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SHORT COMMUNICATION

An Investigation into the Mechanism of Cytotoxicity of Levorphanol

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SUMMARY

Levorphanol, a synthetic analogue of morphine (L-3-hydroxy-*N*-methylmorphinan tartrate), has been reported to inhibit RNA synthesis. The effect of this compound upon the replication of Sindbis virus and upon the host cells in culture was studied. Propagation of the virus as well as synthesis of RNA and protein by the cells was inhibited by the drug. Light microscopic examination of the treated cells revealed extensive morphological alterations at doses lower than those which produced biochemical effects. These results indicate that levorphanol has cytotoxic activity other than its ability to diminish RNA synthesis.

Levorphanol, a synthetic analogue of morphine (L-3-hydroxy-*N*-methylmorphinan tartrate), has been reported to inhibit RNA synthesis in bacteria (1), rat liver[3] (2), and HeLa cells (3, 4). A related morphinan, levallorphan, was reported to inhibit synthesis of Mengovirus RNA (5), based on incorporation studies in actinomycin-inhibited cells. As a result of several of these studies, it has been suggested that the cytotoxicity of these compounds results primarily from interference with RNA synthesis (1, 2). It has been suggested recently, however, that these agents may affect other metabolic pathways, resulting secondarily in inhibition of RNA synthesis (3, 4). There is evidence that levorphanol affects the membranes of *Escherichia coli* and the squid axon (6, 7).

To gain further insight into the mechanism of action of levorphanol, the effect of this agent was determined in cultured mesenchymal and epithelial cells and on Sindbis virus propagation in chick embryo cells (8).

The use of Sindbis virus enabled us to examine RNA-dependent RNA synthesis. To study the effects of levorphanol on virus yield, duplicate monolayers of primary chick embryo cells were incubated for 45 min in Eagle's minimal essential medium with test levels of levorphanol (see Table 1). The fluids were removed, and Sindbis virus (3 plaque-forming units/cell) was added. After a 1-hr adsorption period, the cultures were washed four times and 2 ml of medium were added. After 10 hr of viral replication, fluid samples were collected. Levorphanol was present at the test level throughout the growth cycle. The samples were assayed for virus yield by plaque formation on chick embryo cell monolayers (8) (Table 1).

Viral yield was unaffected at concentrations below 1 mM. Doses above this level produced a progressive decrease in viral

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[3] F. F. Becker, T. Rossman, B. Reiss, and E. J. Simon, unpublished observations.

yield, although doses as high as 10 mM had only a limited effect.

To determine the effects of levorphanol on chick embryo cells, confluent primary or secondary coverslip cultures were prepared (8). Primary cultures were prepared from cells explanted directly from embryos, whereas secondary cultures were seeded from the primary cultures. The cultures were incubated in levorphanol-containing medium for 3 hr, pulse-labeled with [^{3}H]uridine (specific activity, 2 Ci/mmole), 5 μCi/ml, or [^{3}H]leucine (specific activity, 2 Ci/mmole), 5 μCi/ml, for 45 min, and then extracted (8).

In the secondary cultures no inhibition of RNA synthesis was produced with levels of levorphanol as high as 2.25 mM. At higher doses, a limited decline was detected (Table 2). Primary cultures were more sensitive, and 20–25% inhibition was achieved with 2.25 mM levorphanol. Higher levels, however, had little further effect (Table 2). Inhibition of protein synthesis increased with increasing levels of levorphanol, but was also limited in extent.

Similar experiments were then performed utilizing growing, secondary cultures. Unlike confluent cultures, levorphanol produced increasing inhibition of RNA and protein synthesis in these cultures at higher doses (Table 3).

In a separate group of experiments using rabbit kidney cells, a similar degree of inhibition was produced by comparable levorphanol levels.

Stained coverslip preparations were examined by light microscopy for each experimental situation. In both growing and con-

TABLE 1

Effect of levorphanol on yield of Sindbis virus

Three plaque-forming units of Sindbis virus per cell were added to monolayers of chick embryo cells. After 1 hr of absorption and then washing, the cells were incubated in 2 ml of medium for 10 hr. The concentrations of levorphanol listed below were present during all steps. Fluid samples were assayed for viral yield on other chick embryo fibroblasts.

