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INFLUENCE OF STRAIN AND AGE ON THE INDUCTION OF MAMMARY TUMOURS BY DIETHYLSTILBOESTROL IN C3H MICE

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Abstract—C3H/HeJ and C3H/HeN female mice were fed diets containing targeted concentrations of 320 or 640 ppb diethylstilboestrol (DES) starting at 7 or 11 wk of age and continuing throughout their remaining lifespan. Regardless of the DES concentration there was a faster rate of development and higher final incidence of mammary adenocarcinomas among the C3H/HeN mice than among the C3H/HeJ mice. In C3H/HeN mice started on DES when 11 wk old, mammary tumours developed more rapidly than when treatment was started at 7 wk of age. This was also true for C3H/HeJ mice given 320 ppb DES but not for those treated with 640 ppb DES. Both age at the start of treatment and strain of C3H mice are important factors to be considered in designing experiments to study the tumorigenic activity of oestrogens such as DES.

INTRODUCTION

In earlier studies at this laboratory two strains of C3H mice, both purported to have high titres of the murine mammary tumour virus factor (MMTV), were found to develop mammary tumours at very different rates when fed diets containing diethylstilboestrol (DES). For example, 93% of a starting population of C3H/HeN virgin females fed a diet containing 640 ppb DES had developed palpable mammary adenocarcinomas after 39 wk of DES exposure (Greenman & Highman, 1982) whereas only 29% of a starting population of C3H/HeJ virgin females had developed palpable mammary adenocarcinomas after 39 wk of exposure to 1000 ppb dietary DES (Highman, Greenman, Norvell *et al.* 1980). These studies were not carried out concurrently and, in addition to the differences in strain and dosage, the ages of the mice at the start of DES treatment differed somewhat. C3H/HeN mice were treated from 4–5 wk of age, whereas C3H/HeJ mice were treated from 6 wk of age.

Although separate studies indicate that C3H/HeN (Heston, Deringer & Dunn, 1956) and C3H/HeJ (Richardson, 1973) female mice differ from each other in the incidence and time to appearance of spontaneous mammary tumours, no reports have been found in which these two strains have been evaluated simultaneously under identical environmental conditions for differences in spontaneous or oestrogen-induced mammary tumorigenesis. Neither could we find any reports of studies in which the influence of age at the start of oestrogen treatment on the rate of mammary tumour appearance had been examined in either strain. The current study was designed to determine the relative importance of these two factors, strain and age, in designing protocols for evaluating the carcinogenic potential of oestrogens.

EXPERIMENTAL

Specific pathogen-free C3H/HeN mice were produced at the National Center for Toxicological Research (NCTR); C3H/HeJ mice were obtained from Jackson Laboratories (Bar Harbor, ME) and were quarantined for 2 wk before experimental use. DES, obtained from Sigma Chemical Company (St Louis, MO), was found to be free of impurities when analysed by gas chromatography and high-pressure liquid chromatography (HPLC). The DES comprised virtually 100% of the *trans* isomer. The diet (5010M) was purchased from Ralston Purina Company (St Louis, MO) and was found to contain < 5 ppb (b = 10) DES equivalents when tested by a uterine weight assay. The diets were autoclaved and reground before mixing with DES in a Patterson Kelly V-blender. A solution of DES in 95% ethanol was thoroughly mixed with diet in a ratio of 5 ml/100 g of feed to obtain targeted dietary DES concentrations of 320 and 640 ppb. Ethanol was then removed by mixing under vacuum at 82 C. All batches of feed were analysed for DES by electron-capture gas chromatography or HPLC (King, Nony & Bowman, 1977). Mean (± SD) concentrations for the two target concentrations were 331 ± 56 and 636 ± 47 ppb. The formulated diets were stored at room temperature in stainless-steel containers for 8 wk or less.

Virgin female C3H/HeN-MTV+/Nctr (C3H/HeN) and C3H/HeJ mice were continuously fed diets containing the targeted DES concentrations of 320 or 640 ppb starting at either 7 wk (49–54 days) or 11 wk

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Abbreviations: DES = diethylstilboestrol; DMBA = 7,12-dimethylbenz[*a*]anthracene; HPLC = high-pressure liquid chromatography; MMTV = murine mammary tumour virus factor; NCTR = National Center for Toxicological Research.

