## Morphine Tolerance in a Human Neuroblastoma Line: Changes in Choline Acetylase and Cholinesterase Activities

Cholinergic mechanisms are significantly affected by the pharmacological actions of morphine[1,2]. The influence of the acute and chronic administration of morphine and related drugs on the formation, release and breakdown of acetylcholine has therefore been studied extensively[3-8]. These investigations, like other studies on the biochemistry of morphine tolerance and dependence, were carried out on whole animals or tissues taken from tolerant animals. Some of the phenomena observed might therefore result from effects of secondary factors rather than reflect the primary action of morphine.

Several investigators have observed the development of tolerance to and dependence on morphine in a variety of cultured cells[9-12]. Most of these studies were limited to survival, cell proliferation rate and morphology, and in most cases the cell lines studied were not of nervous origin.

In the present investigation, the short-term and long-term effect of morphine on the survival and proliferation rate of the established human neuroblastoma line IMR-32[13] and on the activities of choline acetylase and cholinesterase in the cells was studied. This cell line was chosen because cultured neuroblastoma cells, despite their malignant origin, retain many of the specialized biophysical and biochemical properties of nervous tissue[14-18].

*Materials and methods.* All tissue culture materials were obtained from the Grand Island Biological Co., Grand Island, N.Y., radiochemicals from New England Nuclear Corporation, Boston, Mass., and biochemicals from Sigma Chemical Co., St. Louis, Mo.

Cultures of IMR-32 (purchased from the American Type Culture Collection, Rockville, Md.) were grown at 36.5 °C by standard methods in Eagle's MEM with Earle's salts and non-essential amino acids, supplemented with 10 % fetal calf serum inactivated for 30 min at 56 °C. Media were changed 3 times weekly and subculture was carried out once or twice weekly. Cell numbers were determined by trypsinization and counting in a hemocytometer. Since the activities of choline acetyltransferase and and of cholinesterase are functions of cell division[15,18], for the determination of the effects of morphine on these enzymes the cultures were used at high cell density, when proliferation was slow. The cells were dispersed and washed 3 times by resuspension in 2–3 ml of ice-cold saline and centrifugation at 500 × g for 10 min. The cell pellet was resuspended in ice-cold water containing 0.5 % Triton X-100 and disrupted in a motor-driven glass micro-homogenizer. Cholinesterase activity was determined according to BLUME et al.[15], Choline-acetyltransferase according to WHITE and CAVALLITO[19] and total protein according to LOWRY et al.[20].


1 A. K. REYNOLDS and L. RANDALL, *Morphine and Allied Drugs* (University of Toronto Press, Toronto 1957), p. 89.
2 M. WEINSTOCK, in *Narcotic Drugs* (Ed. D. H. CLOUET; Plenum Press, New York 1971), p. 254.
3 E. W. MAYNERT, Archs. Biol. Med. exp. *4*, 36 (1967).
4 K. HANO, H. KANETO, T. KAKUNAGA and N. MORIBAYASHI, Biochem. Pharmac. *13*, 441 (1964).
5 D. BELESLIN and R. L. POLAK, J. Physiol., Lond. *177*, 411 (1965).
6 K. JHANANDAS, C. PINSKY and J. W. PHILLIS, Nature, Lond. *228*, 176 (1970).
7 W. A. LARGE and A. S. MILTON, Br. J. Pharmac. *41*, 398 (1971).
8 K. DATTA, L. THAL and I. WAJDA, Br. J. Pharmac. *41*, 84 (1971).
9 S. SEMURA, Folia pharmac. jap. *17*, 3 (1933).
10 M. SASAKI, Arch. exp. Zellforsch. *21*, 289 (1938).
11 G. CORSSEN and I. A. SKORA, J. Am. med. Ass. *187*, 328 (1964).
12 A. GHADIRIAN, Can. psychiat. Ass. J. *14*, 607 (1969).
13 J. J. TUMILOWICZ, W. W. NICHOLS, J. J. CHOLON and A. E. GREENE, Cancer Res. *30*, 2110 (1970).
14 O. AUGUSTI-TOCCO and G. SATO, Proc. natn. Acad. Sci. USA *64*, 311 (1969).
15 A. BLUME, F. GILBERT, S. WILSON, J. FARBER, R. ROSENBERG and M. NIRENBERG, Proc. natn. Acad. Sci. USA *67*, 786 (1970).
16 A. J. HARRIS and M. J. DENNIS, Science *167*, 1253 (1970).
17 P. G. NELSON, J. M. PEACOCK, T. AMANO and J. MINNA, J. Cell Physiol. *77*, 337 (1971).
18 R. ROSENBERG, L. VANDEVENTER, L. DEFRANCESCO and M. E. FRIEDKIN, Proc. natn. Acad. Sci. USA *68*, 1436 (1971).
19 H. L. WHITE and C. J. CAVALLITO, Biochim. biophys. Acta *206*, 242 (1970).
20 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, J. biol. Chem. *193*, 265 (1951).

