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Human macrophage development: a morphometric study*

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INTRODUCTION

In previous investigations of patients with malignant lymphoma (Sokol, Durrant & Hudson, 1980: Sokol, Hudson, Wales & James, 1985: Sokol, Wales & Hudson, 1985*a*), distinctive differences were noted in the morphology of mononuclear phagocytes which seemed consistent with disordered cell maturation. In order to make objective comparisons, it seemed necessary to quantify the changes which normally occur during the maturation of human monocytes into macrophages. There appear to have been no previous stereological studies of macrophage development, although numerous investigators including Sutton & Weiss (1966), Sutton (1967), Fedorko & Hirsch (1970), Van der Rhee (1979), Ackerman, Zuckerman & Douglas (1981), Stevenson *et al.* (1981) and Yoda *et al.* (1984) have described the ultrastructural changes in both man and animals. While Tsukada *et al.* (1985) have provided some relative quantitative data on macrophage development, their studies were not stereological, being carried out on adherent cells.

In the present investigation, the use of a recently described method for culturing mononuclear phagocytes in suspension (Sokol, Wales & Hudson, 1985*b*) has allowed a morphometric analysis to be made during the development of macrophages from human blood monocytes over a period of six days.

MATERIALS AND METHODS

Studies were carried out on blood cells from 6 male and 4 female subjects with mean age ($\pm$standard error) of $39{\cdot}3 \pm 3{\cdot}1$ and $33{\cdot}0 \pm 4{\cdot}7$ years respectively. All enjoyed good health and were not taking any medication.

About 40 ml of venous blood was collected from each and defibrinated with polystyrene granules under sterile conditions. Mononuclear cells were then separated, washed, divided into aliquots and cultured in suspension by the method previously described by Sokol *et al.* (1985*b*). One aliquot of cells was examined without culture (Day 0 specimen) and the others were studied after culture for 1, 2, 4 and 6 days respectively. After assessment of viability by trypan blue exclusion, the cells were washed with sterile isotonic saline. They were then pelleted into beam capsules, fixed with 1·5 % glutaraldehyde in 0·1 M cacodylate buffer at pH 7·2 for 1 hour, washed with 7 % sucrose in buffer and postfixed in 2 % osmium tetroxide at 4 °C for 1 hour. The specimens were then prepared for electron microscopy by standard methods (Sokol, Wales, Norris & Hudson, 1982) and examined in a Philips 400T

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electron microscope. Mononuclear phagocytes were readily distinguishable from lymphocytes (which were also present and showed no evidence of transformation) by their characteristic ultrastructural appearances (Van der Rhee, 1979).

Between 12 and 16 micrographs of nucleated mononuclear phagocytes were obtained in an independent uniform random manner (at magnifications ranging from 7000 to 14000) from each culture, and morphometric analysis was carried out by methods similar to those described by Sokol, James, Wales & Hudson (1985). The following morphometric/stereological parameters were obtained: *for the whole cell*, profile area (A_C), volume (V_C), surface-to-volume ratio ($(S/V)_C$) and surface area (S_C): *for the nucleus*, volume fraction (corrected for nuclear-biased sampling technique using the formula for eccentric nuclei (Mayhew & Cruz, 1973)) (V_{VN}), profile area (A_N), volume (V_N), surface-to-volume ratio ($(S/V)_N$) and surface area (S_N): *and for the mitochondria*, volume fractions within whole cell and within cytoplasm ($V_{VM\text{-}C}$ and $V_{VM\text{-}cyto}$), profile area (A_M), volume (V_M), surface-to-volume ratio ($(S/V)_M$), surface area (S_M) and number of profiles per section, together with the profile area of individual mitochondria (A_m). In this investigation, V_M was calculated from $V_{VM\text{-}cyto}$ to minimise the effect of nuclear-biased sampling; $V_{VM\text{-}C}$ was then derived from V_M/V_C. In addition, the mean diameter of the nucleus (D_N) was derived from A_N, compensation being made for non-equatorial sectioning (Underwood, 1970); the mean diameter of the whole cell (D_C) was derived from V_C. 'Excess surface membrane' was calculated for both cell and nucleus as the difference between S_C (or S_N) and the surface area of a sphere of equivalent volume to V_C (or V_N); an estimate of cell and nuclear 'irregularity' was also obtained by dividing S_C (or S_N) by the surface area of the appropriate sphere of equivalent volume.

