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Effect of Hydrocortisone on Growth and Detachment of Human Heteroploid Cells in Maintenance Media.* (32116)

EMILY F. OMURA,† MELVIN S. SCHWARTZ, RENÉ I. JAHIEL,‡ AND EDWIN D. KILBOURNE

Divisions of Virus Research and Biometrics, Department of Public Health, Cornell University Medical College, New York City

The sustaining, or preserving, effect of hydrocortisone, in physiological concentrations, on heteroploid cell monolayers has been noted(1,2), and has been observed independently in this laboratory. The mechanism of this effect is not understood. In the present study, enumeration of cell nuclei and other quantitative studies were performed in order to evaluate the relative contributions of increased cell proliferation and decreased cell detachment and death to the sustaining effect of hydrocortisone.

Materials and methods. Cells. Two sublines of a cloned substrain (clone 22) of the Wong-Kilbourne influenza virus-susceptible variant of Chang's human conjunctival cells (3) were used. Mycoplasma were isolated by the method of Pollock *et al*(4) in an atmosphere of 95% nitrogen and 5% CO_2 from the subline used in Experiments I and II in 1962, but not from the one used in Experiments III and IV in 1964. *Glassware.* The cells were propagated in 200 ml milk-dilution bottles. Rubber-stoppered, 16 × 125 ml stationary test tubes were used to grow cells for experiments unless stated otherwise. All non-disposable glassware was pyrex and was washed in 7X.§ Disposable DeMuth glass test tubes‖ were used in experiment III. *Media.* Growth medium was mixture 199 (5) with 10% calf serum. Maintenance medium was mixture 199 with 2% horse serum. All sera had been heated for 30 minutes at 56°C. *Propagation and maintenance of cells.* The temperature for growth and maintenance was 37°C. For transfer, cells were dispersed with 0.25% trypsin¶ in phosphate-buffered saline (PBS) in the 1962 experiments or 0.04% versene in 1964. Bottles were seeded with 10^6 cells in 10 ml medium. Medium was changed every 2-3 days. A continuous monolayer was reached in 7 days in 1962 and in 4 days in the 1964 experiments. Test tubes were seeded with 10^5 cells. pH was readjusted to 7.2 with 0.5% sodium bicarbonate as needed. *Hydrocortisone.* Pfizer hydrocortisone diethylaminoacetate hydrochloride (aqueous solubility 28 mg/ml) was added to the maintenance medium. *Enumerations of cell nuclei* were performed with Rappaport's technique(6). *Cell viability* was assessed by the trypan blue exclusion test of McLimans *et al*(7). *Stained preparations.* Cells were grown and maintained on cover slips in Leighton tubes, fixed in Bouin's solution and stained with hematoxylin and eosin.

Results. The effects of hydrocortisone on

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† Present address: Division of Dermatology, Dept. of Medicine, New York Hospital, New York City.

‡ Career Scientist, Health Research Council of City of New York (1-202).

§ Linbro Chemical Co., New Haven, Conn.

‖ DeMuth Glass Works Division, Brockway Glass Co., Parkersburg, W. Va.

¶ Trypsin 300, Difco, Detroit, Mich.

