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PROBLEMS ASSOCIATED WITH THE PREPARATION OF CELL SUSPENSIONS FOR X-RAY MICROANALYSIS HIGHLIGHTED BY THE COMPARISON OF RESULTS WITH THOSE OBTAINED FROM TISSUE SECTIONS

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Abstract

Fully quantitative X-ray microanalysis of freeze dried frozen sections allowed the concentration of monovalent ions in the nuclei of mouse thymocytes in tissue sections to be compared with those from thymocytes which had been isolated by a simple preparation procedure. Isolated cells showed increased concentrations of Na and Cl. This was further investigated in the rat where the size of the thymus allowed comparison between different isolation media using cells derived from the same animal. Use of autologous serum as the final suspending medium gave reproducible results, whereas with suspension in BSS, or 20% dextran the results were more variable. When the results obtained for the cells isolated in serum were compared with values for tissue thymocytes elevated levels of Na and Cl were still apparent although these were not as great as the differences recorded for the mouse. The effect of the drop in temperature which occurs during the isolation procedure could not account for the observed differences.

Key Words: Electron probe, X-ray microanalysis, frozen sections, freeze dried sections, cryoultramicrotomy, thymocytes, cell suspensions.

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Introduction

A major interest in our laboratory is the relationship between structure and function in the thymus gland (Kendall, 1981). A number of immunological tests for thymocyte function are now routinely performed in tissue culture and we wished to be able to establish how these studies relate to thymocyte function in vivo. X-ray microanalysis offers an analytical technique which theoretically should allow us to compare elemental composition of thymocytes in vitro with those in vivo: a situation which is offered by few other analytical methods (Macknight, 1980). Information gained about the elemental levels, particularly the monovalent ions, is of interest as these appear to have a role in cell cycle control in cells in culture (Rozengurt and Mendoza, 1980). We have therefore used frozen sectioning coupled with X-ray microanalysis of the freeze dried sections to compare elemental levels in rodent thymocytes within tissues with those in thymocytes isolated by a simple conventional preparation procedure. Such cell suspensions are commonly used to initiate tissue culture studies. Any results obtained should have wider application in the field of tissue culture where frequently it can be difficult to establish the extent to which the tissue culture cell is representative of its in vivo counterpart.

Materials and methods

Preparation of cell suspensions and tissues for microanalysis.

Mice. Male A2G Swiss mice (approximately 6 weeks old) were killed by intraperitoneal injection of Nembutal. Thymus glands were rapidly dissected out and small cubes of tissue were frozen on stubs in Freon 22 cooled in liquid nitrogen. For the preparation of cell suspensions tissue was pressed through a thin mesh wire gauze and washed through with 10 ml Hanks' balanced salt solution (BSS, 142 mM Na, 5mM K, 145 mM Cl, 0.8 mM Mg, 1.25 mM Ca, 0.8 mM S and 0.8 mM P + 20% fetal calf serum FCS).

Cells were centrifuged at 500 g for 10 min., washed once in 10 ml BSS and then recentrifuged. The supernatant was removed and drops of the thick cell suspension were frozen onto silver stubs as for the tissues.

Rats. Male CSE rats (approximately 250 gms in weight) were killed by chloroform anaesthesia, bled out through the heart, and the blood was retained for the preparation of autologous serum for suspending the cells. The thymus was removed quickly, and thymocyte tissue blocks and suspensions were prepared as for the mouse except that the initial suspension was divided into three. After the first centrifugation (500 g for 5 min.) the pellets were resuspended in BSS, autologous serum, or 20% w/v dextran (Mw 252,000, obtained from Sigma) made up in BSS. The suspensions were recentrifuged and frozen as previously. All samples were stored in liquid nitrogen before sectioning.

Frozen Sectioning and Analysis.

Frozen sections approximately 300 nm thick were cut on a Slee cryoultramicrotome at a temperature of 203 to 208K. The sections were collected using a vacuum suction device onto Formvar coated Ni grids (150 or 300 x 75 mesh). The sections were allowed to freeze dry for three hours in the chamber of the cryostat at the same temperature at which they were cut. They were then placed inside a desiccator and allowed to warm up to room temperature overnight. The grids were carbon coated under vacuum before analysis.

Specimens were analysed in an AEI EMMA-4 electron microscope fitted with a Link Systems 860 series 2 energy dispersive detection system. Analysis proceeded for 100 s live time at 60kV accelerating voltage and 4nA beam current (measured with a Faraday cage). The specimen area was cooled with gaseous N_2 to reduce contamination. Spectra were processed using the Quantem-FLS software (Link Systems Ltd, High Wycombe, Bucks., England). This program has been described in Hall & Gupta, 1982. Quantitation was achieved by comparison with standards made up in gelatin: for full details of the standardisation see Kendall et al. (1985). All of the analyses were conducted with a probe area of 0.8 (rat) or 1.2 μm^2 (mouse).

