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The effect of hydrocortisone on the para-aortic body of the newborn mouse: an *in vivo* fraction of labelled mitoses study

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INTRODUCTION

The largest collection of extra-adrenal chromaffin cells in the mouse has been termed the para-aortic body; it is found adjacent to the aorta and closely related to the renal and superior mesenteric vessels (Coupland, 1965). It differs from adrenal medullary chromaffin tissue in that it is neither surrounded by the cells of the adrenal cortex nor, presumably, exposed to the high concentrations of glucocorticoid hormones secreted by the cortical cells. Lempinen (1964) showed in the rat that corticosteroids lead to an increase in size of postnatal extra-adrenal chromaffin tissue and Wurtman & Axelrod (1966) demonstrated a direct effect of corticosteroids on the catecholamine synthetic pathway. Subsequent work (for review see Monkhouse & Coupland, 1985) has added to the growing body of knowledge concerning the biochemical, developmental and morphological effects of adrenocortical secretions on chromaffin tissue, and Monkhouse & Coupland established that hydrocortisone administered *in vivo* to gravid female mice has a hyperplastic effect on the para-aortic body of both prenatal and newborn animals, causing a marked increase in the S-phase labelling index. In this work we have sought to elucidate this by using the fraction of labelled mitoses technique to establish some of the features of the cell cycle in both the normal and the hydrocortisone-treated para-aortic body. Since in the mouse the body attains its maximum size at or around the time of birth (Monkhouse & Coupland, 1985), newborn animals were used.

MATERIALS AND METHODS

Albino mice (*Mus musculus*, strain CS1), originally part of closed colonies, were supplied by Nottingham University Medical School Animal House and were maintained under standard conditions of temperature, lighting (12 hours light, 12 hours darkness), humidity and hygiene. Animals were allowed free access to water and a normal diet (R & M Breeding Diet, Heygate & Sons Ltd, Northampton).

Hydrocortisone (total daily dosage 40 mg/Kg/day) was given orally to gravid mice from the thirteenth day of pregnancy until term (day 0 of pregnancy was counted as the day marked by the appearance of a vaginal copulation plug). It was prepared and administered as described in Monkhouse & Coupland (1985) except that it was given twice daily at roughly 12 hourly intervals (not thrice daily at 8 hourly intervals).

The newborn mice, as well as an equivalent number of normal neonates, within the first 24 hours of postnatal life were injected intraperitoneally at 10 am with

10 μCi (370 KBq) [6-^{3}H]thymidine, specific activity 21 Ci/mmol (777 GBq/mmol; Amersham International plc). In both normal and hydrocortisone treated groups, newborn mice were killed at various time intervals up to 14 hours (hourly up to 6 hours, then 2 hourly) after injection of tritiated thymidine. Three mice were used at each time interval except at 12 hours in the experimental group when only two were used. Animals were killed by decapitation and were eviscerated; a block of tissue was removed comprising the kidneys, adrenal glands and the inter-renal area. Tissues were fixed by immersion in 10 % buffered formalin (pH 6·8) for 24 hours, washed in buffer, dehydrated in graded alcohols, cleared in xylene and embedded in paraffin wax. Each block was sectioned serially at 6 μm using a Cambridge rotary rocking microtome and sections were dried overnight on to gelatinised glass slides before being dewaxed in xylene and rehydrated. Autoradiographs were prepared by dipping in Ilford K2 photographic emulsion at 43 °C under an Ilford safelight F904. The emulsion was allowed to gel on a cold plate and the slides were exposed in dry conditions for 5 days at 4 °C in light-tight boxes. Autoradiographs were developed for 4½ minutes in Kodak D19 developer at 16 °C, washed in distilled water and fixed for 2½ minutes in ammonium thiosulphate. Sections were stained in Mayer's haemalum for 4½ minutes, blued in tap water for 15 minutes, dried in air overnight and mounted in picolyte resin.

