INDUCTION OF DIFFERENTIATION IN MOUSE NEUROBLASTOMA CELLS BY HEXAMETHYLENE BISACETAMIDE

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Summary: Hexamethylene bisacetamide (HMBA), a potent inducer of erythroid differentiation in murine erythroleukemia cells (1), induces differentiation in mouse neuroblastoma cells, as indicated by the extension of neurites and the development of an excitable membrane. HMBA is effective at concentrations 50-fold lower than dimethylsulfoxide (2), another inducer of differentiation in both mouse neuroblastoma and murine erythroleukemia cells.

Introduction

Compounds of the polymethylene bisacetamide series were found by Reuben et al (1) to be potent inducers of differentiation of Friend virus-infected murine erythroleukemia cells (MELC). The properties of these acetylated diamines were studied by these authors in the course of an attempt to understand the mode of action of dimethylsulfoxide (Me_2SO), as well as other compounds of similar nature, which are able to induce differentiation in the erythroleukemic cells. We have previously shown that addition of Me_2SO to mouse neuroblastoma clone NIE-115 cells, causes differentiation in these cells as judged by growth of neurites and development of a highly excitable membrane (2). We have, therefore, examined the ability of hexamethylene bisacetamide (HMBA), which acts in the Friend cell system at a concentration range 50 times lower than Me_2SO (1), to induce maturation of neuroblastoma cells. Our results indicate that HMBA can, like Me_2SO,

Footnote: Abbreviations - HMBA, hexamethylene bisacetamide
MELC, murine erythroleukemia cells
AChE, acetylcholinesterase
Me_2SO, dimethylsulfoxide

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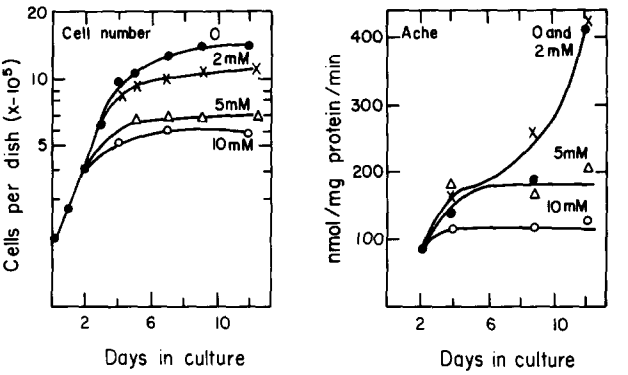

Figure 1. Response of exponentially growing cells of clone NIE-115 to HMBA (2, 5 and 10 mM). A - growth curve; B - AChE specific activity. The inducing agent was added two days after replating. Concentration of HMBA: ●——●, None; X——X, 2mM; △——△, 5mM; o——o, 10mM.

stimulate differentiation, but that there are certain differences in the response of these neuroblastoma cells to the two inducing agents.

Materials and Methods

Mouse neuroblastoma, clone NIE-115, was obtained from Dr. M. Nirenberg, NIH (3). Cells were grown at 37° in Dulbecco's modified Eagle's medium (DMEM) containing 0.12% $NaHCO_3$ and supplemented with 10% fetal calf serum (Gibco). The cultures were maintained under a humidified atmosphere containing 5% CO_2. Cell counts were performed using a hemacytometer and growth curves were constructed without regard to floating cells.

Biochemical Measurements: Acetylcholinesterase (AChE) (acetylcholine hydrolase, E.C. 3.1. .7) activity was determined using sonicated cell suspensions (4). Cells at the appropriate phase of growth were washed x 3 with phosphate buffered saline, pelleted by centrifugation and stored frozen in liquid nitrogen. The packed cells were thawed and sonicated just before the assay of enzymatic activity. One unit of activity corresponds to 1 nmole of acetylcholine hydrolyzed per min.

Efflux studies: Passive and activated efflux of ^{86}Rb (in absence and presence of 10^{-4}M veratridine) were measured as previously described (5).

Results and Discussion

Effect of HMBA on growth and morphology: This work was carried out with cells of the adrenergic clone NIE-115 (3) which were maintained in the exponential phase of growth. Under these conditions most of the cells retain a round shape and only few cells in the culture extend neurites. Adding HMBA to the medium affects growth and cell morphology in a time and concentration-dependent fashion. At a final concentration of 5mM, growth is inhibited within 3-4 days (Fig. 1a) and concomitantly close to 100% of the cells develop neurites.