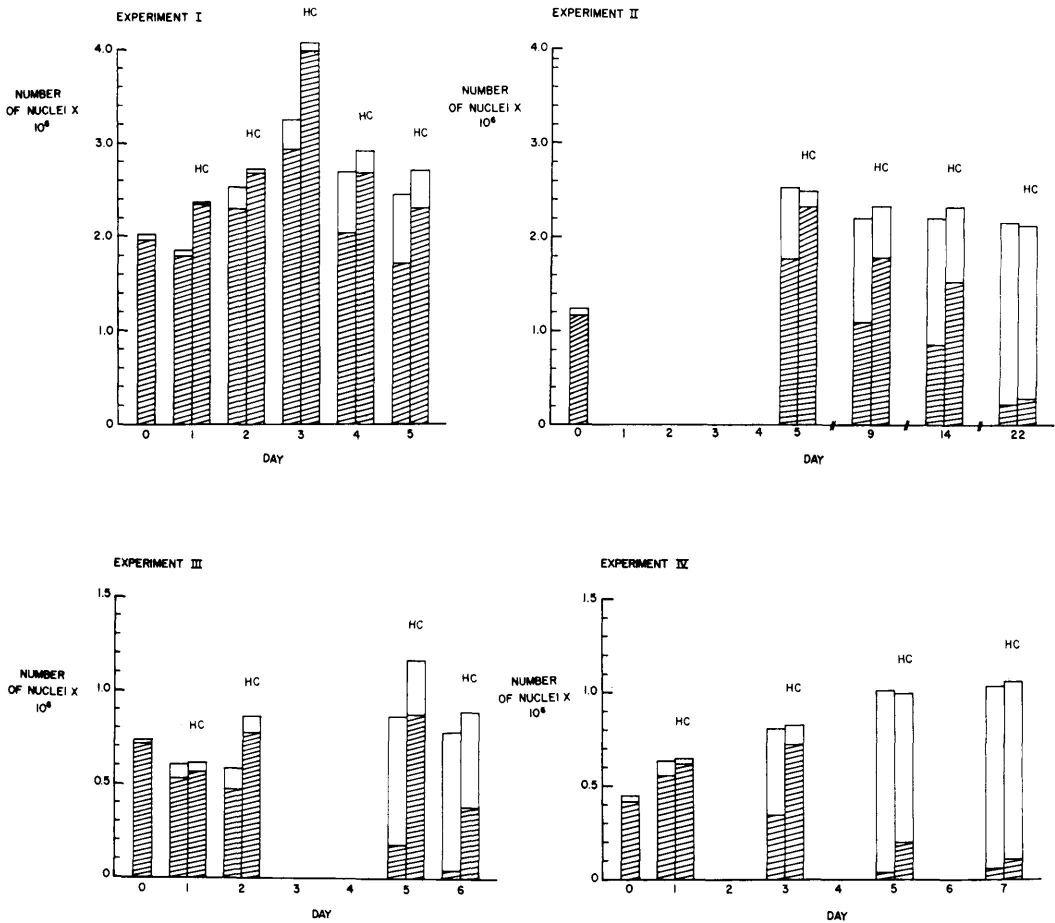

FIG. 1. Effect of hydrocortisone (HC), at a concentration of 1 μg/ml, on number of nuclei per tube (total height of bars) and number of nuclei in monolayer (height of hatched area of bars). The medium contained 2% serum in all experiments.

the total number of cells in the culture tubes and on the number of cells remaining on the glass wall of the tubes were determined in 4 experiments of similar design. Growth medium was removed from tubes containing 0.4-2.0 $\times$ 10^6 cells and replaced with 1 ml of maintenance medium containing 1 μg of hydrocortisone in one group and no hydrocortisone in the other group. Enumeration of cell nuclei was performed on 5 tubes from each group at this time (day 0) and at intervals of 1 to 22 days as indicated in Fig. 1. The nuclei of detached cells (sum of cells in medium and in wash fluid) and of cells present in the monolayer (those detached from the glass wall of the tube with 10% citric acid) were enumerated separately in each tube. The number of nuclei per tube was obtained by adding these counts. The mean numbers of nuclei per tube and of nuclei in the monolayer in the 4 experiments are shown in Fig. 1. Logarithmic transforms of the data were used for statistical analysis, since they gave to the distribution properties of the data (for example the distribution of replicate values) closer agreement with a normal distribution. The mean and standard deviation of the log transforms of the numbers of nuclei per tube and of nuclei in the monolayer in Exp. III are shown in Table I which also includes data on cultures maintained in serumless

TABLE I. Effect of Hydrocortisone on Total Cell Number and on Number of Cells in Monolayer with Time in Maintenance Medium and in the Presence and Absence of Serum.

