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Ultrastructural features of acute monoblastic leukaemia cells: A multivariate morphometric analysis

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Summary. Morphometric studies were carried out on five ultrastructural measures of size or quantity of some of the intracellular organelles in monoblasts obtained from six patients diagnosed as having acute monoblastic leukaemia and also on monocytes from six normal controls. The morphometric measures were analysed using a one way multivariate analysis of variance (MANOVA) to see whether acute monoblastic leukaemic cells differed from those of normals. It was found that there was a highly significant decrease both in the surface to volume ratio of mitochondria and also in the surface to volume ratio of the nucleus. The possible physiological significance of these structural changes is stressed.

Key words: Monoblastic leukaemia – Electron microscopy – Morphometry – Multivariate statistics

Introduction

Acute monoblastic leukaemia is classified as AML M5 by the French-American-British Cooperative Group. It is further subdivided into M5a where more than 80% of all the monocytic cells are monoblasts and M5b where less than 80% of the monocytic cells are monoblasts and where the remainder are predominantly promonocytes and monocytes (Bennett et al. 1985). The diagnosis is based on the characteristic morphology of the leukaemic cells at light level using Romanowsky stains. Additional evidence is provided by fluoride sensitive cytoplasmic α-napthyl acetate (ANAE) and by raised levels of both serum and urinary lysozyme. The expression of monocyte antigens on leukaemic cells using various monocyte associated monoclonal antibodies has also been demonstrated. However, immunological and cytochemical heterogeneity may occur within the category of FAB cells M5 with some typical morphological cases being negative for ANAE and others negative for monocyte monoclonal antibodies (Milligan et al. 1984). A number of studies have described qualitative ultrastructural features found in monocytic leukaemia (Kim et al. 1982; Djaldetti et al. 1984; Darbyshire et al. 1985). The aim of this investigation was to quantify some of the important ultrastructural features of the monocytic M5b cells using morphometric techniques in six patients with M5b. Monocytes from six normal individuals were also studied for comparison. Multivariate statistical analysis was used to compare M5b with normal cells.

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Materials and methods

Clinical material. Six patients with standard haematological criteria of M5b were studied as they presented. The clinical data on these patients is shown in Table 1. Normal controls were randomly selected volunteers in good health with normal peripheral blood counts.

Optical microscopical studies. Cytochemical methods: Standard methods were used to demonstrate peroxidase, non-specific esterase and chloroacetate esterase (Shibata et al. 1985). Serum and urine lysozyme: these were measured by a turbidiometric method based on the lysis of *micrococcus lysodeikticus* by lysozyme (Harrison and Swingler 1971).

Cell surface maker studies. The presence of membrane I_a (OKI_a-ortho) and $UCHM_1$ were assessed using both conventional indirect immunofluorescence and the monoclonal antibodies OKI_a and CD 14.[1]

Ultrastructural studies. Leucocytes were obtained following dextran sedimentation of venous blood. Fixation occurred in 1.5%

[1] Monoclonal antibodies were kindly provided by Dr. P. Beverley and for which the authors are grateful

Table 1. Modes of presentation, clinical features and therapeutic responses[a]

Patient	Sex/Age	Presentation	Splenic enlargement (cms)	Chemotherapy	Remission
T.N.	F71	Chest pains on exertion	2	Complete remission	18 months
E.W.	M47	Tiredness	0	Complete remission	24 months
C.W.	F58	Nasal obstruction	0	No remission	
E.B.	M69	Abdominal pain	2	No remission	
E.K.	F24	Gum hypertrophy and Gingivitis	0	Complete remission	10 months
E.B.	F65	Nosebleed	2	No remission	

[a] Previous illnesses: None except E.B. previously diagnosed as having a small cleaved lymphoma identified by lymph node biopsy. Treated with low dose Chlorambucil for 5 years

glutaraldehyde contained in 0.1 M cacodylate buffer (pH 7.4) for 1 h at room temperature (18° C). The fixed buffy layers were then processed, embedded, sectioned and stained according to our standard laboratory procedures procedures. Electron micrographs were obtained using a Phillips 400 EM. The final magnification of the prints was ×12,000.