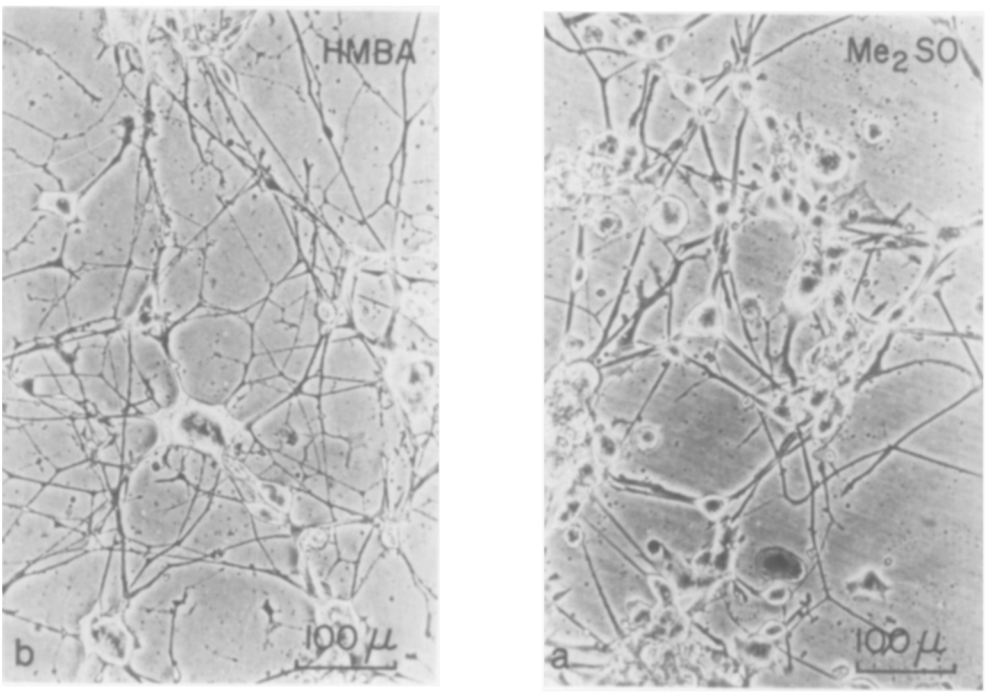

Figure 2. Morphological appearance of NIE-115 treated with (a): 2% Me_2SO and (b) 5mM HMBA. Phase contrast photomicrographs were taken 7 days after addition of the inducer.

An extensive neurite network is established rapidly and the presence of many thin, fragile, branches is clearly evident. The processes resemble those of aminopterine treated cells (6) and are different from the neurites which are formed in the presence of 280mM Me_2SO (2). In the latter case, the neurites are thicker and less branched (Fig. 2).

At a lower concentration of HMBA (2mM) the culture consists of a mixed population of both round and process-bearing cells, and growth tapers off at densities somewhat lower than the untreated control cultures. The proportion of neurite-extending cells at 2mM was, however, at all times higher than that of the control. Higher concentrations of HMBA (10mM) seem to have an adverse effect on the cells. They appear granulated and cell debris was evident in the medium; however, neurite extension was prominent.

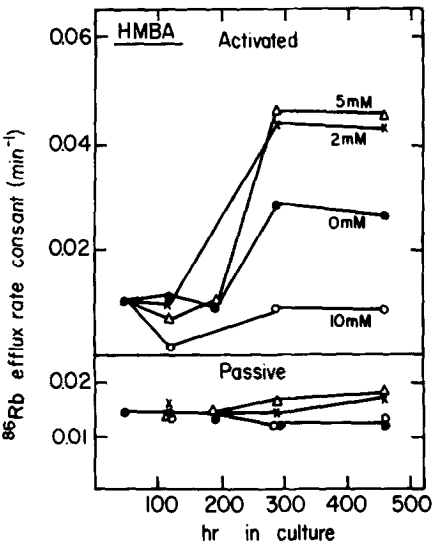

Figure 3. Development of veratridine-induced ^{86}Rb efflux in the presence of various concentrations of HMBA. Treatment of the exponential cultures was started 48 hours after subculture.

Acetylcholinesterase activity: AChE activity in neuroblastoma cells has been found to be inversely related to the growth rate, i.e. it reaches its highest values as cell division declines or when cells are induced to differentiate (7, 8). Fig. 1b, shows that, as expected, AChE in control and 2mM HMBA cultures increases at a time when the cells reach confluency (Fig. 1b). However, in the presence of 5mM or 10mM HMBA the specific activity of AChE remained unchanged and its value remained significantly lower than that of the control cultures or cultures with 2mM HMBA.

Rb^+ efflux studies: We have recently demonstrated in neuroblastoma cells, a veratridine activated K^+ and Rb^+ efflux, which is Na^+ dependent, and tetrodotoxin sensitive (5) and appears to represent passage of ions through the excitable Na^+ channel. We have used this method as an indicator to probe membrane maturation in cells treated with various agents (C. Palfrey and U. Littauer, unpublished results). We now report the response to HMBA. Activation of the veratridine induced release of ^{86}Rb displays a long delay when cells are cultured with HMBA, 2mM or 5mM being equally potent, while 10mM

HMBA completely inhibited the development of veratridine sensitivity. The treatment had no effect on the passive efflux value.