Results and Discussion

Mouse Thymocytes.

X-ray microanalysis has now been used by several groups for the study of cells in tissue culture. When studies are performed on whole cells in the SEM interpretation of data can be complicated by contribution from overlying extracellular medium if the cells are unwashed (James-Kracke et al., 1980, Zierold, 1980, Wrobleski et al., 1983). But washing cells to remove or replace the culture medium has been reported to alter elemental composition of the cells under study (James-Kracke et al., 1980; Wrobleski et al., 1983). The use of cryosections, as opposed to whole cells, should avoid the problem of the overlying medium. Initial studies were carried out on mouse thymocytes (Kendall et al., 1985). Results for the concentrations of the elements Na, Cl and K, and the significance of the differences between their concentrations in the nuclei of cells in tissues and cell suspensions are shown in Table 1. These results indicate that the isolated thymocytes showed apparent increases in Na and Cl, and were significantly different from those of thymocytes in tissue sections. There was a high correlation between the values of Na and Cl in the isolated thymocytes (correlation coefficient 0.84): no such correlation was observed in the tissue cells (correlation coefficient 0.15).

Table 1 Concentrations of Na, Cl and K in nuclei of mouse thymocytes in tissue sections compared with values from nuclei of thymocytes in suspension (mmol/kg dry weight ± se).

	n	Na	Cl	K
Tissues	64	152±9	247±16	552±17
Suspension	115	778±33	937±35	457±18
t-test		***	***	**

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

The most likely explanation for the finding of high values of Na and Cl are, either damage occurring to the cells during the isolation procedure, or some artefact occurring during the later stages of preparation and analysis. If membrane damage had occurred then the high values of Na and Cl should have been accompanied by low values of K. This was not so. Examination of cell suspensions for viability using the vital stain trypan blue indicated that the cells had a viability of > 99%. We also examined cells by microanalysis after incubating them in culture medium for 4h at 310°K (this time should have allowed recovery from any trauma experienced during the isolation procedure). However, in this experiment high values of Na and Cl were still recorded.

A problem of apparent overestimation of Na and Cl when using X-ray microanalysis has been recognised by Tormey and Platz (1979) in studies on sheep red blood cells. They ascribed the problem to electron scatter which occurred when the specimens were examined at low voltage in the scanning electron microscope (SEM). The conditions of analysis which we used, i.e., higher kV and the examination of specimens in a transmission instrument should have eliminated any problems from this source. We feel that the values for Na and Cl are artefactually high and that the correlation between these two elements does suggest that the medium is probably the source of the problem.

Table 2 Concentrations of Na, Cl and K in nuclei of rat thymocytes isolated in the presence of different media (mmol/kg dry weight ± se).

Treatment	n	Na	Cl	K
BSS	22	493±42	724±48	725±29
20% Dextran	18	285±42	361±27	467±59
Serum	25	226±17	369±26	659±39
BSS	25	229±30	369±19	527±22
20% Dextran	24	150±24	464±35	648±61
Serum	25	220±19	271±14	588±33

Table 3 Concentrations of Na, Cl and K in nuclei of rat thymocytes in tissue sections compared with values from nuclei of thymocytes suspended in autologous serum (mmol/kg dry weight ± se).

	n	Na	Cl	K
Tissue	22	123±12	141±8	678±29
Suspension in serum	50	223±13	319±16	623±26
t-test		***	***	n/s

*** $p < 0.001$; n/s = not significant

Composition of bathing medium.

We therefore decided to investigate the effect of varying the bathing medium. For this study we chose to use a larger animal (the rat) as sufficient cells would then be obtained from one animal to enable different treatments to be performed on cells from the same source, thus avoiding any interanimal variation. Levels of the monovalent ions were studied in thymocytes from two different rats. The thymocytes were exposed to BSS (with no fetal calf serum (FCS) which is known to be mitogenic), autologous serum, or 20% dextran prepared in BSS. The results are shown in Table 2. Examination of these results shows that the different treatments apparently affect the Na and Cl concentrations obtained and there was also variation between the animals when BSS or dextran was used. In the case of serum treatment, there were no significant differences between animals for Na and K concentrations but some difference in chlorine levels did occur ($P < 0.01$). The data for the serum from the two animals were pooled and analysis of variance was performed to assess the effect of variation within treatment and animals. The results showed that the variation from one treatment to another was greater than the variation within a sample.