Morphometric methods. Morphometric data was obtained according to previously described detailed methods (James 1983; Hughson 1985; Sokol et al. 1985). Standard morphometric techniques (Underwood 1970; Weibel 1979) were used to obtain measures of mitochondrial volume fractions $V_v - m$ and nuclear volume fractions $V_v - n$ related to whole cells. Surface to volume ratios of mitochondria, nuclei and cell cytoplasm (S/V) were also obtained. Volume fractions were estimated by standard point counting methods to establish areal fractions of cells occupied by mitochondrial and nuclear profiles. Surface to volume ratios were measured using Chalkley's method (Underwood 1970) by calculating the ratio $2I/P$ where I is the profile intersection count per unit length of test probe and P the point fraction of test grid points overlying each of the test organelles.

The morphometric techniques were implemented on specimen blocks randomly selected for microtomy. Since the mixing of blood cells both in the circulation and during the preparative laboratory procedures precludes the presence of any spatial tissue structure only random assemblages of cells in tissue blocks are available. The sampling regime is, therefore, adequate in selecting an *a priori* determined number of cells non-systematically. For each control subject and patient 50 identically and independently drawn cells were selected for analysis following preliminary sample size estimates for type I and type II, errors being set at 0.05 and 0.20 respectively for detecting 10 percent differences in morphometric measures of size. Care was taken to ensure that only one profile per cell was photographed for analysis and that each profile contained some identifiable region of nucleus.

Statistical methods. Since five measures of size are being compared for both normal and leukaemic cells univariate statistics which are appropriate only for single comparisons will not yield true levels of significance for sets of data. The Bonferroni relation (Seber 1984; Sokal and Rohlf 1981) illustrates the overall simultaneous significance level (S) for a set of 5 comparisons to their individual test levels ($\alpha = 0.05$)

$$S = 1 - (1 - \alpha)^5$$

Individual conventional values of 0.05 for each individual test yield an overall level for S of up to 22.6%. A multivariate approach was therefore used in the present study to allow for such multiple comparisons. The test was carried out using an $SPSS_X$ mainframe statistical package (issue ×2.1) implemented on the University of Sheffield Prime 9955-II computer. Raw data volume fractions for nuclei and mitochondria were transformed prior to analysis using the arcsin $\sqrt{V_v}$ transformation where $(0 < V_v < 1)$. Multivariate normality and homogeneity of variances using Bartlett's test were also carried out using the $SPSS_X$ program to satisfy the necessary conditions for valid MANOVA (Seber 1984). Significance values of the difference between the set of control and patient variables are given by the standard tests of Pillais, Hotelling, and also of Wilks and Roy which are listed in descending order of relative power. Since the Hotelling's T^2 test is directly related to a one way MANOVA, this analysis was also carried out for computational convenience to obtain an indication of the relative strengths of the contributions of the variables (measures of size) to the overall significance levels.

Results

Haematological results

Peripheral blood and bone marrow results are given in Table 2 to illustrate the essential diagnostic features. These show that in all six cases the leukaemia is of the FAB type M5b although there is some variability of cytochemical and immunological reactions.

Ultrastructural results

Qualitative results. Sections of cells from the different patients and 1 normal are shown in plates 1 to 7. The main ultrastructural features of the leukaemic cells were: 1. Irregular cytoplasmic outline with numerous pinocytotic vesicles and vacuoles around the periphery of the cell. Some vacuoles contained breakdown products. It is assumed these are phagocytic vacuoles (Figs. 3 and 4). 2. Bundles

Table 2. Results of cytological, fluid assay and chromosome analyses[a]

Patient blood	T.N.	E.W.	C.W.	E.B.	E.K.	E.B.
Haemoglobin g/dl	10.3	6.9	10.2	9.4	8.6	10.7
White count $\times 10^9$/l	10.5	17.6	38.5	12.2	23.7	40.4
Platelets $\times 10^9$/l	169	149	89	47	82	16
Marrow						
Cellularity	+++	+++	+++	+++	+++	+++
% Blasts	93	95	90	90	95	96
Cytochemistry	82% ANAE	80% ANAE	95% ANAE	95% ANAE	3% ANAE 2% ANAE	30% ANA 2% ANA
FAB type lysozyme	M5b	M5b	M5b	M5b	M5b	M5b
Serum µg/ml (N.R. 3.6–10)	650	122	110	650	93	94
Urine µg/ml (N.R. <1)	2,800	440	820	N.D.	940	188
Monoclonal antibodies Ia (%)	75	84	79	95	30	59
Chromosomes	46XX	46XY	46XX	46XY 46XY*	46XX** 47XX 46XX	46XX
Blast cells	monocytic	monocytic	monocytic	monocytic	monocytic	monocytic