The effective concentrations of dimethylsulfoxide (Me_2SO) and hexamethylene bisacetamide (HMBA) as inducers of differentiation in Friend erythroleukemic and neuroblastoma cells are similar i.e. 200-300mM and 2-5mM respectively (1, 2). Also, in both systems, a higher percentage of differentiating cells is achieved with HMBA as compared to that seen with Me_2SO. In Friend erythroleukemic cells, differentiation as monitored by globin synthesis and and morphological maturation is initiated with only a transient, early effect on delaying onset of cell growth(9). However, as in normal erythropoiesis (10) commitment to differentiation is associated with a finite number (4 to 5) of cell divisions resulting in cessation of further cell growth (11). In neuroblastoma, morphological differentiation, manifested by extension of long processes, is likewise accompanied by cessation of growth. Whether neurite outgrowth is related to the ability to mobilize some of its structural components (i.e. tubulin or actin) which also participate in the process of cell division is not yet clear (12).

In both neural and erythroid cells, the induction process is time and concentration dependent. Formation of neurite network, as well as the development of an excitable membrane (measured by Rb^+ efflux) extends over a period of some days, and is not apparent immediately. As was the case for Me_2SO induced neurite development (2), there is not obvious correlation between the levels of AChE and the degree of electrical excitability or extent of neurite outgrowth. These observations suggest that biochemical differentiation may be dissociated from morphological or electrical differentiation in these cells and be specified by separate "gene programs".

The mechanism by which Me_2SO and HMBA induce differentiation in neuroblastoma cells remains to be determined. Alterations of the surface membrane (13,14), changes in the assembly of microtubules and microfilaments (12, 15-17) or distortion of chromosomal structures should be considered. Since the neurite

morphology is different in the two cases: thin and branched in HMBA, thick and less branched in Me_2SO, it is tempting to assume that the two agents act by different mechanisms. It has been demonstrated in murine erythroleukemia cells that the ratios of hemoglobin major to hemoglobin minor during erythroid differentiation varies with the inducing agent employed (18) indicating that in this system, also, different inducers of differentiation can give rise to different programs of differentiation.

Acknowledgments

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References

1. Reuben, R.C., Wife, R.L., Breslow, R., Rifkind, R.A. and Marks, P. (1976) Proc. Nat. Acad. Sci. USA 73, 862-866.
2. Kimhi, Y., Palfrey, C., Spector, Y., Barak, Y., and Littauer, U.Z. (1976) Proc. Nat. Acad. Sci. USA 73, 462-466.
3. Amano, T., Richelson, E. and Nirenberg, M. (1972) Proc. Nat. Acad. Sci. USA 69, 258-263.
4. Wilson, S.H., Schrier, B., Farber, J.L., Thompson, E.J., Rosenberg, R.N., Blume, A.J. and Nirenberg, M. (1972) J. Biol. Chem. 247, 3159-3169.
5. Palfrey, C., Littauer, U.Z. (1976) Biophys. Biochem. Res. Comm. 72, 203-215.
6. Spector, I., Kimhi, Y. and Nelson, P. (1973) Nature New Biol. 246, 124-125.
7. Blume, A., Gilbert, F., Wilson, S., Farber, J., Rosenberg, R. and Nirenberg, M. (1970) Proc. Nat. Acad. Sci. USA 67, 786-792.
8. Lanks, K.W., Dorwin, J.M. and Papirmeister, B. (1974) J. Cell Biol. 63, 824-830.
9. Terada, M., Fried, J., Nudel, U., Rifkind, R.A. and Marks, P.A. (1977) Proc. Nat. Acad. Sci. USA 74, 248-252.
10. Marks, P.A. and Rifkind, R.A. (1972) Science 175, 955-961.
11. Fibach, E., Reuben, R., Rifkind, R.A. and Marks, P.A. (1977) Cancer Res., 37, 440-444.
12. Littauer, U.Z., Schmitt, H., Gozes, I., J. Nat. Can. Inst., In Press.
13. Glick, M.C., Kimhi, Y., Littauer, U.Z. (1973) Proc. Nat. Acad. Sci. USA 70, 1682-1687.
14. Glick, M.C., Kimhi, Y., Littauer, U.Z. (1976) Nature 259, 230-239.
15. Gozes, I., Schmitt, H., Littauer, U.Z. (1975) Proc. Nat. Acad. Sci. USA 72, 701-705.
16. Schmitt, H. (1976) Brain Res. 115, 165-173.
17. Schmitt, H., Gozes, I., Littauer, U.Z., Brain Res., In Press.
18. Nudel, U., Salmon, J., Terada, M., Bank, A., Rifkind, R.A. and Marks, P.A. (1977) Proc. Nat. Acad. Sci. USA 74, 1100-1104.