[^{3}H]Ouabain binding and ^{86}rubidium uptake during the dimethyl sulfoxide-induced differentiation of human promyelocytic leukemia HL-60 cells

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When HL-60 cells were induced to differentiate into granulocyte-like cells by culture in the presence of 1.3% dimethyl sulfoxide, an increase in the rate of ouabain-sensitive ^{86}Rb transport was observed during the first 6 h followed by a constant decrease which became stable on day 4 at about 40% of control level. By contrast, the number of ouabain binding sites remained constant during the first 24 h then decreased with the same kinetics as that of the ^{86}Rb transport rate. These results suggest that alterations in ionic fluxes, through activation of the sodium pump, are part of the early events resulting in HL-60 cell differentiation.

HL-60 leukemic cell *DMSO-induced differentiation* *^{86}Rb transport* *Ouabain binding site*

1. INTRODUCTION

The human leukemia cell line HL-60, which is composed mainly (95%) of promyelocytes, proliferates continuously in suspension culture [1,2]. It can be induced to differentiate in vitro into cells having morphological and functional characteristics of mature granulocytes by exposure to compounds such as dimethyl sulfoxide (DMSO) or retinoic acid [3,4]. Thus this cell line provides a very useful model system for studying the molecular events occurring during granulocytic differentiation.

Several reports have related the proliferation and differentiation of various cell types, including human and murine normal bone marrow cells, to a functional membrane-bound sodium pump (Na^+-K^+-ATPase, EC 3.6.1.3) [5,6]. Authors in [7] have shown that rat reticulocytes, compared to mature erythrocytes, have an enhanced rate of K^+ accumulation which can be quantitatively attributed to an increased number of Na^+-K^+-ATPase molecules. Authors in [8] demonstrated that immature granulocytes from normal human bone marrow and leukemia myeloblasts have higher sodium pump activities than mature granulocytes. Recently, electron microscopic histochemical observations have shown that myeloid or monocytoid leukemia cells have elevated Na^+-K^+-ATPase activity as compared to normal cells [9]. On the other hand, the inhibition of this enzyme activity triggers the proliferation of spinal cord neurons [10], the differentiation of Friend erythroleukemia cells [11] and of a pre-B lymphocyte cell line [12]. In Friend cells, differentiation can be induced by DMSO and this results in a decrease in the rate of ^{86}Rb transport beginning 5 h after stimulation [13]. No information is as yet available on ionic fluxes in HL-60 cells before and during differentiation.

Here we have measured the Na^+-K^+-ATPase activity, reflected by the rate of ^{86}Rb transport and the number of enzyme molecules during DMSO-induced differentiation of HL-60 cells.

2. MATERIALS AND METHODS

The HL-60 cells (kindly provided by Dr T. Breit-

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man, NCI Bethesda, USA) were grown in RPMI 1640 medium supplemented with 15% heat-inactivated fetal calf serum and antibiotics at an initial concentration of 3×10^5 cells/ml. Differentiation was induced by addition of 1.3% DMSO (Sigma), and, at intervals, during 6 days aliquots were taken for the determination of cell viability, the amount of differentiation, ^{86}Rb transport and [^{3}H]ouabain binding. Cell viability was assessed by Trypan blue dye exclusion and was never below 90%. Differentiation was determined by counting the cells having the morphology of mature granulocytes on cytospin (Shandon Elliot) slide preparations stained with May-Grunwald-Giemsa, and the capacity to reduce nitroblue tetrazolium (NBT) [14].

Binding of [^{3}H]ouabain (18 Ci/mmol, New England Nuclear) was performed in Earle's modified medium, where KCl was replaced by NaCl, 0.1% bovine serum albumin (BSA, Fraction V, Sigma) was added and the solution was buffered at pH 7.4 with Tris–HCl. Cells were washed 3 times, resuspended in this medium at a density of 5×10^6 cells/ml and preincubated at 37°C for 30 min. Aliquots (0.9 ml) were then added to plastic microfuge tubes containing 0.1 ml of 5×10^{-7} M [^{3}H]ouabain and incubated at 37°C. The reaction was terminated by centrifuging the cells (10 s at 12000 rpm). The supernatant was discarded by aspiration and the cell pellet was washed rapidly twice with 1 ml of ice-cold medium. The last pellet was dissolved in 0.5 ml aqueous sodium-dodecylsulfate (2%) and transferred to counting vials containing 10 ml Instagel. Radioactivity was measured in a liquid scintillation counter (Kontron Electronics SL 3000). Non-specific binding was defined as the amount of binding not inhibited by 10^{-3} M unlabelled ouabain. Specific binding was defined as the total amount of [^{3}H]ouabain bound minus the non-specific binding.

