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Differentiated HL60 promyelocytic leukaemia cells have a deficient myeloperoxidase/halide killing system

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SUMMARY

Following induction of differentiation by incubation with 1·25% dimethylsulfoxide (DMSO), cells of the HL60 promyelocytic leukaemia cell line acquire certain characteristics of the mature polymorphonuclear leucocyte (PMN) including the ability to produce oxygen radicals and to phagocytose opsonized bacteria. However, these cells are unable to fix ^{125}I during phagocytosis and are only able to kill phagocytosed microorganisms (*C. albicans* and *S. aureus*) to a small degree compared to mature PMN. Further, release of myeloperoxidase (MPO) from cytoplasmic granules occurs to approximately 20% of control levels after 6 days culture with DMSO, and drops to negligible levels by 7 days. These data suggest an immature or inactive MPO/peroxide/halide killing system. Insensitivity to the cyclooxygenase pathway inhibitor indomethacin suggests that there may also be a defect in this pathway.

Keywords promyelocytic leukaemia differentiation myeloperoxidase neutrophil function

INTRODUCTION

The human polymorphonuclear leucocyte (PMN), or neutrophil, is one of the principal effector cells in the bodies defences against infection. The cell biology of the mature PMN has been studied extensively, with particular emphasis on the activation mechanisms leading to the respiratory burst which is responsible for generation of microbicidal agents (Babior, 1984). The recent availability of a stable human promyelocytic leukaemia cell line (HL60) which could be maintained in continuous culture (Collins, Gallo & Gallagher, 1977), has enabled the study of neutrophil differentiation. When cultured in the presence of DMSO for 5–7 days, these cells develop complement receptors and acquire the capacity to respond to chemotactic stimuli, to secrete lysosomal enzymes, to generate reactive oxygen species, and to reduce tetrazolium dyes (Collins *et al.*, 1979). These cells also acquire a glycoprotein differentiation antigen of molecular weight 110,000 which is recognised by the anti-neutrophil monoclonal antibody NCD1 (Cotter & Henson, 1983).

In this report we examine the metabolic responses of DMSO-differentiated HL60 cells, and demonstrate an immature or partially inactive MPO/peroxide/halide killing system. A possible defect in the cyclooxygenase pathway is also described.

MATERIALS AND METHODS

Cells. HL60 cells (kindly supplied by Dr J. Werkmeister, Walter & Eliza Hall Institute,

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Parkville) were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum. Cells were induced to differentiate by the addition of either 1·25% (v/v) DMSO or 20 ng/ml phorbol myristate acetate (PMA; Sigma Chemical Co., St Louis, USA), which was stored at −20°C at 2 mg/ml in acetone and was diluted with PBS prior to use. These cells were maintained at 37°C in a humid 5% CO_2 atmosphere.

Peripheral blood PMN were purified on a discontinuous Percoll gradient (Phillips, Hosking & Shelton, 1983) to greater than 99% purity. Viability of all cell preparations was determined prior to use by exclusion of trypan blue dye.

Chemotaxis. The assay was based on a modified Boyden chamber procedure using a 5 μm filter; migration was assessed by the leading front method (Hosking, Fitzgerald & Shelton, 1977).

Phagocytic index. Cells (2×10^6) in Hanks balanced salt solution without phenol red indicator (HBSS) were incubated with Bakers yeast and pooled human serum (PHS) for 60 min. at 37°C. Cells were washed with PBS, then slide preparations were stained with Wrights stain. At least 50 cells in duplicate preparations were counted, and results were expressed as a phagocytic index, representing the average number of ingested yeast per cell.

Iodination and glucose oxidation. Fixation of ^{125}I during phagocytosis of opsonized Bakers yeast was determined by a micromethod (Pereira, Shelton & Hosking, 1983). Results were expressed as a percentage of the control ^{125}I incorporation.

Activity of the hexose monophosphate shunt was determined by the production of $^{14}CO_2$ by resting and phagocytosing cells in the presence of ^{14}C-glucose (Hosking *et al.*, 1977). Assays were performed in triplicate. Results were expressed as a stimulation index, representing the ratio of counts in test tubes to counts in control tubes.

Enzyme assays. Superoxide production was assessed by reduction of cytochrome c in the presence or absence of superoxide dismutase (Babior & Kipnes, 1977). PMA (4 μg) or opsonized zymosan (OZ; 100 μg) were used as stimulants. Where indicated, inhibitors were incubated with the cells for 10 min. at 37°C prior to addition of cytochrome c and the stimulant. Iodoacetamide (BDH Chemicals, Poole, UK) was prepared as a stock solution of 18·5 mg/ml in HBSS. Indomethacin (Sigma, Chemical Co., St Louis, USA) was prepared at a concentration of 0·5 mg/ml in ethanol, and diluted ten-fold with HBSS prior to use. All tests were performed in triplicate, and activity was expressed as nmoles superoxide produced/10^7 cells/min.

