Discussion

These results indicate that a single dose of low energy X-rays during intrauterine development of mice can induce fetal death and abnormalities. The type of anomaly and the frequency of incidence depended on the developmental stage at which the irradiation was done.

The observation that exposure at 3.5 d.p.c. increased the preimplantation mortality is in agreement with the earlier findings of Russell[10], Rugh and Grupp[11] and Ohzu[3] that preimplantation embryos are highly sensitive to radiation-induced mortality. While Russell[10, 12] reported almost no abnormalities in the survivors to term, Rugh and Grupp[11] and Ohzu[3] found a very low incidence of morphological anomalies which was not statistically significant, even though the doses used in their studies (5 cGy) were higher than in the present study. The present data showed a significant increase in retarded embryos, with reduction in body length and weight, among preimplantation embryos which had received X-rays; however, these changes were transient and complete recovery to normal was effected before 6 weeks of age (publ. elsewhere). The difference in response from those described in earlier reports[3, 11] could be due to a difference in strain sensitivity, as Michel and Fritz-Niggli[7] observed a strain difference in the resorption rates between NMRI and F/A mice after exposure to 1 cGy of 140 kVp X-rays.

Exposure at 6.5 d.p.c. resulted in a significant increase in the number of retarded fetuses, even though all the animals recovered and regained normal size during early postnatal development (unpubl. obs.). Such a transient increase in the number of retarded fetuses was also reported by Michel and Fritz-Niggli[7], after irradiation of 8-day embryos with 1 cGy of X-rays.

The main effect noticed after exposure to low doses of X-rays in the diagnostic range during late organogenesis (11.5 d.p.c.) in the present study was a significant reduction in the head and brain size, which continued through later development (unpubl. obs.) Miller[2] suggested that small head size is the simplest and most sensitive measure of radiation effect on humans which can be detected at birth. The present results show that this may apply to the mouse fetus too, especially during late organogenesis, which is the critical stage for brain damage[13].

These results indicate that there is a greater risk of early intrauterine death by exposure at the preimplantation stage, while this stage and the organogenesis period are equally sensitive to radiation-induced fetal growth retardation; head and brain growth are more susceptible at the late organogenesis period. All these effects could be induced in the mouse embryos by a whole body exposure of the pregnant mother to as low a dose as 9 mGy of diagnostic X-rays.

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Persistence of added retinoids in organ culture media during induction of mucous metaplasia and glandular morphogenesis in hamster cheek pouches

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Summary. The retinoid concentration (determined colorimetrically) did not change significantly in retinyl acetate-supplemented (6 µg/ml) Eagle's Minimal Essential Medium containing 10% fetal calf serum when stored at − 20 or 4 °C over 7 days. After the medium was incubated at 37 °C for 48 h, 37–49% of the retinoid remained, whether or not tissue (neonatal Syrian hamster cheek pouch) was present, and irrespective of explant age. The normal retinoid level in the tissue was approximately 0.25 µg per gram. Therefore, neonatal hamster cheek pouches, incubated in medium with the addition of 6 µg of retinyl acetate per ml of medium and undergoing mucous metaplasia and some mucous gland morphogenesis, were continually being exposed to retinoid levels which, though gradually decreasing, remained well above their normal physiological level.

Key words. Retinyl acetate; retinoid concentration; culture medium; hamster; cheek pouch; organ culture; mucous metaplasia; glandular morphogenesis.

The recent evidence that all-trans retinoic acid, a natural morphogen, can direct gene transcription for development of a normal structure [1–5], has heightened interest in the in vivo and in vitro models of normal and abnormal morphogenesis which are controlled by high doses of naturally occurring retinoids. These include the duplication of structures in the regenerating limbs of urodeles [6], the formation of feathers instead of scales on the feet of embryonic chicks [7], and the formation of branching, mucus-secreting glands in organ culture, either from the vibrissa follicles of embryonic mice [8] or from the developing cheek pouch of the newborn hamster [9, 10]. Doses administered and exposure times have been shown to be critical in many of these systems, yet the levels of retinoids maintained in the medium or the animal model have not been monitored despite the sensitivity of retinoids to light and oxidative degradation. No estimates have been made of the quantity of retinoid existing in a given culture system over the entire culture period in order to fully describe the dose delivered to the responding tissue. In addressing these problems, we adapted a simple colorimetric method to measure retinoid concentrations in medium prepared, stored and incubated under a variety of conditions including use in culture of the newborn hamster cheek pouch. The method used for retinoid estimation has the advantage of providing a single value that includes all the biologically active retinoids which may have been present in the original medium and also those which may have been derived by metabolism during incubation, including retinoic acid [11, 12].