For ^{86}Rb uptake measurements, the cells were washed 3 times, resuspended in Earle's medium containing 5 mM KCl, 0.1% BSA, pH 7.4, at a density of 1.5×10^7 cells/ml and preincubated for 30 min at 37°C with or without 10^{-3} M ouabain. ^{86}Rubidium chloride (Commissariat à l'Energie Atomique-France) at a final concentration of 3 μCi/ml was then added. After 1, 3, 5, and 7 min two 0.2-ml aliquots were taken and the incorporation of $^{86}Rb^+$ stopped by sedimentation of the cells through oil [15].

The cell pellet was resuspended in 1 ml of distilled water and the cell-associated radioactivity was measured from Cerenkow radiation in a liquid scintillation counter. We have checked that $^{86}Rb^+$ was transported as K^+ by using $^{42}K^+$ as a tracer in control experiments and that ^{86}Rb uptake was linear for at least 15 min.

3. RESULTS

The time course of binding of [^{3}H]ouabain (5×10^{-8} M) at 37°C to HL-60 cells before and after 6 days of differentiation in the presence of 1.3% DMSO is shown in fig.1. Total binding increased progressively to reach a maximum after 60 min of incubation. Non-specific binding was about 5% of total binding and remained constant throughout the incubation period. The dissociation of [^{3}H]ouabain was slow with a half-life of 6 h at 37°C (not shown). When HL-60 cells were incubated with [^{3}H]ouabain (6×10^{-9} M) together with increasing concentrations of unlabelled ouabain at 37°C for 60 min, a competitive displacement of [^{3}H]ouabain was observed as shown in

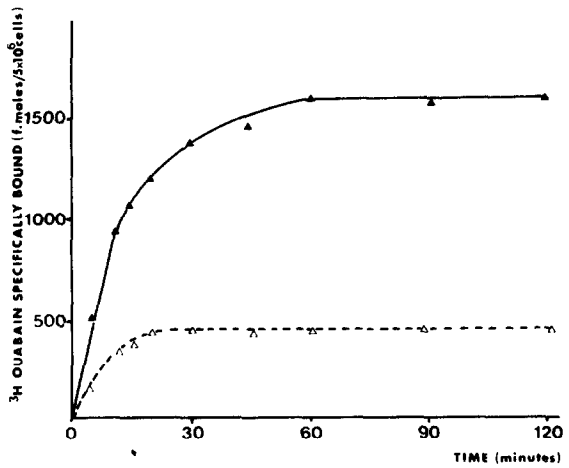

Fig.1. Time course of [^{3}H]ouabain binding on undifferentiated (▲—▲) and differentiated (Δ---Δ) HL-60 cells (see text). [^{3}H]Ouabain (5×10^{-8} M) was incubated at 37°C with cells (5×10^6/ml) in the presence and in the absence of unlabelled ouabain (10^{-3} M). At intervals, aliquots of 1 ml were removed, centrifuged and washed. The radioactivity in the cell pellet was counted. The specifically bound [^{3}H]ouabain was determined as described in section 2. Each point is the average of 3 experiments made in triplicate.

Table 1

HL-60 cells incubated with DMSO: morphological changes, NBT reduction and Na^+-K^+-ATPase modifications

Day after addition of DMSO	% of cells morphologically differentiated	% of cells reducing NBT	Number of ouabain binding sites per cell ($\times 10^3$)	Apparent affinity constant (10^8 M^{-1})	K^+ influx[a] (nmol/min per 10^6 cells)
0 = control	9 ± 6	7 ± 4	192 ± 18	1.3 ± 0.31	1.48 ± 0.06
1	22 ± 4	15 ± 10	176 ± 12	not done	1.05 ± 0.05
3	52 ± 8	50 ± 12	100 ± 6	not done	0.78 ± 0.02
6	85 ± 10	90 ± 7	66 ± 3	1.1 ± 0.3	0.59 ± 0.02

[a] K^+ influx among which 77% ± 5 were not inhibited by 10^{-3} M ouabain

Results are means ± SE of 3–9 different experiments made in triplicate

fig.2. Half-maximal displacement was obtained at 1.5×10^{-8} M ouabain. The Scatchard plot derived from these data (inset fig.2) indicates the presence of a single class of independent binding sites with an apparent affinity constant of 1.3×10^8 M^{-1}. The maximum binding capacity was 320 fmol [^{3}H]ouabain/10^6 cells which corresponds to 192000 sites/cell (table 1).

In HL-60 cells the rate of ^{86}Rb transport, used as a measure of K^+ influx, was equal to 1.48 nmol/10^6 cells per min at 37°C. 79% of this value was inhibited in the presence of 10^{-3} M ouabain and this represents the specific activity of the membrane-bound Na^+-K^+-ATPase.