[a] ANAE = α-naphthyl acetate esterase; CE = chloroacetate esterase; N.R. = normal range; N.D. = not demonstrated. * del 7; ** del 9q (62%) +8 (8%)

of microfilaments were present in numerous cell sections, mainly adjacent to the nucleus (Figs. 1 to 6, inclusive). 3. The nucleus appeared convoluted; some sections presenting numerous nuclear outlines. Nucleoli were often seen. Heterochromatin was distributed mainly around the periphery of the nuclear outline (Figs. 3, 4, 5 and 6). The cell profiles from normal monocyte populations show the same cell structures: granules, microfilaments, convoluted nuclei and peripheral phagocytic vesicles (Fig. 7). The differences appear to be in the relative quantities of structures present and not in qualitative features.

Quantitative results. The raw data for the five measures of size (variables) for both control and leukaemic cells are recorded in Table 3. Nuclear and mitochondrial volume fractions were found to be significantly increased whilst surface to volume ratios of the whole cell, the nuclei and also the mitochondria were found to be decreased in leukaemic cells.

Inspection of individual variables would appear to show differences even at the raw data level. The multivariate significance tests of Pillais, Ho-

Table 3. Primary data on nuclear and mitochondrial volume fractions and other surface to volume measures[a]

No.	Nuclear volume fraction	Cell S/V	Nuclear S/V	Mitochondrial S/V	Mitochondrial volume fraction
	V_v%	µm²/fl	µm²/fl	µm²/fl	V_v%
1	29.6	0.77	1.63	3.41	3.50
2	31.3	0.88	1.75	2.99	5.00
3	31.7	0.76	1.38	3.22	3.80
4	28.5	0.93	1.46	3.64	3.50
5	34.8	0.88	1.70	3.73	3.60
6	29.5	0.81	1.71	3.51	4.30
7	21.0	0.85	2.15	16.2	2.60
8	25.0	0.98	2.04	15.4	2.80
9	21.0	0.89	2.13	15.7	2.70
10	28.0	0.95	1.99	14.9	2.90
11	20.0	0.95	1.99	14.9	3.00
12	26.0	0.94	1.96	15.4	3.00

[a] Percentage data items were transformed prior to analysis using the function arcsin $\sqrt{V_v}$. Upper group of six values obtained from patients; lower group of six values obtained from control subjects