Para-aortic bodies were examined using a ×100 (oil immersion) objective and the numbers of both labelled and unlabelled mitoses noted. A cell was counted as labelled when associated with six or more silver grains. In 51 of the 59 animals, a total of at least 100 mitoses was counted, the sampling system being both independent and random. In the remaining 8 specimens 100 mitoses per para-aortic body could not be found; in these cases all mitoses seen were counted, the lowest number of mitoses being (in one specimen) 61. All stages of mitosis were counted and the fraction of labelled mitoses was calculated as the number of labelled mitoses expressed as a percentage of the total number, both labelled and unlabelled. For each time interval, the mean and standard error of the mean were calculated. The results are shown in Figures 4 and 5; the single line was fitted visually to the plotted data.

RESULTS

The light microscopic appearance of the para-aortic bodies accorded in every way with that reported in Monkhouse & Coupland (1985), those of the experimental group being markedly larger than those of the control group. Labelled and unlabelled cells were readily distinguishable at all stages of mitosis from prophase to telophase (Figs. 1–3).

Control group (Fig. 4)

The fraction of labelled mitoses data produce a curve with a well defined first peak and most of the ascent to a probable second peak. The first labelled mitoses have appeared by 1 hour and therefore the duration of the G_2 phase of the cell cycle, immediately preceding the mitosis, is about 1 hour. The time interval between the 50 % level on the first ascent (point A on Fig. 4) and a similar point on the second ascent (point C) is taken as the total cell cycle time, in this case 8½ hours, and the interval between points A and B represents the duration of S phase, here 2 hours. The duration of mitosis can be taken as the interval between the first appearance of a mitosis and the first peak (about 3½ hours). This leaves the duration of the G_1 phase

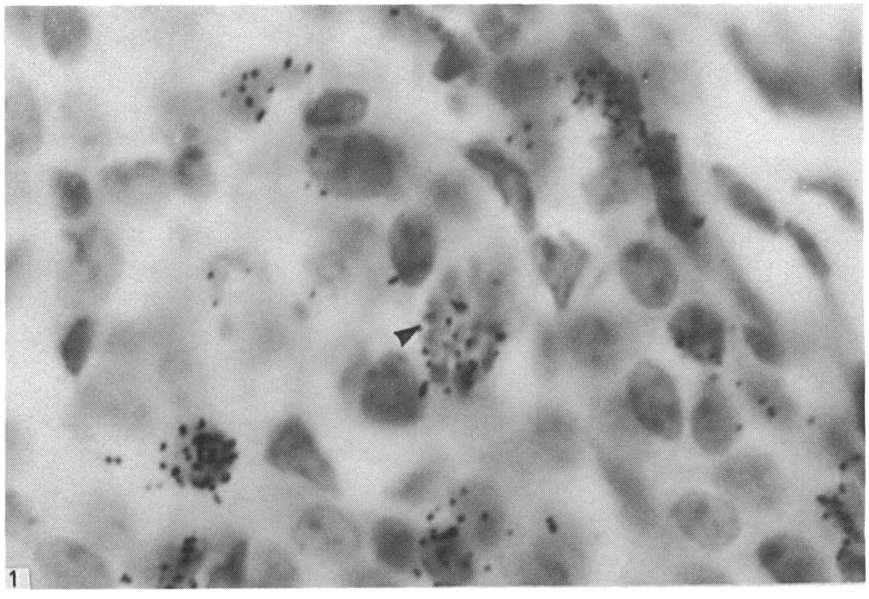

Fig. 1. Autoradiograph of para-aortic body from newborn mouse after hydrocortisone treatment showing labelled mitosis (arrowed) and several labelled interphase nuclei. Mayer's haemalum. × 1925.