Statistical analysis

The data was analysed using a two-way analysis of variance for fixed effects (Sokal & Rohlf, 1981). Our statement of experimental design is given by the expression

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \epsilon_{ijk},$$

where Y_{ijk} is the variate response, μ is the general mean, α_i is the effect due to length of culture, β_j is the effect due to different subject, $(\alpha\beta)_{ij}$ is the effect of interaction between α_i and β_j, and ϵ_{ijk} is the experimental error.

In the present analysis, interaction effects were assumed to be zero (i.e. $(\alpha\beta)_{ij} = 0$) since Tukey's test for additivity (Sokal & Rohlf, 1981) was not applicable. The analysis of variance was taken to be of Type I model, in that subjects and time were regarded as fixed effects and not subject to random variation as in a Type II model. The adoption of a Type I model facilitated the calculation of least significant differences using the error mean square S_e^2. In the first instance, the F test was used to detect the presence of variation of the parameters between culture time intervals. Analysis of the raw data using the W test for normality (Shapiro & Wilk, 1965) and the G test for equality of variances (Cochran, 1941) showed non-normality and significant heteroscedasticity (heterogeneity of variances). Logarithmically transformed data were therefore used in the analysis as the W and G tests showed these to be both normally distributed and to be homoscedastic (similar variances).

Nine parameters were analysed separately at Days 0, 2, 4 and 6 (Day 1 results were not included in this analysis for reasons of time symmetry). The parameters

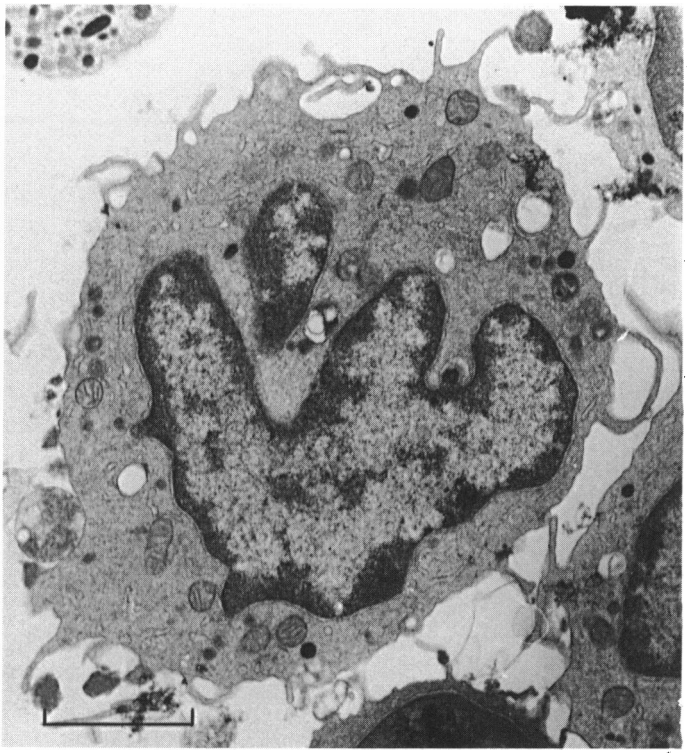

Fig. 1. Monocyte from Day 0 specimen. The surface shows short microvillous projections and numerous pinocytic vesicles. Characteristic lysosomal granules and mitochondria are evident in the cytoplasm. The nucleus is irregularly shaped, showing two profiles and the heterochromatin is peripherally distributed. Bar, 2 μm.

were V_C, V_N and V_M: S_C, S_N and S_M: excess membrane for cell and nucleus: and the number of mitochondrial profiles per section. These parameters were selected as variables independent of one another, the specific aim being to minimise or eliminate the effects of colinearity between variables. An example of such colinearity would be a reduction in the nuclear volume fraction when cell size increased independently of nuclear changes.

