase. When such enzyme treatments are used prolonged periods of incubation (e.g., days) are required to allow cells to recover from the isolation step. The method used for the isolation of the thymocytes is relatively mild and these cells have frequently been used immediately after isolation. However the studies reported in this paper show that a short incubation period after isolation is necessary and results in a decrease in the concentrations of Na and Cl. Similar findings have been reported for rat thymocytes by Jones et al. (1981) who used tracer techniques to show that the estimated concentrations of Na fell after incubation for 1h. Thus even when mild isolation conditions are used this is one stage at which alterations in intracellular concentrations of Na and Cl can occur.

Preparation of cells for freezing

Thymocytes and peripheral blood lymphocytes occur naturally in suspension and do not normally grow as monolayers. Many problems have been encountered when preparing cells grown as monolayers for freezing and

these have already been discussed in detail (Wroblewski and Roomans, 1984). These problems include altered balance of Na and K caused by damage to the cells when they are removed from the substrate (James-Kracke et al., 1980; Norrie et al., 1982) and possible alteration of intracellular balance caused by washing of cells to remove the external medium (Zierold et al., 1979; James-Kracke et al., 1980; Zierold, 1981; Ceder et al., 1982; Wroblewski et al., 1983). These particular problems related to the study of cells grown as monolayers should not be encountered when studying cells in suspension, but for suspended cells other problems may occur. Cells in suspension are usually concentrated by centrifugation prior to freezing (e.g., erythrocytes; Shuman et al., 1976; Andrews et al., 1983; Kirk et al., 1983). It is possible that centrifugation could cause alteration of the ionic balance within cells. There appears to have been no systematic study of this problem. Previous studies on HeLa cells infected with vaccinia virus (Warley and Stephen, 1985) and the results from isolated grossly damaged pyknotic thymocytes reported in this paper suggest that centrifugation will only cause problems if membrane damage has already occurred.

The medium in which the cells are prepared could cause alteration in intracellular concentrations of elements if it is not isotonic or if it contains substances which are biologically active (Von Euler & Wallace, 1985) or which alter the physiology of the cell. In the present studies thymocytes were prepared in the presence of different media which are similar in electrolyte content but which differed in the amount of organic matrix; thus the suspending medium, apart from the one containing dextran, should not have upset the balance of electrolytes inside the cell. High molecular weight additives, such as dextran, form a matrix in the medium which suppresses the growth of ice crystals and helps prevent translocation of elements. When such additives are used it is necessary to establish whether they are compatible with the normal function of a tissue or suspension (discussed in Gupta and Hall, 1982; Barnard et al., 1984). Dextran appears to have no effect on suspensions of erythrocytes (Jones et al., 1979; Tormey, 1981). However, as shown previously (Warley, 1986b) when thymocytes are suspended in medium containing dextran a proportion of the population is susceptible to the presence of dextran. These cells have low K/Na ratios but do not take up vital dye. These results are interesting as many rats are known to be dextran sensitive in life (Mendler et al., 1969; Proctor, 1977). In the population of thymocytes that is unaffected by dextran the concentrations of Na and Cl are similar to values obtained from tissue thymocytes. These observations coupled with those where the presence of I_2 was found in cells isolated on lymphocyte separation medium indicate that in the isolated thymocytes the increased concentrations of Na and Cl are due to non-specific contamination from the medium.

There are various possible sources for this contamination. In our early studies on mouse thymocytes

and rat thymocytes isolated on lymphocyte separation medium where gross contamination of the cells was occurring the problem may well have been related to difficulties in handling a small number of cells. Thymocytes are small cells (approximately 4µm in diameter). Tracer studies marking the external medium show that in a pellet that has been concentrated by low speed centrifugation the cells may occupy only 50% of the volume of the pellet. Thus in addition to their small size the cells are sparse in the pellet in relation to the volume occupied by medium. The large areas unoccupied by cells could prevent the detection of folding occurring during the sectioning procedure and folding could be the cause of the very high concentrations of Na and Cl.