Expt	Levorphanol	Plaque-forming units/culture
	mM	
1	0	1.54×10^{10}
	0.0225	1.52×10^{10}
	0.225	1.57×10^{10}
	2.25	9.7×10^{9}
2	0	9.95×10^{9}
	2.25	1.37×10^{9}
	4.50	1.20×10^{9}
	11.25	1.08×10^{9}

TABLE 2

Effect of levorphanol on RNA and protein synthesis in chick embryo confluent monolayers

Petri dishes containing three coverslips each were seeded with either primary (6×10^6 cells/dish) or secondary (2×10^6 cells/dish) chick embryo cells. These cultures were allowed to grow to confluence. They were then incubated in levorphanol-containing minimal essential medium for 3 hr, pulse-labeled, extracted, and counted. Unlabeled thymidine (0.5 mmole) was added in the experiments with uridine to prevent incorporation into DNA.

Levorphanol	[^{3}H]Uridine		[^{3}H]Leucine
	Secondary	Primary	Primary
mM	*cpm/coverslip*		
0	5580	6063	2633
0.225	5570		
2.25	5612	5152 (84%)	2402 (91%)
4.50		5279 (87%)	2081 (79%)
11.25		4758 (78%)	1546 (58%)
22.5	3643 (65%)		

TABLE 3

Effect of levorphanol on RNA and protein synthesis in growing cultures of chick embryo fibroblasts

Secondary chick embryo fibroblasts (1×10^6 cells/dish) were seeded and allowed to grow for 18 hr. After incubation for 3 hr in minimal essential medium containing levorphanol at the concentrations listed below, the cells were pulse-labeled, extracted, and counted (8). Results are average values of 3 coverslips per dish.

Levorphanol	[^{3}H]Leucine	[^{3}H]Uridine
mM	*cpm/coverslip*	
0	517	717
2.25	449 (86%)	767 (106%)
4.50	295 (57%)	495 (69%)
11.25	98 (18%)	320 (44%)