Materials and methods

A small colony of Syrian hamsters was maintained on a β-carotene-free diet at the University of Guelph's Animal Care facility. Hamsters less than 24 h old were euthanized and the cheek pouch rudiments, approximately 1 mm^2 in area and 0.5 mm thick, were excised. A detailed description of methods for dissection, culture and histological processing of this tissue for morphological analysis has been published elsewhere [10]. In the present study, each culture comprised one intact pouch on a gelatin sponge raft floating on 1 ml of medium in a Leighton tube, and was incubated in 5–7% CO_2 in air at 37 °C. The test medium, consisting of Eagle's Minimal Essential Medium with 10% calf serum and 6 μg/ml of crystalline retinyl acetate (1.8×10^{-5} M), or the control medium, without the retinyl acetate, was replaced every 48 h. Each pseudo-culture consisted of a similar Leighton tube culture assembly incubated with test medium at 37 °C, but without an explant.

Three experiments were conducted. Two sets of cultures were established; in one (Experiment 1), groups of four cultures were terminated after 2, 4, 6, 8 and 10 days of incubation. Because of the number of samples required, a second set of cultures (Experiment 2) was established to allow sampling of groups of cultures at 12, 14, 16, 18 and 20 days. A third experiment (Experiment 3) involved pseudo-cultures only, from which medium was sampled at 2, 4 and 6 days. We wished to simulate procedures commonly used in our laboratory to induce metaplasia and morphogenesis, therefore medium was prepared according to the usual laboratory protocol, i.e. one day prior to culture, and every 8 days thereafter. Thus, medium batches A and B were used in Experiment 1, batches C, D and E in Experiment 2, and batch F in Experiment 3. All batches of medium were stored at − 20 °C, except that half of batch F (F′) was stored at 4 °C. Each batch of medium was sampled immediately following preparation, and just prior to each use in culture. Incubated medium was collected from the cultures terminated after each 48 h, by aspiration from the Leighton tubes and squeezing from the gelatin sponges.

Retinoids were extracted from culture medium and amounts determined colorimetrically by modifications of the methods of Bayfield [13] and Bayfield and Cole [14] as described previously [15] for extraction and quantitation of retinoids from plasma. All experimental procedures were performed under illumination from gold fluorescent light (F40/G0 General Electric, Toronto, Ontario) with all other light excluded to prevent photodegradation [16, 17]. Samples for analysis were protected from ordinary light at all times and were stored at − 60 to − 50 °C.

Using standard histological techniques, selected explants were fixed and sectioned serially to determine the health and degree of differentiation of the tissues.

Results

In the three experiments conducted, none of the samples of control medium taken before or after storage, or after culture, contained measurable amounts of retinoid. All the samples of retinyl acetate-supplemented medium contained considerable amounts of retinoid (fig. 1), although the concentrations were always below the calculated value (6 μg per ml). No trend was apparent in the changes in retinoid concentrations of unincubated medium during storage at − 20 °C (fig. 1 A), and a linear regression analysis of data for each batch of medium showed that there were no significant losses of retinoid over time. Storage at 4 °C resulted in a slight loss over time, although this was not significant at the 5% level. An analysis of covariance of the retinoid concentrations of all the batches of unincubated medium, stored for up to 7 days at − 20 °C, showed no significant change in retinoid levels over this storage period ($F = 0.0330$, $p = 0.8562$). However, the differences in the initial retinoid concentrations between batches were significant in some cases.

When samples of the same batch of medium were stored at − 20 °C and 4 °C in Experiment 3, the mean retinoid levels were slightly lower for samples stored at 4 °C for each period (fig. 1 B). However, the mean values over the