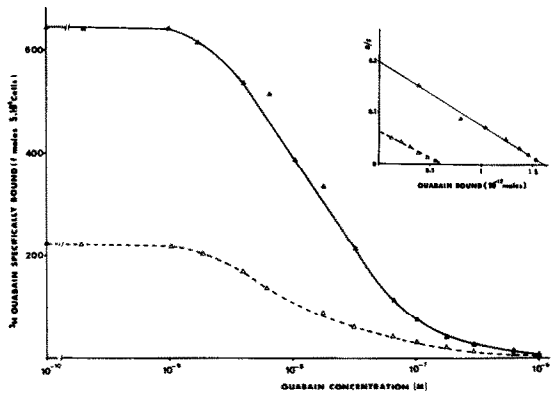

Fig.2. Displacement of [^{3}H]ouabain bound to HL-60 cells before (▲——▲) and after (△---△) differentiation. Cells (5×10^6 cells in 1 ml) were incubated at 37°C with [^{3}H]ouabain (6×10^{-9} M) and various concentrations of unlabelled ouabain (0–10^{-6} M) for 90 min. Cells were then centrifuged, washed and the radioactivity in the pellet counted. The non-specific binding (measured in the presence of 10^{-3} M ouabain) has been subtracted from all values. Each point is the average of 3 experiments made in triplicate. Inset: Scatchard analysis of the above data.

After 6 days of culture in the presence of 1.3% DMSO, more than 90% of the HL-60 cells were differentiated into myelocytes, metamyelocytes and banded and segmented neutrophils. This morphological differentiation correlated with an increase in nitroblue tetrazolium reduction and was accompanied by a net decrease in the rate of cell growth (table 1). Differentiated HL-60 cells have a lower number of Na^+-K^+-ATPase molecules: 66000 per cell (fig.2, table 1) with the same affinity constant 1.1×10^8 M^{-1} as control cells, and a lower rate of ^{86}Rb transport: 0.59 nmol/min per 10^6 cells (table 1).

The time course for the changes in the number of Na^+-K^+-ATPase molecules and in the specific activity of this enzyme when HL-60 cells were incubated with 1.3% DMSO during 6 days is shown in fig.3. The number of ouabain binding sites remains constant during 24 h, then decreases continuously and reaches about 40% of control level by day 4. However, the rate of ouabain-sensitive ^{86}Rb transport increases after addition of DMSO to reach a maximum of 130% of control after 4–6 h (inset fig.3, fig.4). It returns to control level after 10 h and then it decreases to about 40% of control value by day 5. During the whole incubation period with DMSO, the ouabain-sensitive ^{86}Rb transport represents 77 ± 5% of total uptake.

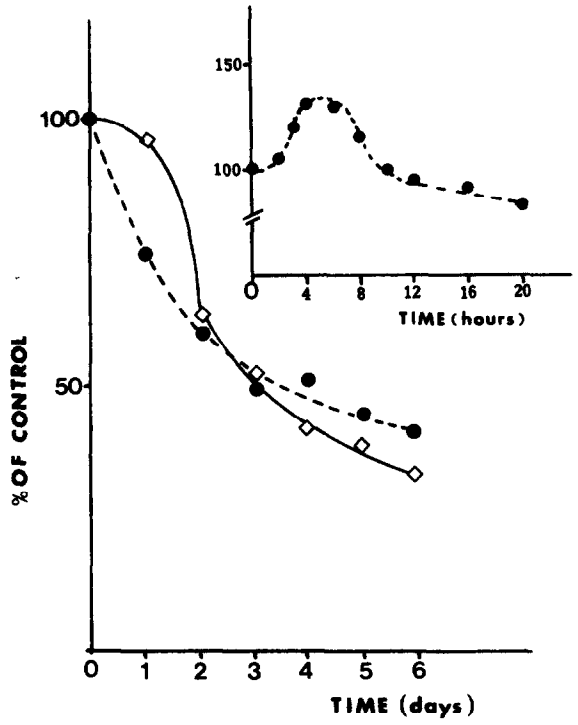

Fig.3. Ouabain binding sites and active ^{86}rubidium uptake during differentiation. Modifications occurring in the number of ouabain binding sites (◇——◇) and in the active ^{86}rubidium uptake (●---●) during 6 days after addition of 1.3% DMSO in the culture medium. Results are expressed in percentages of the control (1.13 nmol/10^6 cells per min measured on HL-60 cells before differentiation). Inset: variation of the active $^{86}Rb^+$ uptake during the first 24 h after addition of the inducer for differentiation.