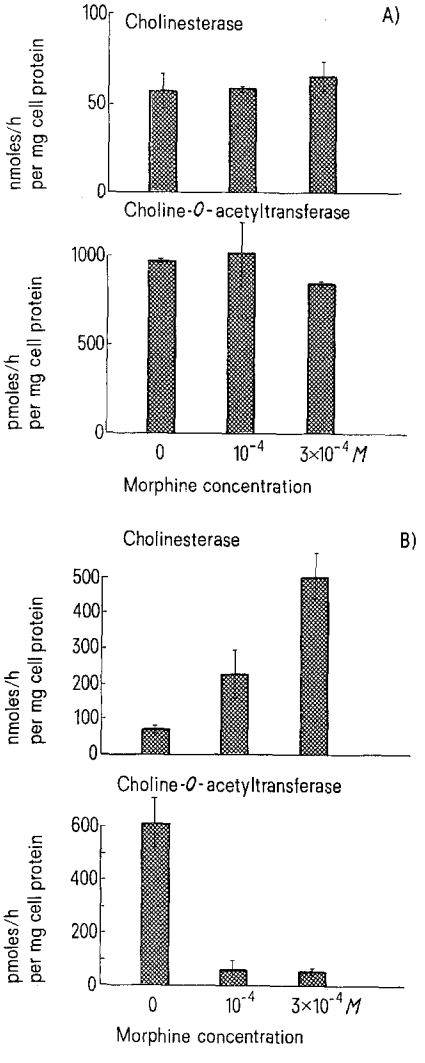

Effect of morphine on survival and proliferation rate of IMR-32 cells

| Morphine concentration ($M$) | Doubling time (h) | Inhibition (%) |
|---|---|---|
| A) Acute exposure (4 days) of cells not previously exposed to morphine | | |
| 0 (control) | 31 | — |
| $3 \times 10^{-5}$ | 31 | 0 |
| $10^{-4}$ | 36 | 14 |
| $3 \times 10^{-4}$ | 53 | 42 |
| $5 \times 10^{-4}$ | No proliferation (cells dead within 7–14 days) | |
| $10^{-3}$ | No proliferation (cells dead within 3–7 days) | |
| B) Chronic exposure (30 generations) to morphine and effect of sudden change of morphine concentration | | |
| $3 \times 10^{-4}$ throughout (control) | 36 | — |
| $3 \times 10^{-4}$, then $5 \times 10^{-4}$ (2 days) | 76 | 53 |
| $3 \times 10^{-4}$, then $5 \times 10^{-4}$ (2 months) | 50 | 28 |
| $3 \times 10^{-4}$, then $10^{-3}$ | No proliferation (cells dead within 14–20 days) | |
| $3 \times 10^{-4}$, then none (2 days) | 44 | 19 |
| $3 \times 10^{-4}$, then $3 \times 10^{-6}$ (2 days) | 36 | 0 |

Cells were dispersed by trypsinization and seeded in 35 × 10 mm disposable Falcon petri dishes at 100,000–200,000 cells/plate. Morphine was added to the desired concentration and cell counts were performed after 1, 2, and 4 days. Doubling time was determined graphically from semilogarithmic plots. (Average of 2 experiments.)