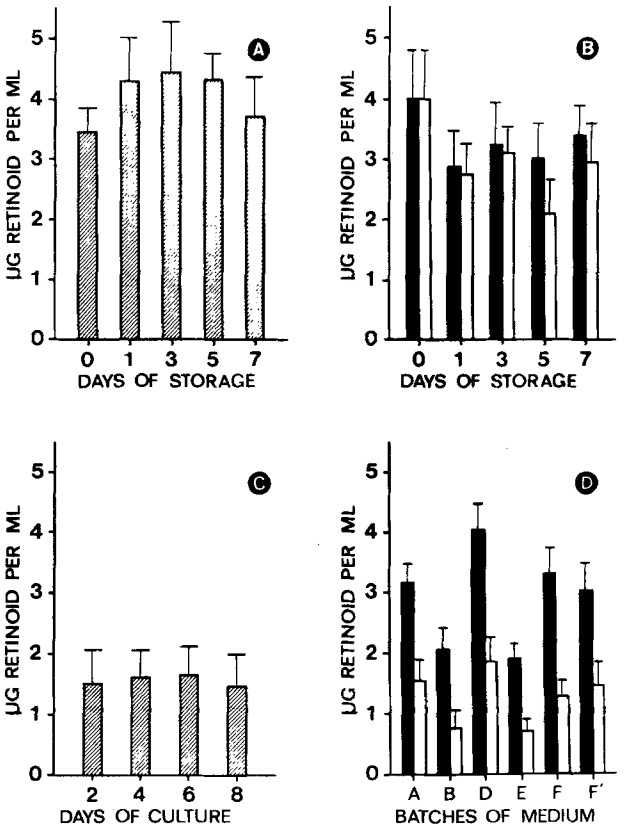

Figure 1. Histograms showing retinoid concentrations in retinyl acetate-supplemented media. (T-bars represent one standard deviation). *A* Unincubated medium stored at − 20 °C. *B* Unincubated medium, stored at different temperatures. (■, Stored at − 20 °C; □, stored at 4 °C). *C* Medium after two days of incubation. *D* Effect of two days of incubation on retinoid concentrations. (Batch F′ stored at 4 °C. All other batches stored at − 20 °C. ■, Prior to incubation; □, after incubation).

seven days for each storage temperature, 3.32 and 3.03 µg per ml respectively, were not significantly different by Student's t-test ($n = 44$, $p = 0.338$, $t = 0.97$).

Figure 1 C shows the retinoid concentration for medium collected after 2 days of incubation with tissue (data from medium batches A, D and E combined). A comparison with figure 1 A illustrates the loss of retinoids due to incubation. Separate regression analyses for batches A, D and E confirmed the lack of a significant relationship of retinoid loss during culture to the duration of prior storage of medium. The average retinoid concentration of the medium after two days of incubation, either with or without tissue, was significantly lower than that of the medium before incubation for every batch of medium tested (fig. 1 D). After incubation with cheek pouch, 37–49 % of the initial retinoid remained. When medium was incubated without tissue (batch F), 39–48 % of the retinoid remained, so a difference caused by the presence of tissue was not apparent.

Another set of regression analyses of the retinoid concentrations for medium batches A, D and E after each 2 days of incubation with tissue showed that increasing age of culture from 2 to 20 days had no significant effect on the rate of loss of retinoids.

In the medium incubated without tissue (batch F), the replacement of incubated with fresh medium did not cause a significant change in the initial retinoid level of subsequent fresh medium replacements, i.e., there was no net loss in the retinoid concentration by dilution of the fresh medium by old medium still remaining in the tube, nor was there a net increase in the retinoid concentration because of retinoids being concentrated in the sponge and then mixing with fresh medium.

Histological observations were made on complete serial sections of representative explants in order to monitor the health of the tissues and verify the effects of retinyl