Contrasts between means for successive time intervals were examined using a multiple comparison procedure. For each stereological parameter the least significant difference (l.s.d.) was found using the relation (Federer & McCulloch, 1984):

$$\text{l.s.d.} = t_{\alpha;f}\sqrt{\left(\frac{2S_e^2}{r}\right)},$$

where $t_{\alpha;f}$ is the tabulated value of the two-tailed Student's t-test, α the size of the test, f the degrees of freedom, r the number of subjects per group and S_e^2 the residual mean square error obtained from the computation of the analysis of variance.

Computations were carried out on a Sharp PC1211 minicomputer.

RESULTS

Satisfactory preparations were obtained from each subject at each interval, and the viability was greater than 90 % in all cases. On visual examination, the mononuclear phagocytes at the earlier intervals showed the morphological features of

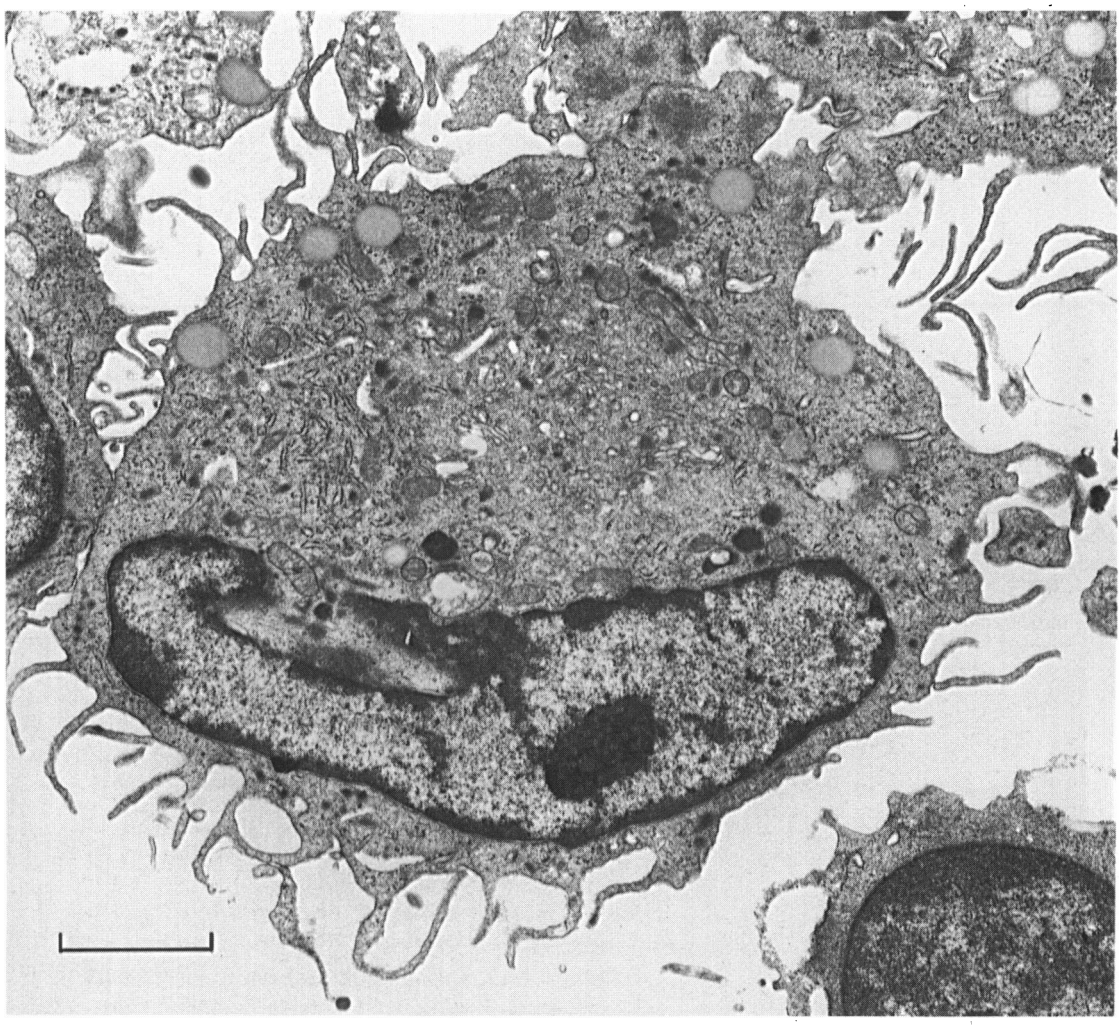

Fig. 2. Mature macrophage from Day 6 culture. The surface shows many long microvilli. The cytoplasm is voluminous and contains a wealth of organelles, including lysosomal granules, mitochondria, Golgi complex, short strands of rough endoplasmic reticulum and some peripherally-placed lipid vesicles. The nucleus is situated eccentrically and shows a nucleolus and a thin peripheral rim of heterochromatin. Bar, 2 μm.

monocytes (Fig. 1), while at the later intervals cells with the appearances of macrophages predominated (Fig. 2). The results of the analysis of variance are shown in Table 1. Table 2 summarises the values obtained for other parameters not included in the formal analysis. In view of the results of the analysis of variance, Student's *t*-tests were used to compare Day 0 and Day 6 values for the cell irregularity estimate ($t = 3{\cdot}5$, $P < 0{\cdot}005$), nuclear volume fraction, V_{VN} ($t = 7{\cdot}0$, $P < 0{\cdot}001$) and area of individual mitochondrial profiles, A_m ($t = 5{\cdot}7$, $P < 0{\cdot}001$) but these differences should be interpreted with caution (see Discussion). The absolute and relative changes in cell, nuclear, mitochondrial and cytoplasmic volumes over the period of culture are shown graphically in Fig. 3.