Since BSS is an isotonic saline the ionic equilibrium of the cells should not be disturbed. Although it is still not clear why we find the variation in results from cells suspended in BSS, we note that other authors who have studied cryosections of cultured cells have found variation in their results too (James-Kracke et al., 1980; Warley et al. 1983a,b ; Zierold et al. 1984). It is also notable that the values for Na and Cl for the BSS treatment in these experiments were much lower than those recorded for the mouse, and in all treatments for the rat we did not see the strong correlation between Na and Cl which was noted in the mouse.

Use of dextran as a high molecular weight cryoprotectant was first proposed by Franks et al. (1977), additionally its presence in the medium should also give physical support to suspended cells especially during the sectioning procedure. It has been used successfully as a supporting medium, particularly in studies on red cells (Jones et al., 1979, Tormey, 1981) at the same concentration which we used (20%). However, Echlin et al. (1977) noted that prolonged treatment of HeLa cells with 25% dextran did cause physiological disturbance, and more recently, Barnard (1982) has also documented functional disturbances in tissues exposed to this substance. Although our cells were exposed to the polymer for less than 10 min. it is possible that the membrane equilibrium may have been disturbed.

Autologous serum appears to give the least variable results, possibly because it, like dextran, should provide support for the cell suspension during sectioning. It also may represent a more natural environment for the cells.

Differences between cells and tissues in rats.

Comparison of tissue levels with the pooled results for cells suspended in serum (Table 3, Fig 1) shows that Na and Cl values were still higher than in tissue sections. One of the dextran treatments did give Na values close to the tissue but Cl levels were high and the results were not as reproducible as the two serum treatments.

Effect of temperature.

It may be suggested that, as the cells were isolated at room temperature, there might have been an elevation of Na due to inactivation of the membrane Na/K ATPase. This enzyme is known to be temperature dependent in red cells of non-hibernating animals (Ellory & Willis, 1983). So we examined the results from cells prepared in serum at 310°K with those obtained under our normal procedure. These studies (Table 4) showed that the drop in temperature which occurs during the normal isolation procedure cannot explain the differences in Na content which we have observed between cells and tissues (although a difference in K values was noted).

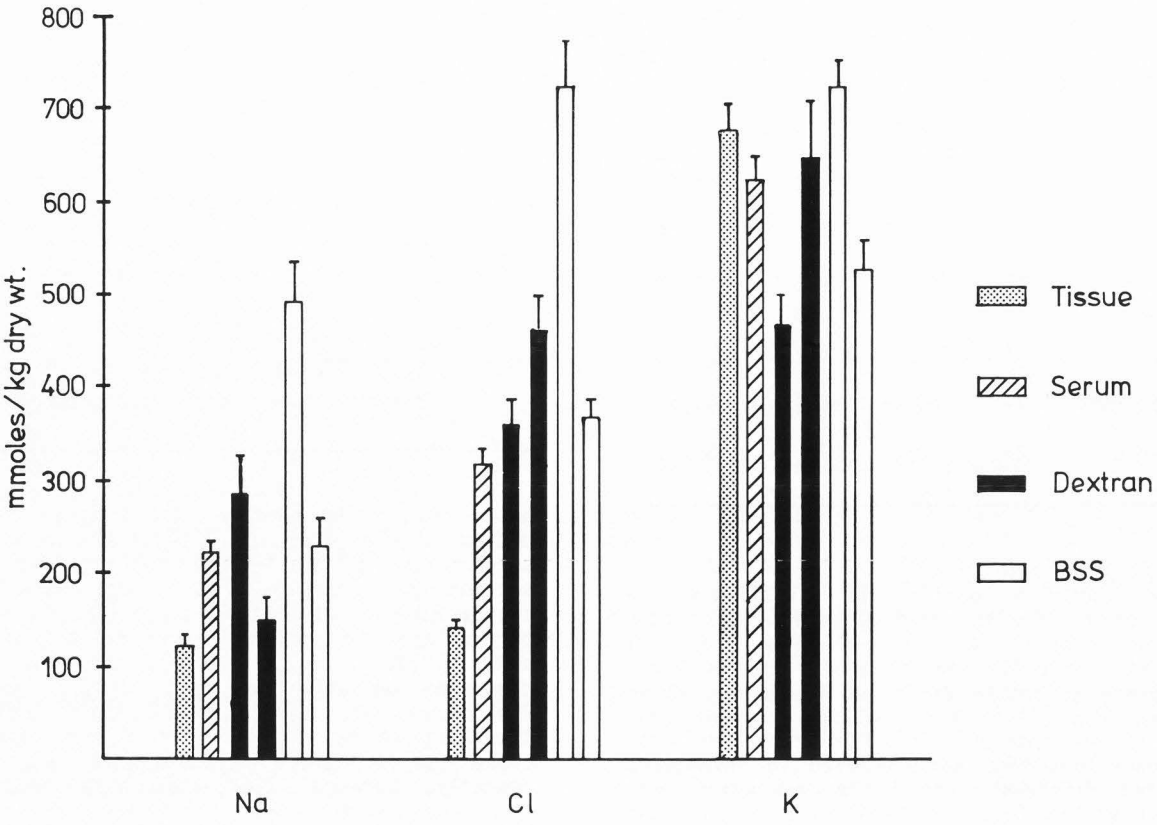

Fig. 1 Concentrations of Na, Cl and K in nuclei from tissues, and the values obtained from cells suspended in different media (mmoles/kg dry wt ± se).