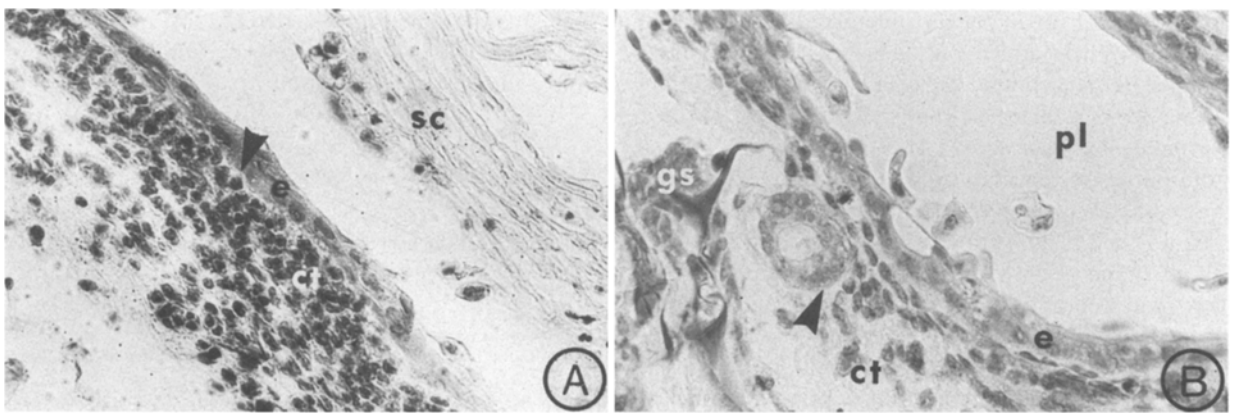

Figure 2. Histological sections of cheek pouches in organ culture. Hematoxylin, eosin. ×400. *A* After 14 days in control medium (Experiment 2), an intact basement membrane (arrowhead) separates the connective tissue stroma from the epithelium (*e*). The epithelium contains 2–3 layers of living cells and a well-developed stratum corneum (*sc*). There is an artifactual separation of the stratum corneum from the adjacent cell layers. *B* After 8 days in retinyl acetate-supplemented medium (Experiment 1), there are no cornified cells in the pouch lumen (*pl*). The epithelial lining (*e*) on both sides of the lumen is of stratified cuboidal type. A small mucous gland (arrowhead) has developed as a downgrowth from the epithelium into the connective tissue stroma (*ct*) and is seen here in cross section. The micrograph shows remnants of the gelatin sponge substrate (*gs*) on which the culture was grown.

acetate, as described elsewhere[10]. These included mucous metaplasia of the epithelium in all explants as well as glandular morphogenesis in some.

Serial sections of explants fixed after 10, 14 and 20 days of culture in control medium were examined. All of the cultures appeared to be healthy and keratinizing normally (fig. 2A), although the stratum corneum frequently separated from the adjacent cellular layers of epithelium at fixation or during tissue processing.

Figure 2B shows a section of an explant, cultured for 8 days with retinyl acetate. Keratinization was totally suppressed and the epithelial cells were mostly low cuboidal, as opposed to squamous, but stratified, being 2–5 cell layers thick. Many superficial cells seemed to be of mucus-secreting type, thus suggesting the mucous metaplasia which has been confirmed by histochemical tests in concurrent experiments[10]. Additionally, a small, multicellular, mucous-like gland was observed, giving evidence of glandular morphogenesis. All explants which were cultured for 10–20 days with retinyl acetate showed evidence of mucous metaplasia and appeared fairly healthy, with only a very few pyknotic nuclei in the epithelium and no necrosis in the stroma.

Discussion

The spectrophotometric analysis of retinoids adapted from that of Bayfield and Cole[14] proved adequate for the samples of retinyl acetate-treated culture medium that were measured. Absorbance readings obtained from standard solutions of retinyl acetate were very similar to those obtained by Parizkova et al.[18], although these researchers worked with retinol as the standard and used antimony trichloride rather than trichloroacetic acid as the chromogen reagent.

In this study, the retinoid remaining after incubation for 48 h of retinyl acetate-supplemented medium containing hamster cheek pouches was 37–49% of the initial level. After organ culture of rat prostate gland for 48 h, 45% of the previously added radioactively-labelled retinoid remained in the medium[19]; the half-life of retinyl acetate in mouse epidermal cell culture medium has been reported to be 3–4 days[20].

In the culture system employed in this study, the amount of retinoid measured in the medium after incubation with tissue was very similar to that in the medium incubated without tissue (39–48% of initial level). However, the cheek pouch cultures could have been taking up a small quantity of retinoid from the medium. The histological observations indicated that the retinoid-treated tissue had undergone transformations in response to excess vitamin A in vitro typical of those reported by other investigators[9,10]. Several other studies using radioactive labelling have demonstrated retinoid uptake by tissue from medium after culture with excess retinoids[20–23]. In the present study, the proportion of added retinyl acetate which was absorbed and/or metabolized by the tissue was presumably too small to be detected with the assay system employed.

The percentage of retinoid remaining in the medium after incubation without tissue in the present experiments was much lower than that reported by Frolik et al.[24] in their study of hamster tracheal organ cultures. The difference may have been due in part to the difference in incubation period (48 h as opposed to 24 h). Some of the difference may also have been due to the fact that Frolik and his co-workers added ascorbic acid to the medium they employed, as an antioxidant. Ascorbic acid was not added to the medium prior to culture in the present study because the objective was to measure the retinoid content of a particular medium as currently used in this and other laboratories, rather than to retain the maximum amount of the retinoid.