When the rat thymocytes were studied, apart for the cells isolated on lymphocyte separation medium where fewer cells were available, the gross contamination with Na and Cl found for mouse thymocytes did not occur. Freshly isolated thymocytes generally had concentrations of Na of around 200 mmoles/kg dry weight and Cl values of around 300–400 mmoles/kg dry weight. These values were reduced by incubation, and further reduced after concentration of the cell pellet in the microhaematocrit centrifuge (see Table 4). The final values for Na and Cl are within the range that has been reported for isolated rat thymocytes and related cells by other groups (see Warley, 1986b). The slightly higher concentrations of Na and Cl found in cells before concentration could be caused by formation of extracellular ice crystals in the medium leading to local alterations in tonicity of the medium, but this should not occur if freezing rates are adequate. Another explanation of these higher concentrations of Na and Cl is redistribution of these ions occurring during the period between freeze drying and carbon coating. Roomans and Seveus (1976) reported that cryosections of yeast exposed to the atmosphere for periods of days deteriorated visibly and redistribution of elements occurred. Other authors (Frederik and Busing, 1981; Zierold, 1984; Barnard, 1985; Hagler and Buja, 1986; Zierold, 1986) have also noted deterioration of morphology in freeze dried cryosections exposed to the atmosphere for varying periods of time. It is perhaps not surprising that deterioration of sections occurs after prolonged exposure to the atmosphere, but in the experiments recorded by both Barnard (1985) and Hagler and Buja (1986) the time the sections were exposed to the atmosphere was relatively short. These observations on redistribution must be balanced by the many studies (e.g., see section on applications) in which cryotransfer procedures were not used, but in which physiologically interesting results were obtained from sections in which there was no gain in Na or Cl.

Although the results discussed in this paper show that for isolated thymocytes removal of excess medium results in sections in which contamination from excess Na and Cl does not occur, they cannot distinguish between the various explanations for the contamination. Various groups who have studied cells grown as monolayers remove excess medium by blotting (James-Kracke et al., 1980; Buja et al., 1985) and blotting has been used to remove excess medium from suspensions of plant cells (Warley et al., 1985). However, animal cells in suspension are not rigid enough to withstand this procedure. Centrifugation at higher g force may be applicable as a general preparative step when analysing cells in suspension. Apart from the studies on isolated macrophages (Bell et al., 1979; Masters et al., 1979; Smith et al., 1985) the results presented in the present paper indicate that it can also be used for the study of white cells isolated from peripheral blood. These latter results are interesting as Hook et al. (1986b) studying Mg concentration in peripheral blood mononuclear cells sprayed onto supports had also noted the presence of an I_2 peak in cells which had been prepared on lymphocyte separation medium. These results coupled with our early investigations suggest that the separation medium is not completely removed by standard washing procedures. The later studies on peripheral blood cells which have been prepared by higher speed centrifugation indicate that the separation medium probably does not penetrate the cells but may remain adsorbed to the cell surface. The results from studies on isolated thymocytes show that it is possible to prepare cells in suspension for X-ray microanalysis which give a reproducible baseline from which it is possible to undertake studies such as those reported in this paper for thymocytes from diabetic animals.

One of the problems found when studying thymocytes is variability in elemental content which occurs in the normal thymocyte population. This is a very heterogeneous population in terms of cell type and 10–20% of the cells may be actively dividing (Jordan & Robinson, 1981). The differences in Na concentration noted between cells prepared in the presence of Hanks and those prepared in the presence of serum may be related to differences in the numbers of dividing cells. Most of the cells which have been studied in suspension are the formed elements of the blood; these cells are not undergoing rapid cycles of division. Studies on fibroblasts (Proll et al., 1979) and on HeLa cells (Warley et al., 1983a,b) have indicated that differences in elemental content occur in rapidly dividing cells. More recently altered permeability of the cell membrane to Na has been described in non confluent primary cultures of both fibroblasts and proximal tubule cells (Abraham et al., 1985; Larsson et al., 1986) and Chang et al. (1986) have also shown increased levels of Ca in rapidly dividing bacteria. Clearly if studies are undertaken on cells that are rapidly dividing the effects of division will need to be taken into account.

Comparison of isolated thymocytes with thymocytes in tissue

The intention of undertaking X-ray microanalysis of isolated thymocytes was to determine whether the results obtained for thymocytes in vivo and in vitro would be comparable. The results which have been presented in this paper for normal thymocytes

suggest that this is so. The investigations on thymocytes from diabetic animals show how the study of isolated cells can add to the information derived from the study of cells in tissues. The finding of low concentrations of K without a rise in the intracellular concentration of Na in pyknotic thymocytes from diabetic animals was initially surprising. Loss of K from the cell associated with an increase in intracellular concentrations of Na and Ca is usually associated with cell death in cells in culture (Roomans et al., 1981). The fact that uptake of either Na or vital dye does not occur in the isolated pyknotic thymocytes indicates that the membrane is not damaged in these cells and that the isolated cells resemble their tissue counterpart. The decrease which occurs in all elements in the pyknotic cells suggests that overall shrinkage is occurring in these cells; the decrease in cations may be associated with this. These studies indicate the value of including a vital dye which is detectable on analysis in the suspension medium to mark those cells in which membrane damage has occurred. This technique was introduced by Walker et al. (1984) but appears to have been little used.

To date few comparisons have been made between cells in vitro and their counterparts in vivo despite the observations of James-Kracke et al. (1980) that there were similarities in cytoplasmic concentrations of K, Cl, Ca, Mg and S between cultured vascular smooth muscle cells and adult vascular smooth muscle. In other studies Wroblewski et al. (1983) have described major differences in elemental composition between chondrocytes in vitro and chondrocytes in auricular cartilage.