Days in maintenance medium	Hydrocortisone* in maintenance medium	Serum† in maintenance medium	Cell nuclei per tube (log 10) $\bar{x} \pm$ S.D.‡	Cell nuclei in monolayer (log 10) $\bar{x} \pm$ S.D.*	Mean log cell nuclei in monolayer minus mean log cell nuclei per tube§
0	0		5.86 ± .07	5.85 ± .07	—0.01
1	0	+	5.78 ± .08	5.72 ± .10	—0.06
”	+	+	5.77 ± .14	5.74 ± .14	—0.03
2	0	+	5.76 ± .07	5.67 ± .08	—0.09
”	+	+	5.93 ± .03	5.89 ± .04	—0.04
5	0	+	5.93 ± .06	5.21 ± .26	—0.72
”	+	+	6.06 ± .06	5.93 ± .09	—0.13
”	0	0	5.92 ± .04	4.62 ± .32	—1.30
”	+	0	6.16 ± .14	6.03 ± .19	—0.13
6	0	+	5.89 ± .10	4.58 ± .20	—1.13
”	+	+	5.94 ± .13	5.49 ± .35	—0.45

* 1 ug/ml.
† 2%.
‡ $\bar{x}$: mean of log transform of 5 counts (1 count per tube); S.D.: standard deviation.
§ This has a maximum value of 0 if all the cells in the tube are in the monolayer and decreases as the proportion of cells remaining in the monolayer decreases.

medium.** There was a continuous monolayer on day 0 in all experiments, but the cells were more crowded in Exp. I and II than in Exp. III and IV. The mean initial number of cells per tube was 1-2 $\times$ 10^6 in Exp. I and II and 0.4-0.7 $\times$ 10^6 in Exp. III and IV. The number of nuclei per tube increased to about twice its initial value in all 4 experiments during the first 3-7 days and then remained stationary or showed a slight decrease. It tended to be slightly higher in the hydrocortisone treated tubes. However, analysis of variance showed that this difference was not significant ($p>0.2$), except in Exp. I where it reached the 0.05-0.01 level. Furthermore, analysis of the variation in number of nuclei with time suggested that hydrocortisone treated cultures had a lower rate of increase in number of nuclei per tube than controls in 3 out of 4 experiments and that the higher number of nuclei per tube in hydrocortisone treated cultures was due to a slightly higher initial number of nuclei in these tubes as compared to controls.

**The pH of serumless maintenance medium showed considerably more variation during incubation in rubber-stoppered tubes than that of medium with 2% serum. Therefore, in order to maintain a more uniform pH, all the cultures used in Exp. III were grown and maintained in metal capped test tubes in an atmosphere of 2.5% CO_2 in humidified air.

The fraction of the number of nuclei per tube which belonged to cells in the monolayer decreased in all experiments in untreated tubes. On the fifth day, this relative decrease was much more marked in Exp. III and IV than in Exp. I and II. On that day the absolute number of nuclei belonging to cells in the monolayer was in the same range as on day 0 in Exp. I and II and had decreased to less than a fourth of the number of nuclei on day 0 in Exp. III and IV. The number of nuclei of cells remaining attached to glass (nuclei of cells in the monolayer) was consistently higher in the hydrocortisone treated tubes than in the controls. The fraction of nuclei per tube that was present in the monolayer was also consistently higher in the hydrocortisone treated tubes (Table I, Fig. 1). This effect appeared after one day of treatment, reached a maximum about the fifth day and was still present as late as the 22nd day (Fig. 1). Co-variant analysis performed on log transforms of the data showed that the differences between hydrocortisone treated and control tubes in absolute number of nuclei in the monolayer, with the total number of nuclei per tube as the co-variant factor reached the 0.001 level of significance at the time of maximum effect. In general, the greater number of nuclei in

the monolayers of the hydrocortisone treated tubes, compared to those present in the control tubes amounted to 16%; this percentage is adjusted for any difference in average total number of cells present in control and hydrocortisone tubes. This effect of hydrocortisone was most marked where there had been a rapid and marked decrease in the number of nuclei in the monolayers of untreated tubes, for instance on day 5 of Exp. III and IV.

In cultures enumerated on the 5th day in Exp. III (Table I), the number of nuclei per tube was slightly higher in the tubes with hydrocortisone than in the controls. There was no significant difference between the tubes with and without serum. The tubes without either serum or hydrocortisone had the smallest number of nuclei in the monolayer. The number of nuclei in the monolayer was significantly greater than this in the tubes with serum but without hydrocortisone, and was largest in the tubes with hydrocortisone, whether or not serum was present.

In another experiment, hydrocortisone was added to the maintenance medium in concentrations of 10 and 100 μg/ml. The effects on numbers of nuclei per tube and in the monolayer were similar to those observed when a concentration of 1 μg/ml was used.