Storing the retinyl acetate-supplemented medium either at − 20 °C or at 4 °C for up to 7 days did not cause a significant reduction in the retinoid content, although there was a slight downward trend in retinoid content observed in the medium stored at 4 °C. Fell and Mellanby[25] reported a 28% loss of retinyl acetate in fowl plasma stored at 0 °C over a 7-day period, but this represented only a single observation and so is difficult to assess. Additionally, although the plasma was reported to have been stored in the dark, the conditions during the media preparation, media handling and the assessment of the retinoid levels were not specified.

The age of the tissue explant (from 2 to 20 days) had no consistent effect on the percentage of retinoid left in the medium. For this culture protocol, of all the factors evaluated, only the incubation for 48 h at 37 °C consistently caused a decrease in retinoid levels in the medium. In order to compare the amount of retinoid in the medium to the amount normally found in the tissue, 226 neonate cheek pouches, weighing 0.957 g, were analyzed as one sample, yielding 0.25 µg of retinoid per g[26]. This shows that in our culture system the tissue is exposed throughout the culture period to a retinoid level which, though falling off by the end of every two days, remains well above the physiological level.

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A spontaneous benomyl-resistant mutant of *Podospora anserina* exhibiting a diurnal growth rhythm

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Summary. A spontaneous mutant of *P. anserina* isolated by screening for benomyl resistance exhibited a diurnal growth rhythm dependent on light-dark cycles. The rhythmic character, the benomyl resistance and a growth rate reduced to 50% of that of the wild type were inherited together over more than 10 generations. The locus was mapped on linkage group II, 0.35 map units distal to the locus *z* (= 0.81 map units from the centromere).
Key words. Podospora anserina; benomyl resistance; mutant; mapping; diurnal growth rhythm; light-dark cycles.

Benomyl resistance has been found in *Aspergillus nidulans*[1], *Verticillium malthousei*[2], *Neurospora crassa*[3] and *Venturia inaequalis*[4–6]. The resistance is attributed to alterations of the fungal tubulin[1,7,8]. Spontaneous mutagenesis occurs regularly in *Podospora anserina*. As a result, mutants resistant against D-galactose, a non-metabolized hexose[9] and similarly against benomyl[10] have been obtained. In *P. anserina*, the benomyl-resistant strains are interesting in that they exhibit a rhythmic mycelial growth[11]. Rhythmic growth in fungi is related to the permeability of hyphal membranes[12,13], and hence ionic fluxes[14,15] and energy metabolism[16].
One of these strains differs from other clock mutants of *P. anserina*[17], and from the remainder of the isolated benomyl-resistant variants, in that its rhythm is synchronized to light-dark-cycles. This paper reports on further investigations with this particular strain.

Materials and methods

Fungus. The benomyl-resistant mutant *mbc*^{r} of *P. anserina* was isolated from an agar culture containing an MBC gradient. In the gradient agar technique[18], a layer of minimal-thiamine agar supplemented with 2 µg/ml benomyl was allowed to solidify while the petri dish was held in an inclined position. An equal amount of benomyl-free agar was then added which hardened while the plate was level. The two complementary slanted layers established a graded MBC concentration across the plate. The resistant strain grew ahead of the inhibited front of the sensitive mycelium.
The wild type s_1 and the reference strains of *P. anserina* (Ces. ex Rbh.) Niessl were obtained from the collection of *Podospora* strains in Bochum, FRG.

Media and cultures. The following media of Esser[17] were used: corn meal agar for crosses; corn meal agar containing 4.4 g/l ammonium acetate for germination; minimal-thiamine medium with 0.1 mol/l fructose as carbon source in tests.
Colonies for SEM observation were grown on aluminium-coated coverslips, partially covered by 1.5% malt extract solution adjusted to pH 6.0 with potassium hydroxide.
Benomyl, of which MBC (= methyl-benzimidazol-2-yl-carbamate) is the hydrolyzed derivative in aqueous solution, was a gift or Dr P. Wallnöfer (Munich, FRG). It was first dissolved in a solution containing 80% (v/v) ethanol and 20% (v/v) propylene glycol acidified with 10 ml/l 10 M HCl. The benomyl stock was added after the medium was cooled down to 40 °C following autoclaving, to give final concentrations of 1 µg/ml