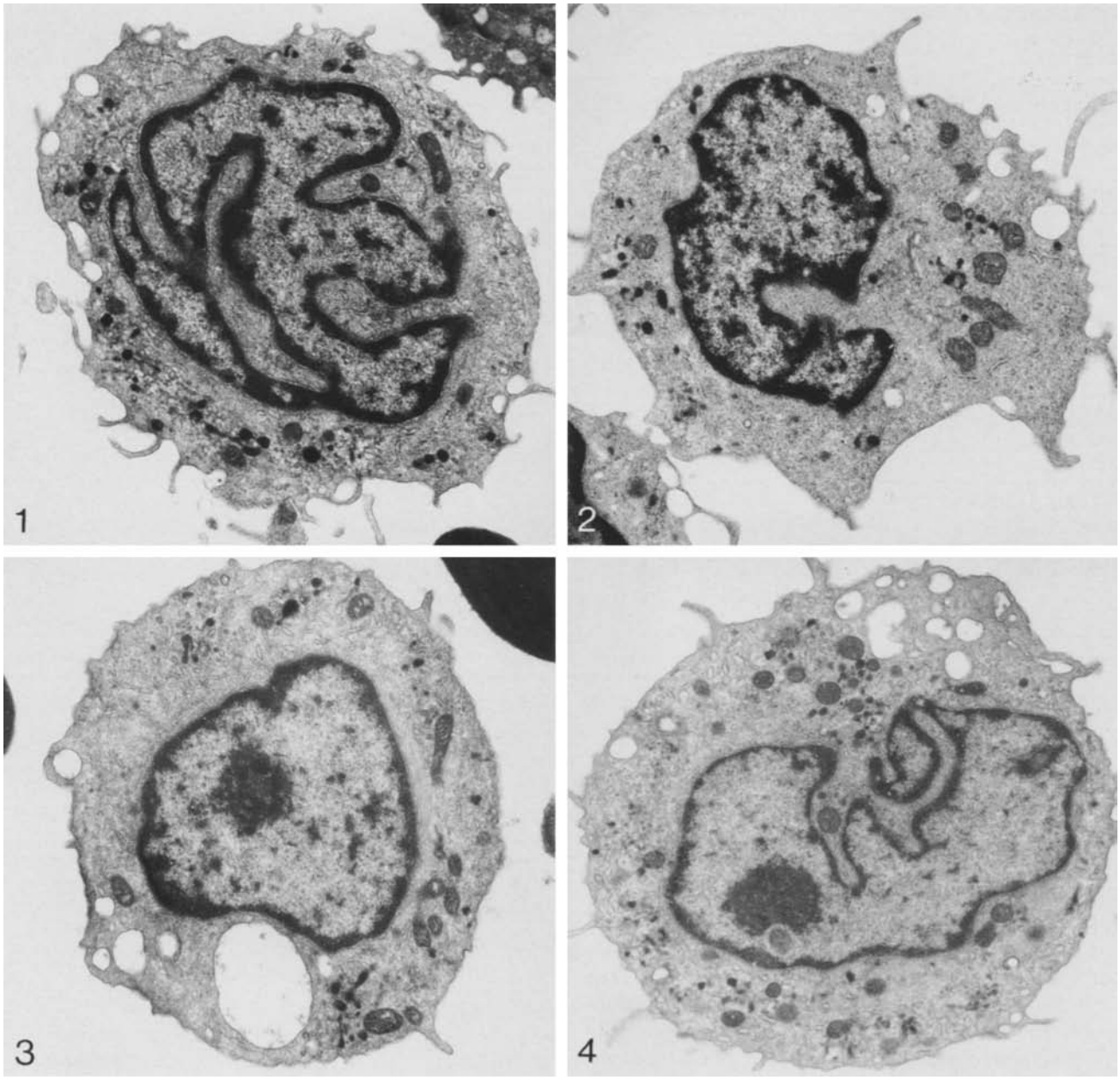


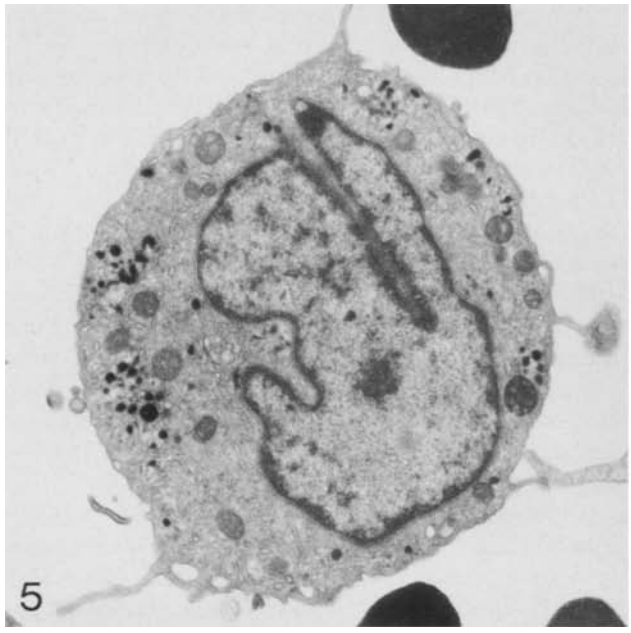
Table 4. Univariate hypothesis and error mean squares for individual measures illustrating their relative contributions to the overall multivariate significance

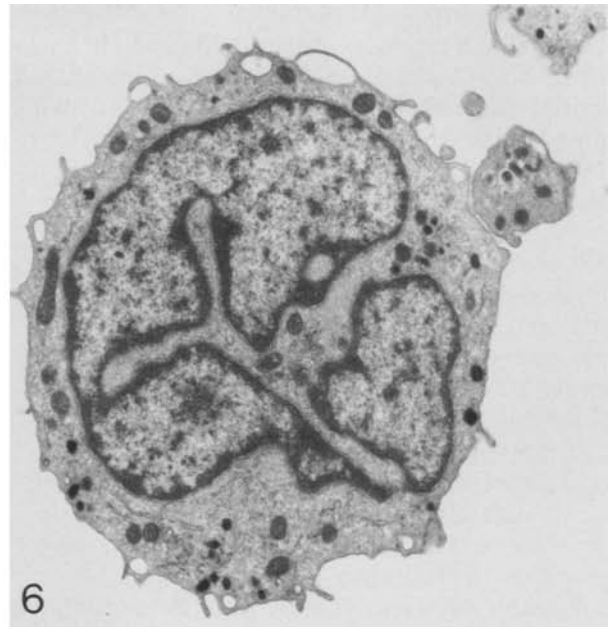
Variable	Hypothesis mean square	Error mean square	F value	Significance level
V_v nucleus	32.795	5.846	5.609	0.039
S/V cell	0.023	0.003	6.723	0.027
S/V nucleus	0.576	0.014	39.784	0.0001
S/V mitochondria	431.999	0.160	2690.143	0.0001
V_v mitochondria	9.205	0.402	22.849	0.0001