Applications

There can be no doubt that the value of X-ray microanalysis is that it couples the resolving power of the electron microscope with the ability to undertake, simultaneously, chemical analyses. Elemental concentrations can be measured both at a cellular level and at the level of organelles within cells and new information can be gained which is not available from other techniques.

X-ray microanalysis has been used successfully to study membrane transport during erythropoeisis (Lee and Kirk, 1982; Kirk et al., 1983). In some animals mature erythrocytes have low concentrations of K and high concentrations of Na. The precursors of these cells have high K and low Na. Kirk et al. (1983) were able to show that the switch from high to low K occurs before the formation of reticulocytes. This study relied on the ability to distinguish cell types according to their morphology in an isolated population of mixed cells; the studies on pyknotic thymocytes also rely on this ability. Another application has been in the study of heavy metal induced toxicity in macrophages (Bell et al., 1979; DeVries et al., 1983). In these studies X-ray microanalysis has been used to show the subcellular localisation and presumable site of action of Cd and

Pb. Another interesting use of X-ray microanalysis for the study of heavy metals is that reported by Pedersen et al. (1981) on the uptake of heavy metals by algae. Smith et al. (1985) studied elemental concentrations in isolated macrophages before and after the ingestion of bacteria. They were able to show increases in Na, Cl, and Ca and loss of K after ingestion of bacteria. These results support the idea that stimulation of cells by the bacteria results in altered permeability of the plasma membrane.

An application which demonstrates the importance of being able to localise elements at a subcellular level is that of Lew et al. (1985). These authors were able to demonstrate in sections of sickle cells the presence of intracellular vesicles capable of accumulating Ca. The presence of these vesicles could account for the elevated concentration of Ca that has been recorded in these cells and therefore question theories which suggest that Ca has a role in the development of irreversibly sickled cells.

Conclusions

There have now been several papers in which the preparative procedures for the study of monolayers of cells in culture by X-ray microanalysis have been detailed (Wroblewski and Roomans, 1984; Zierold, 1986). The development of methods of growing cells as monolayers on a substrate such as collagen which can be sectioned along with the monolayer (e.g., Buja et al., 1985) may circumvent some of the problems which have been encountered in earlier studies. Some of the problems which may be encountered when studying cells in suspension have been documented in the present paper. The studies on isolated thymocytes summarised in this paper indicate that it is possible to make valid comparisons between thymocytes in vivo and thymocytes in vitro. Several interesting papers have now been published from studies on cells in suspension. There can be no doubt that in the future studies on primary cell cultures both as monolayers and in suspension will contribute to our understanding of cell physiology, and the mechanisms by which this is altered in pathological and toxicological situations.

Acknowledgements

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Discussion with Reviewers

G. Hook: After having compared the elemental concentration of thymocytes in suspension using various preparation methods, what is the best method for preparing these cells for X-ray microanalysis?

Author: For preparing thymocytes for X-ray microanalysis I would recommend incubation of the isolated cells for a minimum period of 1h in medium such as buffered RPMI 1640 followed by concentration in a microhaematocrit centrifuge through Hanks or RPMI 1640. When following this procedure one should bear in mind that some separation of cell populations may occur.

J. Wroblewski: What is the main advantage of the present method of preparation in comparison with cells cultured on thin substrate directly on the specimen holder?

Author: The procedures described relate to the preparation of cells in suspension for X-ray microanalysis, I would not apply these techniques to monolayer cells, because it would be necessary to remove the cells from the monolayer. I feel that it is always necessary to work out the best preparation procedures for a given specimen.

G.M. Roomans: Could the iodine signal in the cells from Table 1 result from medium taken up by the cells by pinocytosis?

Author: Erythrosin B is present in the medium at a final concentration of 0.02%. I think that at this concentration uptake by pinocytosis would not result in a sufficient concentration of dye inside the cell to give a detectable signal for iodine.

G.M. Roomans: Have you investigated whether any changes in volume occurred as a result of the different preparative procedures? An increase in cell volume combined with increased Na and Cl concentrations would be indicative for uptake of these ions by the cells from the medium?

Author: I have not looked at volume changes in the freshly isolated thymocytes. I agree that it would be interesting to establish whether any change in volume occurs in the freshly isolated cells which could account for the high levels of Na and Cl immediately after isolation.

Reviewer I: Would the author please comment on how the methodology utilized for the cited thymocyte studies might or might not eliminate/introduce element redistribution? What specific experiments have been performed to determine which freeze drying method is suitable?

