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Quantitative enzyme cytochemistry during human macrophage development

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ABSTRACT

Integrating microdensitometry was used to study changes in the intracellular activity of 4 enzymes during macrophage development. Suspension cultures of blood monocytes from 19 healthy human subjects were examined at 0, 2, 4 and 6 d. Mononuclear phagocytes were harvested by glass adherence and standard methods were used for cytochemical staining for NADH dehydrogenase, succinate dehydrogenase, acid phosphatase and α-naphthyl butyrate esterase. All specimens from all subjects were stained at the same time and staining intensities in individual cells were measured at appropriate wavelengths. A highly significant increase in enzyme activity with culture time was found for all 4 enzymes. These increases in mitochondrial, lysosomal and ectoenzyme activities during development indicate the increasing functional capabilities of the macrophages.

INTRODUCTION

As part of our ongoing studies of human macrophage development, 6 d suspension cultures of blood monocytes have previously been used to quantitate changes in (1) ultrastructure using morphometric methods (Sokol et al. 1987), (2) in cellular dry mass using scanning and integrating microinterferometry (Sokol et al. 1991*a*) and (3) function using immune-mediated erythrophagocytosis (Sokol et al. 1993). It now seemed important to quantitate changes in cellular enzyme activity in this system. As far as we are aware, the only previous work measuring the enzyme activity of individual macrophages related to murine peritoneal cells and employed computerised scanning cytophotometry (Papadimitriou & van Bruggen, 1981). In the present investigation, integrating microdensitometry (Goldstein, 1977, 1981) was used and the activity of 4 different enzymes measured during human macrophage development.

MATERIALS AND METHODS

The study was carried out on 9 male and 10 female subjects aged between 21 and 57 y, the mean age (±S.E.) being 36±2.4 y. All enjoyed good health and stated that they were not taking any medication. The procedures were performed in accordance with the principles in the Declaration of Helsinki and with the approval of the Ethical Committee of the Sheffield Health Authority. Approximately 30 ml of venous blood were taken from each subject and defibrinated with glass beads under sterile conditions. Mononuclear cells were separated using lymphocyte separation medium (Flow Laboratories Ltd) and, after washing twice in Hanks' balanced salt solution, resuspended in medium containing 10% heat-inactivated autologous serum to give a concentration of $1–2 \times 10^6$ cells per ml. The specimens were divided into aliquots, gassed with 5% carbon dioxide in air and cultured in suspension at 37 °C (Sokol et al. 1985). Samples for quantitative cytochemistry were examined prior to incubation (d 0) and after 2, 4 and 6 d, cell viability of > 90% on the trypan blue exclusion test being confirmed in all cases. A few drops of suspension culture at each interval were placed on 12 glass slides which were then kept in a wet chamber at 37 °C for 1 h to allow the mononuclear phagocytes to adhere. The specimens were washed with phosphate-buffered saline (pH 7.0) to remove nonadherent cells (mainly lymphocytes), briefly air dried and stored frozen at −80 °C until required.

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Specimens were removed from the freezer, allowed to come to room temperature and staining procedures carried out, appropriate negative controls being included in all cases. To ensure uniformity, cytochemical staining was performed on all specimens from all subjects at the same time using the same batch of stain. The procedures were standardised following preliminary tests to show that staining would occur progressively and be likely to satisfy the requirements of stoichiometry (i.e. an amount of final reaction product linearly proportional to the amount of biological test substance).

For NADH dehydrogenase (EC 1.6.99.3; International Union of Biochemistry, 1965)), specimens were incubated for 12 min at 37 °C in freshly prepared staining medium containing 2 g/l β NADH in 5 g/l of the tetrazolium salt TNBT. After thoroughly washing with hot (60 °C) phosphate buffer solution at pH 5.3, they were mounted in glycerine jelly.