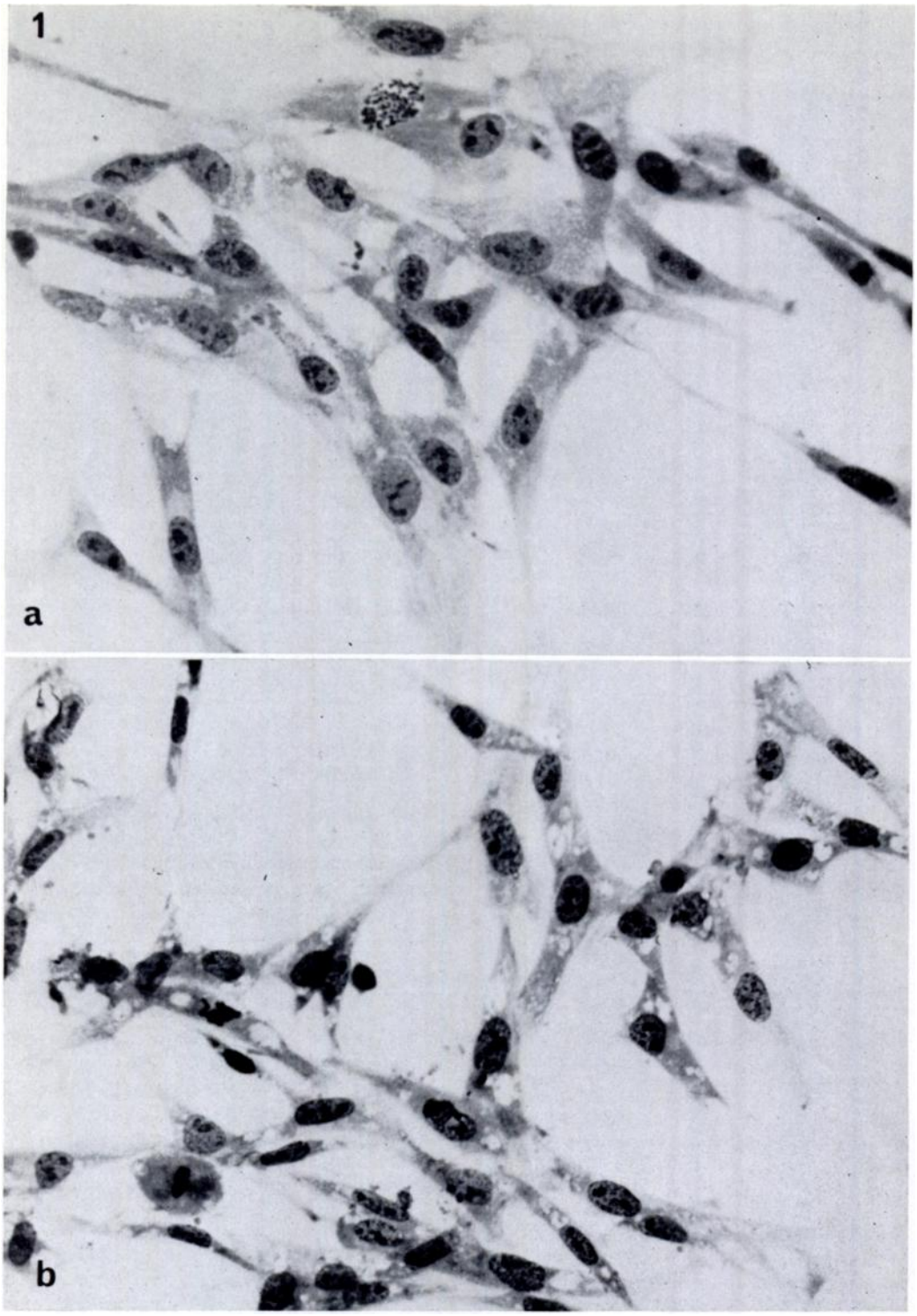

FIG. 1. *Wright-stained coverslips of chick embryo fibroblasts*
After 18 hr of growth, these were incubated for 3 hr in either minimal essential medium alone (a) or minimal essential medium containing 2.25 mM levorphanol (b) and then stained. Extensive vacuolization of the cytoplasm may be seen in (b). Mitotic activity was apparent in both preparations. × 500.

fluent cultures, a severe, dose-related vacuolization of the cytoplasm occurred at levels as low as 0.1 mM (Fig. 1). At levels above 2.25 mM, many cells rounded up, some appeared to be lost from the glass, and mitotic activity was no longer noted in growing cells.

These results indicate that levorphanol has cytotoxic activity other than its ability to diminish RNA synthesis. Thus, at 0.225 mM levorphanol, which had no detectable effect on RNA synthesis, extensive cytoplasmic vacuolization occurred. It may be argued that these morphological alterations were artifacts of fixation or staining. However, they appeared only in levorphanol-exposed cells, and then at extremely low concentrations. They suggest alterations in the cell membranes, possibly as a result of metabolic alteration or direct drug-surface interaction.

It should also be noted that protein synthesis was at least as sensitive to levorphanol as was RNA synthesis, and, at higher drug levels, even more so. The cell alterations observed in the present experiments could have resulted from levorphanol-induced depletion of ATP, as postulated by Greene and Magasanik (9). However, even this depletion of ATP may be secondary to alterations in cell surfaces, as suggested by experiments using *E. coli* and the squid axon (6, 7).

The effects of levorphanol on RNA synthesis and Sindbis virus replication presently reported were less pronounced than those demonstrated for Mengovirus RNA and the RNA synthesis of its host cells (5). Several batches of levorphanol were used in our experiments, and each of these produced severe inhibition of RNA synthesis in rat liver.[3] The morphological alterations of our cells occurred at concentrations of levorphanol as low as those previously reported to inhibit RNA synthesis. In the main, the earlier reports used long-standing tumor cell lines while our experiments were all conducted with recently explanted and normal cells, suggesting the possibility of interesting differences in cell lines used in drug testing.

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