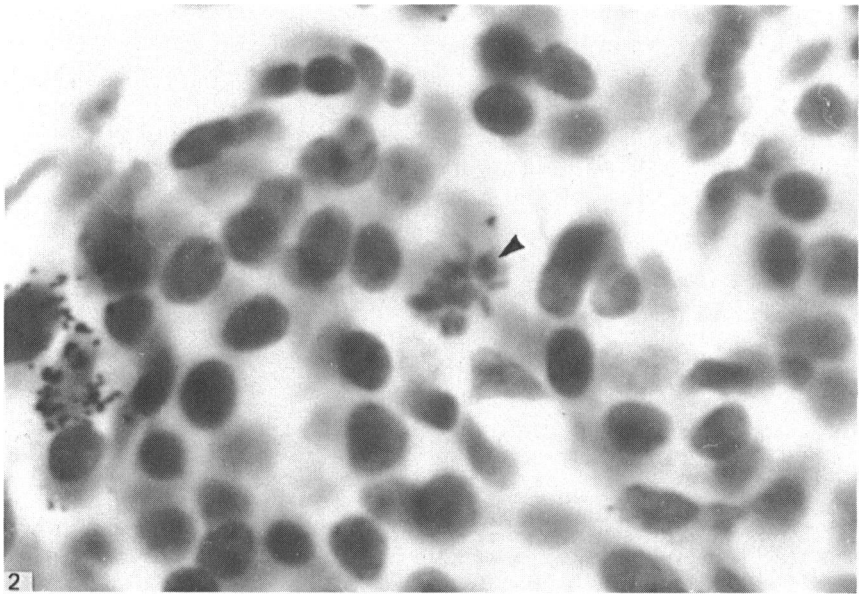

Fig. 2. Autoradiograph of para-aortic body from untreated newborn mouse showing unlabelled prophase (arrowed) and (left) labelled interphase nuclei. Mayer's haemalum. × 1925.

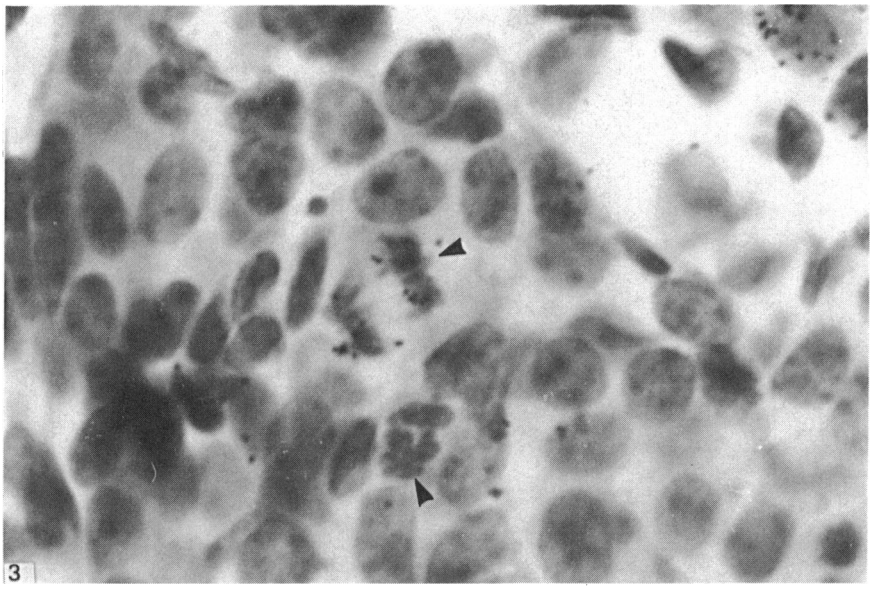

Fig. 3. Autoradiograph of para-aortic body from newborn mouse after hydrocortisone treatment showing labelled anaphase (upper arrow) and unlabelled mitosis (lower arrow). Mayer's haemalum. × 1925.