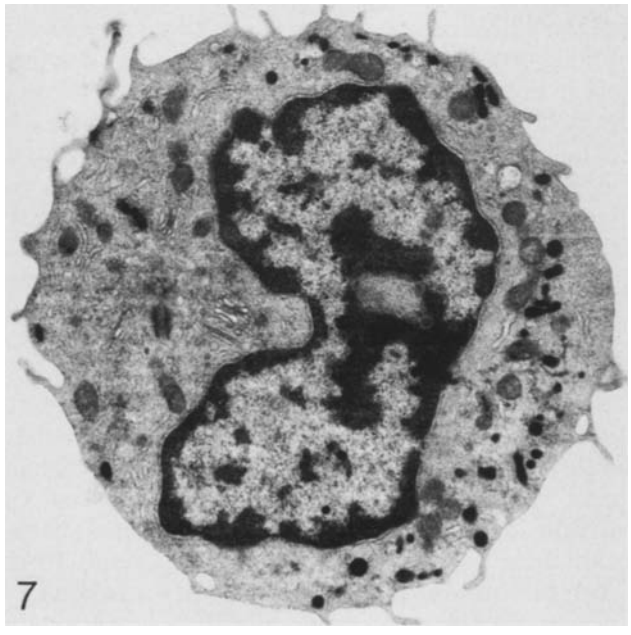
telling, Wilks and that of Roy were found to be 0.998, 912.616, 0.001, 0.998 respectively.

All these tests were highly significant at $p<$ 0.001 and clearly indicate that the set of values for leukaemic cells is markedly different from that of normal monocytes. The relative contributions of each of the 5 measures to the overall significant difference is illustrated in Table 4 which shows the univariate hypothesis and error mean squares, their corresponding F ratios and their significance levels.

The two measures of size which seem to be contributing most strongly to the overall level of







Figs. 1–6. Electron micrographs obtained from each of the 6 patients, × 8,500

Fig. 7. Electron Micrograph obtained from section of normal control monocyte, × 8,500

multivariate significance are S/V for mitochondria and S/V for nuclei. The next most important contributing variable is the V_v for mitochondria whilst the S/V for the cell and V_v for the nucleus seem to be contributing least. All, however, are highly significantly different as univariate measures.

Discussion

This is a study of 6 patients with pure monocytic leukaemia (M5b) established by clinical and haematological methods including cytochemical tests and cell surface marker studies. The ultrastructure of the leukaemic cells is consistent with previously published accounts (Kim et al. 1982; Darbyshire et al. 1985; Djaldetti et al. 1984). An interesting feature of the cells is the apparent maturity of the cytoplasm: granules, vacuoles, mitochondria, smooth and rough ER are all present. Bundles of microfilaments occur frequently. These structures are all found in normal peripheral blood monocytes. Using MANOVA for the 5 variables studied our results indicate that the leukaemic and normal monocytes show significant differences overall, within the confidence limits set by the tests of Pil-

lais, Wilks and Roy. Of particular interest are the variables which show the greatest changes. In our studies the outstanding differences lay in the mitochondrial surface to volume ratios. For normal monocytes the values range from 14.9 $\mu m^2/fl$ to 16.2 $\mu m^2/fl$ whereas in the leukaemic population values range from 2.99 $\mu m^2/fl$ to 3.64 $\mu m^2/fl$. Similar methods were used in a study by Sokol et al. (1985) comparing blood monocytes from patients with Hodgkin's Disease, Non Hodgkin's Lymphoma and normal blood. The surface/volume ratio of mitochondria in normal monocytes was found to be 14.8 ± 0.36 $\mu m^2/fl$ in close agreement with the values found in the present study. A further study by Sokol et al. (1985) on cultured human macrophages finds a surface/volume ratio of mitochondria in a mononuclear phagocyte cell suspension to be 12.6 ± 0.3 $\mu m^2/fl$. There have been no previous quantitative studies on monoblasts. The reduced surface to volume ratio of mitochondria in monoblasts compared to monocytes is of considerable interest. This reduction could be due to several factors such as an increase in average mitochondrial size even if there is no change in mitochondrial shape or changes in mitochondrial shape from a filamentous to a more globular form. Other changes are also possible though in the absence of specific knowledge of three dimensional mitochondrial shapes obtainable from two dimensional micrographs further studies are required. On the whole the change could reflect a larger total mass of mitochondria. The postulate that monoblasts have a larger total mass of mitochondria than monocytes would be consistent with more recent studies of leukaemic cell proliferation. McCulloch (1984) indicates that physiological studies of the proliferative status of blast cell populations in acute myeloid leukaemia showed that they were uniformly in the active cell cycle. Resting cells were not identified in these studies, results conflicting with previous conceptions of leukaemic cell maturation. Our quantitative studies in monoblasts would support the view that these cells also are in an active proliferative phase requiring the energy derived from mitochondrial activity. A quantitative study of mitochondria during the cell cycle in HeLa cells by Posakony et al. (1975) suggested that the estimated number of mitochondria per cell increased in an essentially continuous manner. They found there was continuous growth of the mitochondrial outer membrane during interphase. Their results also pointed to a stable mitochondrial shape distribution. Our quantitative results are however not directly comparable with those of Posakony et al. (1975) due to differing methodologies. It is interesting however that similar interpretations of mitochondrial role are possible.