Table 4 Concentrations of Na, Cl and K in nuclei of rat thymocytes isolated in serum at 37°C and room temperature (mmol/kg dry weight ± se).

	n	Na	Cl	K
Room Temp.	26	287±22	366±20	713±29
37°C	24	296±29	391±21	600±36
t-test		n/s	n/s	*

* $p < 0.1$; n/s = not significant

In conclusion, we would draw attention to the fact that isolation procedures appear to result in increased concentrations of Na and Cl in sectioned suspensions of thymocytes compared to their tissue counterparts. In our hands the use of autologous serum as a suspending medium gave reasonably reproducible results, although Na and Cl were still higher than in tissue sections. At present it is not clear why this is so, but we feel that the origin of this difference needs to be better understood before meaningful comparisons can be made between the in vitro and in vivo situation.

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References

Barnard T. (1982). Thin frozen dried cryosections and biological X-ray microanalysis. J. Microsc. 126, 317-332.

Echlin P, Skaer HLeB, Gardiner BOC, Franks F, Asquith MH. (1977). Polymeric cryoprotectants in the preservation of biological ultrastructure. II. Physiological effects. J. Microsc. 110, 239-255.

Ellory JC, Willis JS. (1983). Adaptive changes in membrane transport systems of hibernators. Biochem. Soc. Trans. 11, 330-332.

Franks F, Asquith MH, Hammond CC, Skaer HLeB, Echlin P. (1977). Polymeric cryoprotectants in the preservation of biological ultrastructure. I. Low temperature states of aqueous solutions of hydrophilic polymers. J. Microsc. 110, 223-238.

Hall TA, Gupta BL. (1982). Quantification for the X-ray microanalysis of cryosections. J. Microsc. 126, 333-345.

James-Kracke MR, Sloane BF, Shuman H, Karp R, Somlyo AP. (1980). Electron probe analysis of cultured vascular smooth muscle. J. Cell Physiol. 103, 313-332.

Jones RT, Johnson RT, Gupta BL, Hall TA. (1979). The quantitative measurement of electrolyte elements in nuclei of maturing erythrocytes of chick embryo using electron-probe X-ray microanalysis. J. Cell Sci. 35, 67-85.

Kendall MD. (1981). (ed). The Thymus Gland, Academic Press, London: New York.

Kendall MD, Warley A, Morris IW. (1985). Differences in apparent elemental composition of tissues and cells using a fully quantitative X-ray microanalysis system. J. Microsc. 138, 35-42.

Macknight ADC. (1980). Comparison of analytic techniques: chemical, isotopic and microprobe analyses. Fed. Proc. 39, 2881-2887.

Rozengurt E. Mendoza SA. (1980). Monovalent ion fluxes and the control of cell proliferation in cultured fibroblasts. Ann. N.Y. Acad. Sci. 339, 175-190.

Tormey JM. (1981). Preparation and use of erythrocyte sections as methodology for validating procedures for X-ray microanalysis of electrolytes, in: Microprobe analysis of biological systems, T.E. Hutchinson and A.P. Somlyo (eds), Academic Press, New York, 177-195.

Tormey JM, Platz RM. (1979). Origin of artefactual quantitation of electrolytes in microprobe analyses of frozen sections of erythrocytes. Scanning Electron Microsc. 1979; II: 627-634.

Warley A, Stephen J, Hockaday A, Appleton TC. (1983a). X-ray microanalysis of HeLa S3 cells. I. Instrumental calibration and analysis of randomly growing cultures. J. Cell Sci. 60, 217-229.

Warley A, Stephen J, Hockday A, Appleton TC. (1983b). X-ray microanalysis of HeLa S3 cells. II. Analysis of elemental levels during the cell cycle. J. Cell Sci. 62, 339-350.

Wrobleski J, Roomans GH, Madsen K, Friberg U. (1983). X-ray microanalysis of cultured chondrocytes. Scanning Electron Microsc. 1983; II: 777-784.

Zierold K. (1980). X-ray microanalysis of tissue culture cells in SEM and STEM. Scanning Electron Microsc. 1981; II: 409-418.

Zierold K, Schafer D, Pietruschka F. (1984). The element distribution in ultrathin cryosections of cultured fibroblast cells. Histochem. 80, 333-337.