*Results and discussion.* The effect of morphine concentrations up to $10^{-3}$ *M* on survival and proliferation of IMR-32 cells not previously exposed to morphine can be seen in Table A. Morphologically, the cells appeared normal up to and including $10^{-4}$ *M* morphine while concentrations of $3\times10^{-4}$ *M* led to degenerative changes such as vacuolization, rounding up and clumping.

After continued exposure to $3\times10^{-4}$*M* morphine for 4–5 weeks (8–10 generations) or more, the morphological appearance of the cells became more normal. Table B shows that cells exposed to morphine for 30 generations had a close to normal proliferation rate in the presence of $3\times10^{-4}$ *M* and tolerated the perviously lethal morphine concentration of $5\times10^{-4}$ *M*. $10^{-3}$ *M* morphine, however, was still lethal. Also, cell proliferation slowed down in such cultures when morphine was completely withdrawn. In most cases the cells from whom morphine had been withdrawn regained normal morphology and proliferation rates within a few days.




A) Cholinesterase (top) and choline-*O*-acetyltransferase (bottom) activities in IMR-32 cultures exposed to morphine for 5 days (no subcultures). B) Cholinesterase (top) and choline-0-acetyltranferase (bottom) activities in IMR-32 cultures maintained and subcultured in the presence of morphine for 8 weeks (12 subcultures). Enzyme activities are expressed as nmoles of product formed per h/mg of cell protein for cholinesterase and as pmoles of product formed per h/mg of cell protein for choline-0-acetyltransferase.

Figure A shows that a 5-day exposure of the cells to morphine at $3\times10^{-4}$ *M* had no significant effect on the activities of either cholinesterase or choline-acetyltransferase. On the other hand (Figure B), long-term exposure to morphine caused a 200–600% increase in cholinesterase and a 90% decrease of choline-acetyltransferase activity at $10^{-4}$ and $3\times10^{-4}$ *M* morphine. These changes persisted in cultures habituated to $10^{-4}$–$3\times10^{-4}$ *M* morphine for at least 2 weeks after the morphine was abruptly withdrawn.

In several experiments, acetylcholinesterase and butyrylcholinesterase activities were determined separately by selective inhibition of the acetylcholinesterase with BW 284 C 51 j[21]. The results showed that in untreated cells 40–50% of the total cholinesterase activity is acetylcholinesterase, and that acetylcholinesterase and butyrylcholinesterase are about equally stimulated by prolonged exposure to morphine.

Our observations, made on cells of an established line, do not necessarily reflect the mechanisms of morphine habituation in the normally non-dividing cells of the nervous system in intact animals. Also, the morphine concentrations applied in our experiments were higher than those reported in the brain of narcotized animals[22]. However, our results do show that morphine tolerance can be induced in neurogenic cells without the mediation of other tissues or organs and that it is accompanied by significant changes in the activities of enzymes involved in neurotransmission. The nature of these changes, while not proof of, is compatible with proposed mechanisms of morphine tolerance and dependence, which postulate that these phenomena are caused by selective enzyme induction or repression to compensate for the acute effects of morphine[23–26].

*Zusammenfassung.* Nachweis der Entstehung einer Morphin-Toleranz und -Dependenz sowie von Veränderungen (Steigerung der Cholinesterase, Verminderung der Cholin-*o*-Acetyltransferase) von Enzymaktivitäten nach länger dauernden Morphingaben bei Neuroblastom-Kulturen.

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[21] L. AUSTIN and W. K. BERRY, Biochem. J. *54*, 695 (1953).
[22] S. MULÉ, in *Narcotic Drugs* (Ed. D. H. CLOUET; Plenum Press, New York 1971), p. 99.
[23] A. GOLDSTEIN and D. B. GOLDSTEIN, Biochem. Pharmac. *8*, 48 (1961).
[24] L. SHUSTER, Nature, Lond. *189*, 314 (1961).
[25] V. P. DOLE, A. Rev. Biochem. *39*, 821 (1970).
[26] L. SHUSTER, in *Narcotic Drugs* (Ed. D. H. CLOUET; Plenum Press, New York 1971), p. 408.
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