For succinate dehydrogenase (SDH; EC 1.3.99.1), specimens were incubated at 37 °C for 1 h in a staining solution containing 16 g/l sodium succinate and 0.77 g/l ïNBT. After draining briefly, they were fixed in buffered formalin at room temperature for 15 min, washed in running water for 5 min, air dried, dehydrated and cleared before mounting in polymount.

For acid phosphatase (EC 3.1.3.2), specimens were fixed for 30 s in citrate-acetone-formaldehyde fixative, rinsed in deionised water and incubated at 37 °C for 10 min in a staining solution (Sigma Chemical Company, Poole, UK) containing naphthol AS-BI phosphoric acid, fast garnet GBC base and sodium nitrite. They were rinsed for 30 s in deionised water, air dried and mounted in glycerine jelly.

For α-naphthyl butyrate esterase (no designated EC number), specimens were fixed and rinsed as for acid phosphatase. They were then incubated at 37 °C for 40 min in a staining solution (Sigma Chemical Company) containing α-naphthyl butyrate, pararosaniline and sodium nitrite, rinsed for 2–3 min in deionised water, air dried, dehydrated, cleared and mounted in polymount.

All specimens were examined in a Vickers M85A integrating microdensitometer using a ×100 oil immersion lens (with a numerical aperture of 1.3) and a spot size of 0.5 μm as measured in the object plane. Wave lengths of 640 nm and 535 nm were employed for measuring the activities of NADH dehydrogenase and SDH respectively. For both acid phosphatase and α-naphthyl butyrate esterase the staining intensity of individual cells was routinely measured at a wave length of 580 nm, but with some of the more densely stained specimens where the area readings were above the threshold setting (Goldstein, 1981), measurements were made at 650 nm, a correction factor being used to equate them with the readings at 580 nm. In all cases, the relative measurements of integrated absorbance were recorded in arbitrary machine units of optical density (glare being routinely measured and offset; Goldstein, 1970). Values were obtained for approximately 20 individual cells for each enzyme at each time interval for each subject, over 6000 cells being measured in total.

Multivariate analysis of variance (MANOVA) was carried out on the results to examine the effects of culture interval, age and sex, the data being transformed before computation in order to improve conformity to model assumptions of multivariate normality and homoscedasticity. Univariate analysis of variance (ANOVA) was carried out in relation to the results for the different enzymes. These analyses were performed on an IBM 3083 main-frame computer using a current SPSS statistical package. Statistical significance was set a priori at the 5% level for the MANOVA and the 1% level for the ANOVA, respectively. The more stringent requirement for the latter was to take account of the 4 sets of comparisons (k); using the relation $S = 1-(1-\alpha)^k$ an individual significance level (α) of 0.01 was required to achieve an overall significance level (S) of < 0.05 (Sokol et al. 1987).

RESULTS

The MANOVA showed a highly significant overall effect of culture time on enzyme activity ($P < 0.001$); ANOVA demonstrated that this effect was operative for each of the 4 enzymes. The Table summarises the results. MANOVA also showed a significant effect attributable to subject age ($P < 0.01$), ANOVA indicating that this only related to α-naphthyl butyrate esterase ($P < 0.05$) for which older subjects had generally higher values. There was no significant effect attributable to the sex of the subjects.

DISCUSSION

It seems reasonable to regard the mononuclear phagocytes studied (and the results obtained from them) as representative. The ability of mononuclear phagocytes to adhere to a glass surface is a characteristic widely used for their isolation (van Furth et al. 1972) and previous work showed that our methods give a good yield of esterase-positive cells (Sokol et al.

Table. *Enzyme activity in developing macrophages**

Enzymes	Period of culture				ANOVA: Effect of time (P)
	Day 0	Day 2	Day 4	Day 6	
NADH dehydrogenase	13.0±1.5	18.3±2.0	23.4±1.5	35.9±2.8	< 0.01
Succinate dehydrogenase	10.2±0.6	14.6±0.6	19.3±0.7	26.7±0.6	< 0.01
Acid phosphatase	4.3±0.5	18.1±3.4	50.2±8.3	105.6±16.6	< 0.01
α-naphthyl butyrate esterase	36.2±9.0	54.7±12.7	59.6±11.0	77.4±10.5	< 0.01

* In optical density units: means±S.E.M.