DMSO is well-known as a membrane active agent. Thus, its effect on ATPase activity could be due to a direct action on the HL-60 cell membrane. To check out this possibility we also measured the kinetics of ouabain-sensitive ^{86}Rb transport over a 12-h period after addition of two other inducers, butyric acid (Sigma) (5×10^{-4} M) and *trans*-retinoic acid (Aldrich) (10^{-6} M). Fig.4 shows that each inducer provokes an increase in the ouabain-sensitive ^{86}Rb uptake during the first few hours following its addition to the medium. It reaches about 140% of the control after 3 h with butyric acid and 160% after 6–9 h with retinoic acid after which, in both cases, it returns to the control level and then decreases slowly.

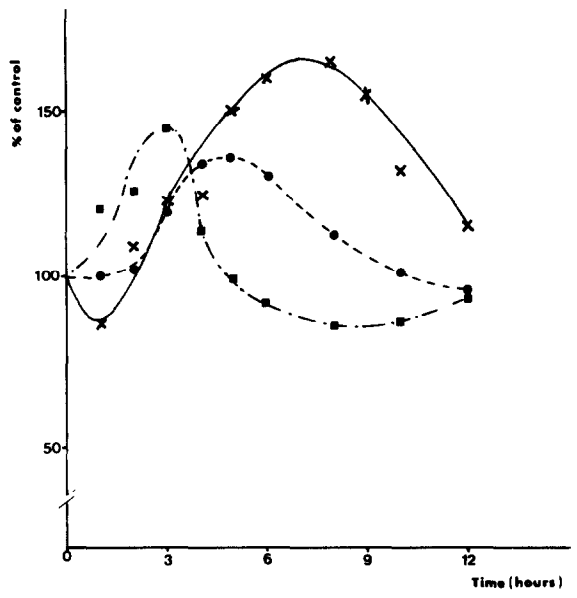

Fig.4. Ouabain sensitive ^{86}rubidium uptake during differentiation. Modifications occurring in physiological conditions during the first 12 h after addition of DMSO (1.3%) (●---●), butyric acid (5×10^{-4} M) (■---■) and retinoic acid (10^{-6} M) (×—×). All these 3 inducers differentiate HL-60 towards granulocyte-like cells. Results are expressed in percentages of the control.

4. DISCUSSION

After exposure to DMSO, HL-60 cells undergo a programme of terminal granulocytic differentiation which resembles the process of normal human myelopoiesis [3]. These cells thus represent a useful model system for studying the regulation of myelopoiesis at the molecular level.

The mechanism of action of DMSO in inducing terminal differentiation of HL-60 cells remains unknown. However, this compound can stimulate Friend erythroleukemic cells to undergo erythroid differentiation in vitro [16], and was shown to cause an early inhibition of Na^+-K^+-ATPase-mediated ^{86}Rb influx [17].

Here we show, for the first time, that DMSO addition to HL-60 cells brings about an early increase in the initial rate of ^{86}Rb transport. This increase occurs in the absence of any change in the amount of ouabain binding sites and is thus a consequence of a change in the activity of the sodium pump rather than a change in the number of pump sites.

However, after a few hours the ^{86}Rb transport decreases as does the number of pump sites. Thus the sodium pump is apparently regulated in two ways in relation to HL-60 cell differentiation: a rapid increase in activity and a slower decrease in pump sites.

The activity of the membrane-bound Na^+-K^+-ATPase can be stimulated in several ways: (1) by an increase in the intracellular Na^+ level; (2) by a decrease in Ca^{2+} level; (3) by a modification in cyclic AMP level [18]; (4) by changes in the environment, especially in the lipid environment of the enzyme [19].

The early stimulation of the sodium pump following DMSO-induced differentiation of HL-60 cells leads to an accumulation of K^+ into the cells and to the pumping of Na^+ out of the cells since the ratio of Na^+ to K^+ transport is tightly coupled: 3 Na^+ out for every 2 K^+ in [20]. Changes in the intracellular levels of Na^+ and K^+ have been related to the initiation of DNA synthesis [22] and to the translation of mRNA into proteins [23], thus leading to the control of gene expression. In this respect it is interesting to note that authors in [24] found that DNA synthesis was enhanced in the first hours of exposure of HL-60 cells to DMSO.

The induction of HL-60 cells to differentiate requires a sequence of events including DNA synthesis [24], changes in the plasma membrane lipids [20] and proteins [25] and a fall in the intracellular level of cyclic AMP [26]. The data presented in this study show that one of the earliest changes is an increase in the activity of the membrane-bound Na^+-K^+-ATPase which, as our results suggest, is not dependent on the nature of the inducer; DMSO, butyric and retinoic acids all led to early increases in enzyme activity.

It has recently been shown that the first DMSO-induced modification of Na^+-K^+-ATPase activity during differentiation of Friend's cells is a stimulation of its activity [27] and was only observed after preloading of the cells with Na^+. Therefore, the early increase on the active K^+ transport due to the Na^+-K^+-ATPase may be an important step in the cascade of events leading to the final differentiation.

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