Effect of hydrocortisone on multinucleation. The greater number of nuclei found in hydrocortisone-treated monolayers, when compared to control monolayers, could be due to a greater number of cells or to a greater number of nuclei per cell. In order to distinguish between these two possibilities, a study of the effect of hydrocortisone on the occurrence of multinucleated cells was performed. Cells were grown and maintained with and without 1 μg/ml hydrocortisone on cover slips in Leighton tubes, and the number of multinucleated cells (any cell with 2 or more nuclei) per 1000 cells was counted at daily intervals from 1 to 4 days. In both groups, most multinucleated cells were binucleate. The hydrocortisone-treated monolayers had initially present a greater number of multinucleated cells. The proportion of multinucleated cells increased with time, the increase being greater in the control tubes, reaching nearly 4% by the 4th day. There were at most 0.8% more multinucleated cells in the hydrocortisone treated group than in the controls. This difference can account only for a negligible part of the difference in number of nuclei between hydrocortisone treated and control monolayers. Thus the increased number of nuclei in the monolayers of hydrocortisone treated tubes is due chiefly to an actual increase in the number of cells.

Effect of hydrocortisone on cell viability. Tests with the trypan blue exclusion technique showed that nearly all the cells in the medium and in the wash fluid became colored (*i.e.*, were presumably necrotic) while most of the cells in the monolayer did not take the stain. Thus, there was an association between detachment and necrosis of cells.

Observations with other cells and experimental conditions. Less detailed and less systematic observations of other cell lines in this laboratory during the past 4 years have been confirmatory of the studies reported above. Prolonged preservation of monolayers has been noted with HEp 2 cells in tube culture with 1.0-100 μg/ml of hydrocortisone, and with BSC cells in petri dish cultures under agar with 10 μg/ml. Better preservation of the monolayer under agar has also been seen with a conjunctival cell clone (3 days longer than controls) with 1 or 10 μg of hydrocortisone incorporated in the primary overlay.

Discussion. The results indicate that the sustaining effect of hydrocortisone was associated with a decreased detachment of cells from the monolayer. This effect was demonstrated under a relatively wide variety of environmental conditions, including differing initial cell counts, presence and absence of demonstrable mycoplasma, the use of pyrex or DeMuth soda-lime glass tubes, and the presence or absence of serum. This effect was more marked in the absence of serum. It was also more marked in certain experiments (Exp. III and IV) in which untreated tubes showed a marked detachment of cells from the monolayer. It is likely, therefore, that the magnitude of this effect of hydro-

cortisone was related to the extent of cellular detachment in untreated tubes. The effect of hydrocortisone on the absolute number of cells in the monolayer was greatest when the detachment of cells from the control tubes was intermediate. The causes of variation in detachment of cells from the monolayer in these experiments are unknown: they may be related to differences in the 2 sublines, in the glass of the tubes, or in the initial number of cells or to the presence or absence of mycoplasma contamination.

The trypan blue exclusion test showed that the detached cells were necrotic. The causes of necrosis of cells in maintenance medium are not understood. Cell death could be due to slowly developing nutritional inadequacy of the medium or to the slow accumulation of cytotoxic products. In this case, necrosis would precede detachment. It is also conceivable that detached cells might lose the ability to reimplant, which requires active cellular processes(8), and become necrotic secondarily, while in suspension. The sustaining effect of hydrocortisone could thus be due either to a protection against cytotoxic effects or to a modification of cell surface or metabolism affecting the ability of a cell to detach or to reimplant.

There is evidence for both possibilities. Hydrocortisone inhibits the release of lysosomal enzymes induced by various agents such as heat or UV irradiation(9). Hydrocortisone inhibits the cell or tissue lesions induced by specific agents which activate lysosomes, such as those induced by excess vit A in cartilaginous limb-bone rudiments in organ culture(10), by rat antiserum in a line of rat fibroblasts(11) and by lauryl sulfate in primary human amnion cell cultures (12). Hydrocortisone also defers attachment of cells induced by specific antiserum(11) and, after prolonged treatment for several weeks, that induced by trypsin and versene (12). The mechanisms that have been suggested for these effects of hydrocortisone include the prevention of modification of ground substance(10) and cell surface(11) by lysosomal enzymes and direct action of the hormone on the cell membrane(12). Any of these 3 types of mechanisms could have played a role in the present experiments.