Table 1. *Parameters of cultured cells subjected to analysis of variance (means* ± S.E.)

Parameter	Day 0		Day 2		Day 4		Day 6
Cell							
V_C (fl)	299±25	*	427±58	*	756±158		945±223
S_C (μm^2)	398±17		441±30	*	740±101	*	956±147
Excess cell membrane (μm^2)	183±11		171±9	*	353±51	*	510±82
Nucleus							
V_N (fl)	101±10		115±11		154±21		163±32
S_N (μm^2)	158±13		162±11	*	212±17		210±26
Excess nuclear membrane (μm^2)	54±6		49±5	*	75±7		70±11
Mitochondria							
V_M (fl)	11·5±0·6	*	17·2±1·6	*	33·1±5·3		42·9±6·7
S_M (μm^2)	144±7	*	210±20	*	352±50		437±68
Profiles/section	12·7±0·5		14·6±0·8	*	18·0±1·4		19·4±1·3

Conjoined underlined values belong to homogeneous groups, significant differences ($P < 0·05$) being indicated by asterisked intervals in the underlining.

Table 2. *Other parameters of cultured cells (means* ± S.E.)

Parameter	Day 0	Day 1	Day 2	Day 4	Day 6
Cell					
A_C (μm^2)	44·5±2·3	48·5±1·3	57·0±5·0	83·5±11·6	97·4±14·6
D_C (μm)	8·2±0·2	8·6±0·1	9·2±0·4	10·9±0·7	11·7±0·8
$(S/V)_C$ (μm^2 fl^{-1})	1·38±0·07	1·24±0·04	1·10±0·06	1·08±0·06	1·14±0·08
'Irregularity'	1·87±0·06	1·77±0·05	1·65±0·04	1·90±0·04	2·14±0·04
Nucleus					
V_{VN} (%)	32·2±0·1	30·3±0·1	27·8±0·1	22·4±0·1	18·3±0·1
A_N (μm^2)	17·3±1·2	17·3±0·4	18·8±1·2	22·8±2·1	23·3±2·9
D_N (μm)	5·7±0·2	5·7±0·1	6·0±0·2	6·5±0·3	6·6±0·4
$(S/V)_N$ (μm^2 fl^{-1})	1·61±0·06	1·52±0·03	1·45±0·06	1·47±0·09	1·42±0·09
'Irregularity'	1·52±0·04	1·46±0·03	1·43±0·04	1·57±0·05	1·50±0·04
Mitochondria					
$V_{VM\text{-}C}$ (%)	3·9±0·2	3·9±0·1	4·2±0·3	4·7±0·4	5·0±0·3
$V_{VM\text{-}cyto}$ (%)	5·9±0·3	5·6±0·2	5·8±0·4	6·0±0·5	6·1±0·4
A_M (μm^2)	1·59±0·06	1·73±0·07	2·15±0·15	3·46±0·37	4·23±0·39
$(S/V)_M$ (μm^2 fl^{-1})	12·6±0·3	12·8±0·2	12·3±0·3	11·0±0·4	10·5±0·6
A_m (μm^2)	0·13±0·004	0·13±0·002	0·15±0·005	0·19±0·01	0·22±0·02