Activity of the lysosomal enzyme MPO was measured by a modification of the method of Bretz & Baggiolini (1974). Cells (1×10^6) in HBSS were incubated with either PMA or OZ at 37°C for 10 min or 30 min respectively, in a total volume of 200 μl. The cells were pelleted by centrifugation, and 100 μl of supernatant added to the reaction mixture (0·32 mM o-dianisidine (Sigma Chemical Co., St Louis, USA) in 0·1 M citrate buffer pH 5·5). Reaction was initiated by addition of H_2O_2 (final concentration 80 μM), and was stopped after 5 min at room temperature by addition of 1 ml 35% (v/v) perchloric acid. Activity was expressed as international units (μmoles o-dianisidine oxidised/10^6 cells/min) using an extinction coefficient of 20040 $M^{-1}cm^{-1}$ (Bretz & Baggiolini, 1974).

Assessment of microbicidal activity. Killing of phagocytosed *Candida albicans* organisms was assessed by incorporation of methylene blue dye (Pereira & Hosking, 1984). Results were expressed as a killing index, representing the average number of dead ingested organisms per cell.

Killing of phagocytosed *Staphlococcus aureus* organisms was determined by measuring the ability of live ingested organisms to reduce the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Brooke, Shelton & Hosking, unpublished results). Briefly, cells and bacteria were incubated in culture medium 199 (M199) with PHS for 10 min at 37°C, then were washed in PBS and resuspended in M199 containing antibiotics (penicillin and streptomycin) to kill non-ingested organisms. Samples were taken after this time interval ($t = 0$) and at 30, 60 and 120 min intervals. All samples were washed and sonicated to disrupt the cells, then were diluted out in a microtitre plate. This was incubated at 37°C for 7 h, then 25 μl of 1·5 mg/ml MTT solution were added to each well. Reduction of the dye by live organisms resulted in the formation of blue crystals, which were dissolved with 50 μl 0·04% HCl in isopropanol; the plates were then read using a Titertek Multiscan ELISA reader at 492 nm.

RESULTS

Neutrophil Function Test

HL60 cells cultured with DMSO for 6 days showed normal phagocytic capabilities and a chemotactic response of about 50% that of control PMN (Table 1). Glucose oxidation was measured at a level of 60% that of control PMN, and the reaction to the inhibitors indomethacin and iodoacetamide were the same as the action on control cells, suggesting a mature functioning hexose monophosphate shunt. This result was confirmed by the detection of normal levels of superoxide generation (see below). However, the iodination response was totally deficient (Table 1), suggesting a defect in the MPO/peroxide/halide killing system.

Myeloperoxidase release

A possible explanation for the lack of ^{125}I fixation during phagocytosis was lack of MPO in the cytoplasmic granules. To test this hypostheis, release of MPO following stimulation with PMA or OZ was assayed. Cells were tested at both 6 and 7 days after induction of differentiation in case there was delayed maturation of this pathway. The data in Table 2 show that DMSO-differentiated HL60 cells are able to release small quantities of active MPO, with a maximal release of 35% of the control

Table 1. Comparison of neutrophil function tests on normal PMN, control HL60 cells and HL60 cells cultured for 6 days with either DMSO or PMA

Cell type	Chemotactic Response (μm)	Phagocytic index	Iodination (%)	Inhibitor*	Stimulation Index
PMN	112	2·0	100	—	7·6
				5·6 μM (IM)	7·8
				4·0 mM (IA)	1·0
HL60	0	1·4	1·2	—	1·4
				5·6 μM (IM)	1·8
				4·0 mM (IA)	0·6
HL60+DMSO	56·4	2·7	2·0	—	4·5
				5·6 μM (IM)	3·2
				4·0 mM (IA)	0·7
HL60+PMA	2·6	0·5	1·2	nd	nd

* IM Indomethacin
IA iodoacetamide
DMSO dimethylsulfoxide
PMA Phorbol myristate acetate
nd Not done.

Table 2. Release of myeloperoxidase from stimulated PMN or HL60 cells.

Cell type	Treatment	MPO Activity at 6 days*	MPO Activity at 7 days*
PMN	PMA	14·6†	37·7†
	OZ	14·6	10·5
HL60	PMA	0	0
		0	0
HL60+DMSO	PMA	5·2	3·1
	OZ	3·1	0

* Increase in activity over unstimulated cells
† Activity of fresh PMN on the day of assay of HL60 cells

level when stimulated with PMA after 6 days culture, and only 8% of control release after 7 days culture. When stimulated with OZ, release after 6 days culture was only 20% of control levels, and was negligible after 7 days culture (Table 2). This experiment was repeated on several occasions, with similar results. These data suggest that DMSO-differentiated HL60 cells have an inactive MPO/peroxide/halide microbicidal system. Whether this is due to lack of enzyme synthesis or to production of an inactive form of the enzyme dye to a gene mutation is unclear. It is also possible that an inhibitory compound be activated or synthesized, or that a number of these events have occurred.