In the present study (in contradistinction to the ones referred to above), the nature of the specific agents or deficiencies which cause cell necrosis and detachment is not known. The sustaining effect of hydrocortisone on heteroploid cell monolayers is comparable in this respect to the longer maintenance of structural integrity found in organ cultures of skin(13) and cartilage(14) treated with hydrocortisone. It is worth considering the possibility that some or all of the agents or deficiencies responsible for the necrosis and detachment of cells in monolayers and those responsible for the disorganization of organ cultures might be the same, or, if not, might exert their action through a common hydrocortisone-sensitive mechanism.

Cell proliferation was not markedly affected by hydrocortisone in the present study. The inhibition of cell proliferation by small doses of hydrocortisone observed in some cell strains but not in others(15) was only minimally present in this study.

Our results as well as similar observations on HeLa cells by Wheeler *et al*(1) suggest that hydrocortisone may be of value when it is desirable to prolong the viability of a monolayer, as, for instance, in studies of viruses with slowly developing plaques. The results obtained in the experiment with serumless medium indicate that hydrocortisone may fulfill certain functions of serum proteins, as has been suggested by Ambrose (16) in the case of lymphoid tissue cultures.

Summary. Nuclear enumeration studies of a human heteroploid conjunctival cell clone in monolayer cultures were performed in order to investigate the sustaining effect of near-physiological concentrations of hydrocortisone on cells in maintenance media. This effect was associated with a slight and inconstant decrease in the limited cell proliferation that occurs in maintenance medium and with a considerable and significant decrease in the detachment of cells from the glass surface.

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Intestinal Absorption of Sugars in Semi-Starved Rats.* (32117)

G. ESPOSITO† (Introduced by T. Z. Csáky)
Department of Pharmacology, University of Kentucky College of Medicine, Lexington

The intestinal epithelium is constantly renewed in normal, well fed animals(1). After a period of starvation, however, this tissue undergoes morphological(2) and biochemical(3) alterations resulting in the reduction of absorption of glucose and fructose(4), as well as other changes. If the food intake is restricted for a period ("semi-starvation"), the animal loses weight and its intestinal wall becomes thin. Kershaw *et al*(5) reported that the active intestinal transport of sugars and amino acids increases markedly in semi-starved rats, both *in vivo* and *in vitro*. The increased absorbing ability of the intestine disappears rapidly, however, when the rats are fed in normal fashion.

The mechanism of enhancement of the intestinal transport in semi-starved animals is not clear. It is of particular interest to ascertain whether semi-starvation modifies the function of the intestinal carrier or of the pump, or whether the enhanced transport reflects the modification of the epithelial substrate metabolism. To gain insight into this problem, the rate of disappearance from the intestinal lumen of two sugars, glucose and 3-0-methylglucose (3 MG), was compared in normally fed and semi-starved animals. 3 MG is known to be transported in the intestine in the same way that glucose is, yet it is not metabolized(6,7).

Methods. Sprague-Dawley male rats, weighing about 200 g, were housed in individual cages. A standard pelleted laboratory diet (Purina rat and mice ration, Laboratory Chow) was fed either *ad libitum* or 8 g per day per rat. As the normal daily food intake of male rats weighing 200 g was found to be approximately 15 g, the latter group was semi-starved. In fact, these rats lost an average of 22% of their initial body weight during 15 days of feeding.

At the end of 15 days of special feeding, the animals were ready for absorption experiments. These were performed *in vivo,* perfusing a loop of the upper jejunum in urethan-anesthetized animals with 50 ml of isosmotic Na_2SO_4 solution containing either glucose or 3 MG. The absorption (disappearance of sugar from the perfusate) was calculated per gram dry weight of the perfused loop, assuming arbitrarily that the dry weight was proportional to the absorptive surface. The methodology was the same as Method I, as described by Csáky and Ho(8).

Results. Both glucose and 3 MG were present in the perfusing fluid in 2 initial concentrations: 50 mg % (about 3.1 mM or

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† Visiting scientist. Permanent address: Inst. of General Physiol., Univ. of Milan, Milan, Italy.