DISCUSSION

The results shown in Tables 1 and 2 and Figure 3 relate to the structure of a model cell and it is assumed that the nuclei formed a monodispersed population with respect to size and were approximately spherical. The latter assumption clearly introduces inaccuracy into the system, although it may be pertinent to note that blood monocyte volumes, obtained by Schmid-Schönbein, Shih & Chien (1980),

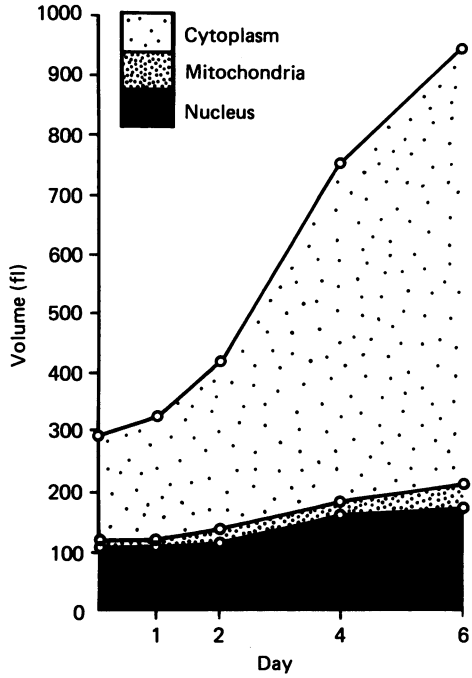

Fig. 3. Volume changes during culture. The upper line indicates the changes in whole cell volume between Day 0 and Day 6. The relative contributions of the nucleus, mitochondria and cytoplasm to the cell volume are shown.

independent of nuclear measurements, were of the same order as those obtained with the present technique (Sokol *et al.* 1985). However, the need for stringency in statistical analysis and caution in interpreting small changes is self-evident.

Over the six day period of suspension culture, a 3–4 fold increase in whole cell and cytoplasmic volume was shown. This quantitates a change which has previously been noted in non-stereological studies by numerous investigators including Sutton & Weiss (1966), Sutton (1967), Fedorko & Hirsch (1970), Van der Rhee (1979), Ackerman *et al.* (1981), Stevenson *et al.* (1981) and Tsukada *et al.* (1985). A similar increase in mean cell size was found in morphometric studies of stimulated rodent peritoneal macrophages by Mayhew & Williams (1971, 1974) and Williams & Mayhew (1973), but this may not be comparable with the present observation on macrophage development in humans.

The cell surface area more than doubled during culture (Table 1). This partly reflected the larger cell volume but the significant change in excess cell membrane (Table 1) indicated that surface irregularity increased as well. This is also shown in the irregularity estimate (Table 2) and by the fact that the fall in cell surface-to-volume ratio over the culture period (17 %) was considerably less than would have been expected (51 %) from an increase in cell volume alone. The increase in surface area is in keeping with the more prominent surface ruffling and the many long microvilli noted in fully developed macrophages by Fedorko & Hirsch (1970) and Van der Rhee (1979). In contrast, increased cell roundness was a feature of stimulated peritoneal macrophages in the studies of Williams & Mayhew (1973) and Mayhew & Williams (1974).

In the present work, the nuclear changes in Table 1 were relatively small and

there was a marked drop in the nuclear volume fraction of the developing cells (Table 2; Fig. 3). These observations are in keeping with the findings of the earlier non-stereological investigations of macrophage development mentioned above but contrast with the morphometric studies of Williams & Mayhew (1973) and Mayhew & Williams (1974) on stimulated rodent peritoneal macrophages, where the nucleus increased in size maintaining a constant nucleocytoplasmic ratio.