The other highly significant difference in our study was the reduction in surface to volume ratio of the nucleus from a range of 1.96 to 2.15 $\mu m^2/fl$ in normal monocytes to a range of 1.36 to 1.75 $\mu m^2/fl$ in the monoblasts. This may also reflect an increase in nuclear size or the possibility of additional membrane changes resulting in a less convoluted nuclear outline in monoblasts. Objective criteria for the simple measurement of nuclear membrane convolutions is both difficult and time consuming and further studies on such curvature may have to await the development of both more advanced theoretical and more automated empirical techniques.

The greater degree of cellular activity involved in more rapid mitoses and growth would necessarily need a correspondingly greater level of nuclear activity which would possibly be reflected in increases of nuclear size or volume fraction relative to the whole cell or in alterations to nuclear membrane structure. It would be of considerable interest in future studies to determine whether the expected greater nuclear activity is associated with a fall in the eu- to heterochromatin ratio found in other types of malignancy which is thought to indicate significantly increased cellular activity (Ghadially 1975).

Increases in nuclear volume fraction probably reflect real increases in nuclear size. Whilst any alteration in volume fraction such as an increase in a test organelle, V_v, could be generated by either an increase in size of that organelle or a decrease in the cell cytoplasm it would appear unlikely that differences in normal compared with leukaemic cells would be due to such differences between organelle or cytoplasmic sizes. Similarly the mitochondrial volume fractions probably result from real increases in the absolute mass of mitochondria present in the leukaemic cells. It is unfortunate that at the present no convenient methods exist for measuring numbers of mitochondria per unit volume of cell since the problem in mathematically intractable. A major reason for the intractability is that re-entrant (concave) surfaces allow single mitochondria to have two or more profiles which cannot be differentiated from those of separate mitochondria. Any attempts to overcome this problem, for example, using a relatively thick section technique (even if technically feasible) for direct counting could be invalidated by possible three dimensional regional gradients within cells.

Quantitative characterization of organelle structure represents an important development in

all areas of cell pathology. Only by quantification can the non obvious differences between cell populations be detected. Numerical differences are necessarily objective and correlations of organelle changes can be more readily made with known alterations in function.

The aim of the study was to use quantitative methods to establish whether numerical differences could be demonstrated between leukaemic and normal monocytes. Morphometric methods are increasingly used in pathology to quantitate differences in cells and tissues (Baak and Ort 1983). Before one can compare the results obtained by different workers it is important that the statistical methods used to analyse the results are established as sound. The advantages of using multivariate statistics where a relatively large number of measures of size or quantity are estimated are worth stressing. First is the avoidance of numerous type I statistical errors in which significant differences are found when, in fact, the appropriate null hypothesis of no difference should be accepted. Second, the use of Hotelling's T^2test allows a discriminant function to be calculated. Such a discriminant function will allow future cases to be diagnosed as belonging either to a normal or pathological group using this simple but objective multivariate rule (Baak and Oort 1983). Ultimately, the discriminatory power of discriminant analysis and similar statistically based methods using the type of quantitative data reported in the present study will facilitate the development of automatic measurement devices to detect diagnostic changes routinely. There is a need, in view of the ever increasing complexity of the classification of the leukaemias and the impact of such classifications on treatment schedules for objective quantitative data on cell structures.

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