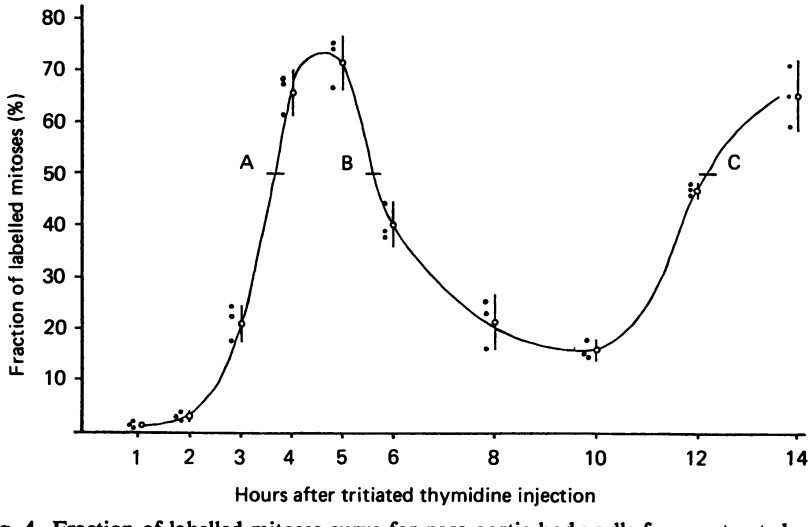

Fig. 4. Fraction of labelled mitoses curve for para-aortic body cells from untreated newborn mice. Solid circles show results from individual animals, open circles show mean with vertical bars as 95% confidence limits (1·96 × standard error of mean). Curve fitted visually.

as $8\frac{1}{2}-(1+3\frac{1}{2}+2) = 2$ hours. However, differences of opinion exist concerning this method of establishing the duration of mitosis, and these will be discussed.

Hydrocortisone-treated group (Fig. 5)

The data for this group produce a curve which begins in a similar manner to that for the control group, and thus the durations of the G_2 and mitotic phases of the cell cycle can be assumed to be similar to those of untreated mice (about 1 hour and

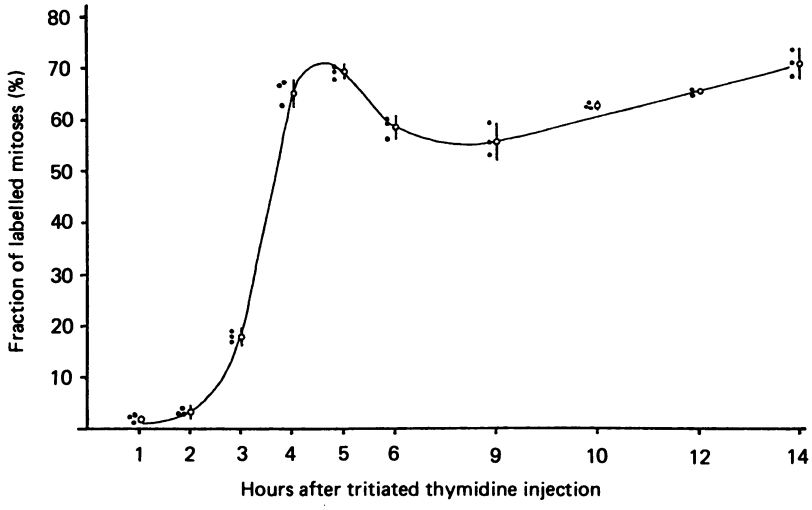

Fig. 5. Fraction of labelled mitoses curve for para-aortic body cells from newborn mice after hydrocortisone treatment. Solid circles show results from individual animals, open circles show mean with vertical bars as 95 % confidence limits (1·96 × standard error of mean). Curve fitted visually.

3½ hours respectively). However, after the first peak the curve exhibits no discernible pattern with values remaining well over 50 % after 5 hours. The total cell cycle time and the lengths of the S and G_1 phases can therefore not be derived.