The mitochondrial volume showed a substantial increase over the culture period (Table 1; Fig. 3) and kept pace with the growth in cytoplasmic volume as shown by the approximately constant volume fraction ($V_{VM\text{-}cyto}$ in Table 2); there were similar significant increments in mitochondrial surface area (Table 1). It is also apparent that with increasing maturity there were more profiles per section (Table 1) and that individual mitochondrial profiles were larger (A_m in Table 2). These changes presumably reflect the increased energy requirements of the more mature forms of mononuclear phagocytes in relation to their increased functional capacity. In animals, increasing numbers of mitochondrial profiles were noted by Van der Rhee (1979) during macrophage maturation, and by Nichols & Bainton (1975) and Mayhew & Williams (1974) following peritoneal stimulation. However, caution needs to be exercised when comparing results from rodent material with the present findings in human cells. Scaling differences are associated with different metabolic rates and consequent differences in organelle content. This is particularly relevant to observations on mitochondria but also applies in the other comparisons referred to above.

It is of interest to note that the Day 0 values for mean cell and nuclear size recorded in Table 1 are somewhat larger than those recorded for blood monocytes by Chien *et al.* (1984) and Sokol *et al.* (1985). This may be attributed to the fact that the Day 0 cells had been suspended in tissue culture medium during preparation.

The results would appear to provide an insight into the sequential morphological changes which take place during the maturation of normal monocytes into macrophages, and will allow future comparison with cells from patients with malignant disease, where mononuclear phagocyte dysfunction may be present (Sokol & Hudson 1983). The experimental system may have applications in the study of various factors on macrophage development such as co-cultured lymphocytes, lymphokines and other serum components.

The importance of the statistical procedures is also worthy of comment. In morphometric studies, the method of statistical analysis is of particular relevance because of the large numbers of possible comparisons, the normality or otherwise of distributions and the population variances. The use of a multiple comparison approach is necessary to avoid the likelihood of false or seriously flawed conclusions. For example, a single comparison procedure at the 5 % significance level (such as the *t*-test) would not be valid at the 5 % level for the simultaneous testing of two or more morphometric measures. The relation between the individual parameter significance level (α) and the overall significance level (S) for comparing K parameters is given by the Bonferroni relation:

$$S = 1-(1-\alpha)^K.$$

Thus, if the *t*-test had been used on each occasion in the present study, overall significance levels of over 50 % would have resulted in a considerable likelihood of error. Three alternative procedures can be used to avoid this difficulty, namely analysis of variance, multivariate analysis (often requiring main-frame computer facilities), or the Dunn-Šidak procedure (Sokal & Rohlf, 1981) where the overall

simultaneous significance level S is set at 0·05, and the individual levels set for each morphometric measure (α) at:

$$\alpha = 1-(1-S)^{1/K}.$$

The validity of any future comparisons with the present work will therefore be dependent both on similar methods of morphometric analysis and on similar numerical procedures being followed. Combination of significance levels from different approaches within the three alternatives given above is always possible using minimum likelihood summation procedures (Sokal & Rohlf, 1981).

SUMMARY

The development of macrophages from the blood monocytes of ten normal subjects has been studied at intervals over a six day period. Suspension cultures were used to obtain randomly orientated cells and morphometric measurements were made on electron micrographs. In order to meet the requirements for normality of distribution and homoscedasticity, data were logarithmically transformed. A two-way analysis of variance was then carried out, taking subjects and time intervals as fixed effects, and using a least significant difference procedure to detect variations between culture time intervals.

The whole cell and cytoplasmic volumes showed 3–4 fold increases during culture. The cell surface area more than doubled; this was partly attributable to the larger cell volume and partly to increased surface irregularity. The mitochondrial volume also showed a similar significant increase, attributable to an increase in both number and size of mitochondrial profiles, the cytoplasmic volume fraction remaining approximately constant. Although there was a statistically significant increase in nuclear surface area, the nuclear changes were relatively small.

The results and the application of appropriate statistical methods have thus provided basic morphometric data for human macrophage development in culture. The experimental system should permit further investigation of factors governing impaired macrophage development in malignant disease.

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