Microbicidal activity

The ability of DMSO-differentiated HL60 cells to kill ingested organisms should reflect the status of their major microbicidal pathways, namely NADPH oxidase and the MPO/peroxidase/halide system. To this end, killing of phagocytosed *C. albicans* and *S. aureus* was assessed. The results of the methylene blue assay (Table 3) show that the differentiated HL60 cells after 6 or 7 days in culture are only able to kill ingested Candida to a level of about 50% that of control PMN. The reduced killing ability is probably due to the observed lack of MPO activity, with the measured killing being due to oxygen radicals produced by NADPH oxidase which is internalized from the plasma membrane into the phagosomal membrane during phagocytosis (Babior, 1984). DMSO-differentiated cells show no ability to kill ingested *S. aureus* compared to control PMN, as measured with the MTT killing assay (Fig. 1), confirming the observed defect in the killing mechansims of these cells.

Sensitivity to metabolic inhibitors

Activity of the plasma membrane-bound NADPH oxidase of HL60 cells was tested after 4 or 6 days culture in the presence of DMSO. Reactions to the cyclooxygenase pathway inhibitor indomethacin and the sulfhydryl reagent and potent enzyme inhibitor iodoacetamide were also assessed.

Table 3. *C. albicans* killing assay (Methylene blue)

	6 days*		7 days*	
Cell type	PI	KI	PI	KI
PMN	3·25†	0·69†	2·89†	1·0†
HL60	1·0	0	0·18	0
HL60+DMSO	1·59	0·37	0·92	0·52

* Time in culture with DMSO
† Activity of fresh PMN on the day of assay of HL60 cells

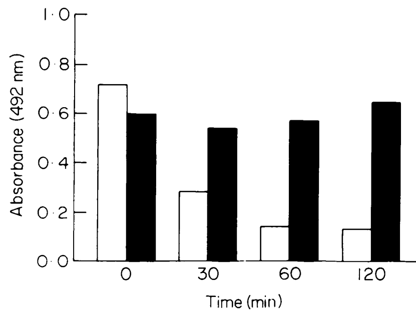

Fig. 1. Comparison of MTT reduction by *S. aureus* ingested either by normal PMN or DMSO-differentiated HL60 cells after 6 days in culture. (■) HL60-DMSO; (□) Control PMN.

Table 4. Effects of iodoacetamide and indomethacin on superoxide production by PMN and HL60 cells

Cell type	Treatment	Inhibitor	Units		
			0 Days*	4 Days*	6 Days*
PMN	—	—	2·8	—	—
	+PMA	—	16·2	—	—
	+PMA	1·0 mM IA	16·6	—	—
	+PMA	2·0 mM IA	2·4	—	—
	+PMA	4·0 mM IA	0·4	—	—
PMN	—	—	3·0	—	—
	+PMA	—	15·9	—	—
	+PMA	1·4 μM IM	15·8	—	—
	+PMA	2·8 μM IM	11·4	—	—
	+PMA	5·6 μM IM	1·6	—	—
	+PMA	11·2 μM IM	1·9	—	—
HL60	—	—	—	2·1	0
	+PMA	—	—	0	0
HL60+DMSO	—	—	—	0·7	0
	+PMA	—	—	1·0	18·2
	+PMA	5·6 μM IM	—	—	21·6
	+PMA	4·0 mM IA	—	—	2·4

* Days in culture with DMSO.
† PMN were used fresh on the day of assay of HL60 cells.

Superoxide production by these cells following PMA stimulation was normal, and was inhibited to the same degree as control PMN by iodoacetamide (Table 4). However, differentiated HL60 cells were unaffected by indomethacin at a concentration which markedly inhibited superoxide generation by control PMN.

DISCUSSION

The data reported in this paper confirms previous reports that DMSO-differentiated HL60 promyelocytic leukaemia cells acquire functions of the mature neutrophil after 6 days in culture. However, we were unable to detect significant levels of MPO activity, either by direct release into the supernatant following stimulation or by indirect methods such as fixation of ^{125}I or killing of ingested microorganisms. This is in contrast to a previous report (Cotter & Henson, 1983) which demonstrated significant MPO release following stimulation with either C5a or the chemotactic peptide FMLP. It is unliklely that the different stimuli used in this report would account for these contrasting results, but must be considered as a possible explanation.

Another possibility is that of mutation or chromosome loss in our cells. Cytogenetic studies on 6 day HL60-DMSO cultures (data not shown) revealed the normal chromosome complement reported for these cells (Collins *et al.*, 1977); whether a mutation has occurred which results in a less effective MPO system has not been determined. We were unable to ascertain whether the actual level of enzyme was lower in these cells, or whether an inactive, or less active, form of MPO was being produced, possibly as the result of a random mutation during culture. Further studies are in progress.

The data in this report also suggests a defect in the cyclooxygenase pathway, in that superoxide production by DMSO-treated HL60 cells was unaffected by indomethacin at a concentration which

abrogated the superoxide response in control PMN. The cause of this defect is unclear, and is under investigation.

The HL60 promyelocytic leukaemia cell line has proved useful for studies of neutrophil differentiation and activation. However, this report suggests that it may not be as stable in culture as previously thought (Collins *et al.*, 1977). It is recommended that the activity of several metabolic pathways be monitored at regular intervals in order that possible chromosomal aberrations be detected as soon as possible.

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