DISCUSSION

Some methodological considerations

Monkhouse & Coupland (1985) showed that the method of drug administration, using a vehicle of water and evaporated milk administered through a feeding tube, was itself without effect on the para-aortic body. It was therefore assumed that a similar method would be appropriate in this work without the need to incorporate a control group of animals that had received the milk and water mixture only, without hydrocortisone. The dosage of tritiated thymidine used in this study is high. It was dictated to an extent by the minimum volume it was found possible to deliver to a newborn mouse without having to dilute the thymidine, a procedure which may risk contamination and subsequent degradation of thymidine. Also, the comparative size of even the smallest needle, coupled with the nature of newborn mouse skin, leads almost inevitably to leakage around the injection site. The relatively high dose meant that the exposure period of the autoradiographs was short, and this was advantageous in keeping down the number of background grains. With such a dose, radiotoxic effects must also be considered. These have been shown to be responsible for elongations of cell cycle phase lengths in *in vitro* studies (Beck, 1981), but they are unlikely to cause significant disruption after a pulse label in *in vivo* studies. Fliedner, Haas, Stehle & Adams (1968) reported surprisingly few side effects in the offspring of pregnant rats exposed to high doses of tritiated thymidine throughout gestation. In any case, the data obtained from the untreated group (Fig. 4) form an adequate basis for comparison with those from the hydrocortisone-treated group, and their relatively small standard errors suggest that radiotoxic effects were insignificant.

It might be expected that each peak of labelled mitoses would rise quickly to 100 % and fall equally quickly to 0 %, and in theory this should indeed be the case. However, variations in cell cycle times lead inevitably to damping and to the obscuring of this theoretical pattern. In addition, the nature of the autoradiographic sections will mean that not all labelled cells will register as such (Monkhouse, 1985), and some early prophases and late telophases will have been counted as interphase nuclei.

The results from untreated mice

No previous work has been performed which attempts to analyse the cell cycle of extra-adrenal chromaffin tissue, and surprisingly little has appeared on prenatal or immediately postnatal tissues. Our values bear comparison with those from other murine tissues (Altman & Katz, 1976; Baserga, 1985) and the total cell cycle time is similar to that for marrow erythroblasts (Tarbutt & Cole, 1972). The S phase duration in the present work (2 hours) is shorter than that for many other tissues (for example, 5½ hours for marrow erythroblasts), but variation is wide (Baserga, 1985).

The observation that the first labelled mitoses appear at one hour, and therefore that G_2 lasts for this length of time does not preclude some cells having G_2 phases longer than this. Neither can it exclude the phase being shorter since no animals were killed less than one hour after the injection of tritiated thymidine. Other values quoted for the length of G_2 in murine tissue have ranged from 0·4 hours to over 4 hours. This lack of certainty has implications regarding the duration of mitosis which is not always accepted as being the length of time between the first appearance of a labelled mitosis and the first peak. In many studies the duration of mitosis, rather than being derived from the fraction of labelled mitoses curve, is arbitrarily assumed to be 1 hour, and some workers (Thomas, 1974) take the time from the beginning of the experiment to the 50 % point on the first ascent (point A in Figure 4) as $G_2 + \frac{1}{2}M$. In fraction of labelled mitoses experiments the values for S phase and total cell cycle time are less questionable and therefore if, in this work, we assume the mitotic phase to last 1 hour and G_2 to last 1 hour, the length of G_1 becomes, by subtraction, 4½ hours. This phase (G_1) is accepted to be the most variable part of the cycle, being almost zero in rapidly proliferating populations and very prolonged in others which are either not proliferating or are doing so very slowly.

The results from hydrocortisone-treated mice

One of the most obvious ways in which hydrocortisone could bring about its hyperplastic effect on extra-adrenal chromaffin tissue is simply by an acceleration of the cell cycle and its constituent phases. It is impossible to interpret the results of this work in such a way, and therefore this notion is discarded. Despite the uncertainty which surrounds the interpretation of fraction of labelled mitoses data in respect of the lengths of G_2 and mitosis, it is noteworthy that in these results the data concerning them are very similar to those of the untreated group, indeed for the first 4 hours the fitted curves could be superimposed, and it is accepted that these two phases of the cell cycle are the least variable. The nature of these experimental data could be accounted for in at least two ways. It could be that the cell cycle is lengthened after hydrocortisone treatment and that even by 14 hours the first descent has not begun. This would mean that S phase duration had increased to 10 hours or more and that the values between 5 and 14 hours were all part of the first peak. This can

surely be disregarded: the values over this 10 hour period show wide variation (53 % at 8 hours, 74 % at 14 hours), and in any case it is incompatible with the increase in labelling indices induced by hydrocortisone reported by Monkhouse & Coupland (1985).