Author: The thymocytes after centrifugation at 500g are in a pellet in which medium may occupy 50% or more of the volume (this has been measured using radioactive tracers to mark the extracellular space). Therefore the medium, high in Na and Cl, is in excess, and this will create a situation in which problems are more likely to occur. Centrifugation to remove as much medium as possible will help prevent contamination by any means whatever. The sections are surrounded with molecular sieve from the sectioning stage onwards, even including the coating procedure. This should help minimise redistribution if it occurs.

As I do not have cryotransfer apparatus available I am unable to determine whether freeze drying in the microscope would be the best procedure to adopt. However the results for the thymocytes after centrifugation at 10,000g are reproducible for a given medium and are in line with the published values for these cells.

B.L. Gupta: I am concerned about inexplicable inconsistencies in your data for Cl. For example in Table 3 the Cl value of 242 ± 18 is completely out of the trend for the change in Na and K in serum medium. Have you considered the problem of Cl adsorption during the drying of sections? The source of Cl could be Freon!

Author: I have wondered about the results for Cl. Contamination with Cl has been documented as a problem in microanalysis and it is possible that Freon could be the source. On the other hand if non specific contamination was occurring I would expect more variability between the different data than is seen. On the whole the results for serum in table 3 are odd in

that Na is also higher than expected (in relation to the other media) and this is coupled with a lower value for K. Foetal calf serum is known to have stimulatory properties and to avoid this autologous serum was used. However serum does contain many growth factors, and thymocytes in vivo are isolated by the presence of a blood-thymus barrier, so this observation could be caused by the cells being stimulated by serum.

B.L. Gupta: Regarding your comments on high molecular weight Dextran as an additive to the suspension medium, it seems from the data in Table 3 that the Dextran sample shows the minimum rise in Na and Cl. Why did you not use Dextran for the data in Table 4?

Author: The problem with the data from Table 3 is that normally if one sees freeze damaged cell one tends not to analyse them. My purpose when undertaking the present experiments was to develop a procedure which could be used as a basis for experiments. It became clear when examining cells suspended in Dextran that in this medium a greater number of cells (about 30%) were freeze damaged than in the other media, these cells had abnormal levels of Na and K but did not take up vital dye. I concluded that some portion of the population is susceptible to the presence of Dextran and because of this did not use this medium any further.

G. Hook: Clinically, elemental analysis of peripheral blood mononuclear cells may be useful for evaluating the cellular mineral status and immunological function of patients. How long must the Ficoll-hypaque isolated peripheral blood mononuclear cells be in culture before the cells are free of I and Na? Recently, we developed an arabinogalactan (Stractan) density gradient method for isolating peripheral blood mononuclear cells that does not seem to contaminate the cells (Microbeam Analysis 1986, pp.217-220). Have you analysed Stractan isolated peripheral blood mononuclear cells?

Author: I have not done any further experiments on the cells isolated in Ficoll or tried the Stractan gradient described by you. I think that the results presented here back up your observations on Ficoll gradients and should alert those who routinely use the Ficoll method. On the other hand the results using a microhaematocrit centifuge suggest that for clinical observations it should be possible to collect the buffy coat directly and thus obviate the need for separation procedures.

J. Wroblewski: As thymocytes are rather small did you consider the possibility of contribution of the cytoplasm or medium to the section?

G. Hook: Your elemental data on normal thymocytes is from analysis of nuclei. What elemental concentrations would you find from analysis of cytoplasm in normal thymocytes?

Author: As 300nm sections of thymocytes were used for analysis it is unlikely that underlying medium would contribute to the spectrum. The heterochromatin of the cell is characteristic and easily identified so contributions from cytoplasm would be minimal. In previous studies the cytoplasmic concentrations of elements have been documented (Warley, 1986b) and apart from lower concentrations of P and K they are similar to those found in the nucleus.

J. Wroblewski: How did you evaluate that 1h was sufficient time for freeze-drying the sections?

Author: In the cryostat that we use 1h represents the minimum time for freeze drying the cryostat remains cold for a long period of time during the warm up and freeze drying will continue.

J. Wroblewski: High pressure during centrifugation at high speed is bound to have some side effects on the cells, may any ionic changes be expected?

Author: Although one may feel that high speed centrifugation ought to upset the ionic balance in these cells I have been unable to find evidence that this occurs unless, as mentioned, the membrane of the cell is damaged prior to centrifugation. The fact that the results for isolated thymocytes which have been subject to high g forces are in the same range as tissue thymocytes which have not been subject to centrifugation indicate that the centrifugation step is probably not causing problems.

G. Hook: What application do you see for digital elemental X-ray imaging in studying normal and diabetic thymocytes?

Author: I think that this technique will be useful as it will give some insight as to whether the levels of elements in cells of the lymphoid series reflect those of other tissues in the body. This is important in diseases such as diabetes where loss of tissue elements can occur.