1985), approximately 290×10^6 monocytes being obtained per litre of blood with no apparent loss over the period of suspension culture.

The activity of all 4 enzymes studied underwent highly significant increases over the 6 d of development ($P < 0.001$; see Table). The most dramatic change was in the acid phosphatase activity which increased on average more than 20 fold between the day 0 monocyte and the d 6 macrophage stages, while the other enzymes showed mean increases of between 2 and 3 fold. This appears to be the first time that measurements of enzyme activity have been made on individual cells during macrophage development, although others have reported increases in activity of acid phosphatase and other lysosomal enzymes in monocyte culture populations (Cohn & Benson, 1965; Cline, 1970; Musson, 1983). Greater numbers and prominence of lysosomes have also been noted (Sutton & Weiss, 1966; van der Rhee et al. 1979). The present findings are similarly consistent with reports of increasing staining reactions of various enzymes in macrophages with time in skin window preparations (Wulff, 1963; Braunsteiner & Schmalzl, 1970) and in monocyte cultures (van der Meer et al. 1982), although peroxidase activity was shown to decrease (Stevenson et al. 1981; van der Meer et al. 1982). The appearance of new isoenzymes has been described during the differentiation of mononuclear phagocytes (Parwaresch et al. 1981; Radzun et al. 1983*a*, *b*).

Our findings can be interpreted as being part of the developmental process which fits the cell for its macrophage functions. The increases in NADH and SDH (Table) reflect the increasing mitochondrial energy requirements for these functions. The energy requirement of phagocytic mechanisms, for example, has been illustrated by an increase in SDH activity in rodent peritoneal macrophages after quartz treatment (Dogra & Kaw, 1988), while increased cellular content of NADH and SDH have been shown in exudative as compared with resident macrophages (Papadimitriou & van Bruggen, 1981). Mitochondrial changes during macrophage development have already been highlighted by ultrastructural morphometry, striking increases in total volume, surface area and numbers being observed over a 6 d period of culture using methods identical to those described here (Sokol et al. 1987).

The increasing functional capability of the developing cells is also reflected by the increases in acid phosphatase and α-naphthyl butyrate esterase activities (Table). Acid phosphatases are located in the lysosomes before release and are known to be important in cytotoxic and lytic activities. The nonspecific esterases which are used clinically to characterise cells of mononuclear phagocyte lineage (International Committee for Standardization in Haematology, 1985) are now generally thought not to be localised in lysosomes but to be associated with the plasma membrane as ectoenzymes (Bozdech & Bainton, 1981; Morahan & Miller, 1984; Rademakers et al. 1989; Drexler et al. 1991). An increase in cell surface area has been shown in developing macrophages using ultrastructural morphometry (Sokol et al. 1987) and it was of the same order as that found for α-naphthyl butyrate esterase (2–3 fold). There is good evidence that macrophage esterases are involved in cytotoxic activity (Oertel et al. 1985; McCormick et al. 1991) and other proposed functions relate to the inactivation of toxic products, to diapedesis and migration through the tissues in inflammation (Monahan et al. 1981) and to pinocytosis (Cohn et al. 1987).

Previous morphological and functional studies have shown that in malignant lymphoma, there are disorders of macrophage development (Sokol et al. 1991*b*, 1992) and there is evidence that monocyte esterase deficiency (which can have a familial tendency; Markey et al. 1986; Bell et al. 1992) may predispose to the development of lymphoid neoplasia (Markey et al. 1986, 1987, 1990). Further work using

the present methods to study macrophage enzyme development in such disorders would therefore seem merited. Specific growth factors such as recombinant M-CSF (Bajorin et al. 1991) might also be included in the culture system to characterise the changes.

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