A more likely explanation is that the data represent the superimposition of the cell cycles of two or more populations, each apparently with similar G_2 and mitotic phase lengths; one of these could even possess cell cycle characteristics which were the same as those in the control group, and hydrocortisone might have brought about change in a sub-population of these. This will now be considered in the light of the action of hydrocortisone on the para-aortic body.

Glucocorticoids have been shown to be necessary for the maintenance *in vitro* of healthy adrenal medullary chromaffin cells (Doupe, Landis & Patterson, 1985), and *in vivo* they certainly delay, although they appear not to prevent, the degeneration of the para-aortic body (Monkhouse, 1986), which takes place during the first few weeks of postnatal life. In this work on newborn mice the para-aortic body will have attained its maximum size, but it is conceivable that already there are degenerating cells present. If this were so, the results from the control group would reflect only the remaining viable cells, whereas in the experimental group the moribund cells may have been revived by glucocorticoid administration. This may be clarified by repeating the study in younger, prenatal, mice (for example 16 days old fetuses) since at this stage the para-aortic body is still expanding; there would therefore be no (or at any rate fewer) moribund cells which may have complicated these results from the newborn.

Not dissimilar to this is the hypothesis that hydrocortisone may cause a change in another cell population, recruiting it into the proliferating chromaffin cell group. This type of transformation has been observed *in vitro*: when cells from newborn rat superior cervical ganglion were grown for 3 weeks in the presence of glucocorticoid, pure cultures of small intensely fluorescent cells developed (Doupe, Patterson & Landis, 1985). These cells synthesised and stored catecholamines and morphologically resembled adrenal chromaffin cells. If a phenomenon similar to this should have taken place in the present study, it is likely that such transformed cells would be indistinguishable from the chromaffin cells of the para-aortic body. This new population may possess cell cycle characteristics different from those of the original chromaffin cells, whose cell cycle phase lengths (other than S and G_2) may also have been altered by hydrocortisone treatment. The fraction of labelled mitoses data would thus be a combination of those from at least two populations.

It is clear that in the newborn mouse para-aortic body the hyperplastic effect of hydrocortisone is not brought about simply by an acceleration of the cell cycle. Its effect is more complex and it may involve the incorporation into the proliferative compartment of cells previously either moribund or non-proliferating.

SUMMARY

Information about the cell cycle of the mouse para-aortic body within the first 24 hours of postnatal life was derived from a fraction of labelled mitoses study. The total cell cycle time was 8½ hours, being made up as follows: S phase – 2 hours; G_2 phase – 1 hour; M phase – 3½ hours (by analysis of the results, not by assumption) and G_1 phase – 2 hours (by subtraction). Problems are discussed regarding the length of G_2 and M phases and the consequences for G_1. After hydrocortisone

administration (40 mg/kg/day) to female mice for the last seven days of pregnancy, the pattern in newborn mice was disrupted. Values for G_2 and M were similar to those of the untreated group, but no values were obtainable for the other phases of the cell cycle or for the total cell cycle time. These results after hydrocortisone treatment could be explained by the superimposition of the cell cycles of two or more different groups of cells. They are discussed with regard to the life span of the para-aortic body, and their implications are considered in the light of previously reported glucocorticoid-induced transformations of small granule cells from cervical sympathetic ganglia into catecholamine-storing chromaffin cells. The established hyperplastic effect of hydrocortisone on the para-aortic body is therefore not the result simply of an acceleration of the cell cycle, but it may involve the incorporation into the proliferative compartment of cells previously either moribund or non-proliferating.

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