(51–52 days) of age and continuing throughout their remaining lifetimes. Groups of 72 and 48 mice of each strain were used at the 320 and 640 ppb levels, respectively. C3H/HeN and C3H/HeJ mice were kept on different cage racks. Each column on a rack was composed of six cages and was randomly assigned *in toto* to a given treatment group so that mice in all cages in a column would receive the same experimental diet and animals for a given treatment group would be equally distributed on each shelf level. Mice from a given strain were assigned to cages randomly in a way that virtually eliminated the housing of littermates in the same cage. The mice were housed four per cage on hardwood chip bedding in plastic 'shoe-box' type cages with spun polyester filter tops. The animal room was maintained at 21.1–23.3 C and 40–60% relative humidity with fluorescent lighting 12 hr/day (06.00–18.00 hr). Ventilation provided 10–15 room air changes/hour.

Feed and pasteurized ultrafiltered water were provided *ad lib*. Daily checks were made for dead or moribund animals. At weekly intervals animals were weighed and examined for clinical signs of disease or significant changes in their appearance and then transferred to clean cages with clean feeders and water bottles. Mice were killed and autopsied when palpable body masses (presumptive mammary tumours) attained a 1-cm diameter. During the autopsy of mice that were removed from the experiment because they died, became moribund or developed 1-cm masses, the mammary glands and palpable subcutaneous masses were excised and fixed for 24 hr in Bouin's solution. They were then trimmed, washed, processed in an automatic tissue processor on a 4-hr schedule, embedded in paraffin blocks, sectioned at 5 μm and stained with haematoxylin and eosin (Frith, Highman & Konvicka, 1976). The classification system of Dunn (1959) was used to identify mammary adenocarcinomas.

Probability distribution curves for time of removal from experiment with palpable, histologically-verified type A, B and/or C mammary adenocarcinomas, adjusted for removal by competing risks, were estimated (Kodell, Shaw & Johnson, 1982) and plotted against time. Tests to determine dose-related effects (Peto, Pike, Day *et al.* 1980) were performed. All estimators and statistical tests were calculated using NCTR's SAS procedure CHRONIC (Kodell, Haskin, Shaw & Gaylor, 1983).

Strain comparisons were one-sided since previous studies at NCTR indicated that C3H/HeN mice might be removed with tumours earlier than C3H/HeJ mice. These earlier studies also indicated that mice started on DES at a young age might develop tumours more quickly than mice started at an older age. Thus, although the current study does not substantiate this finding, it was decided *a priori* that statistical comparisons of the effect of age at the start of treatment would be one-sided.

RESULTS

Virtually all of the mammary adenocarcinomas were of types of A or B. In general, type B adenocarcinomas appeared earlier and were more predominant than type A adenocarcinomas. In C3H/HeN mice the B:A ratio was between 7.9:1 and 10.1:1 whereas in C3H/HeJ mice this ratio was 3.3:1. Strain differences in mammary tumorigenesis are illustrated in Fig. 1. Differences between strains in the rate of development of mammary tumours were highly significant ($P < 0.00005$ with one-sided test) at both dietary concentrations of DES (320 or 640 ppb) and regardless of the age at which DES feeding was initiated (7 or 11 wk). In all cases, mammary tumours developed earlier in the C3H/HeN mice than they did in the C3H/HeJ mice. Although the time until the first mouse was removed with a palpable tumour in a given dose group was only 40–70 days earlier in C3H/HeN than it was in C3H/HeJ mice, the subsequent rate of development of mammary tumours was much slower in C3H/HeJ mice. Thus, the estimated time to a 50% incidence of mammary tumours was at least 260 days greater in C3H/HeJ than it was in C3H/HeN mice for a given dietary concentration of DES. There was a dose-related reduction in time of removal with mammary tumours when DES treatment was started at 7 wk (Fig. 1a), but not at 11 wk of age (Fig. 1b).

It is clear from Figs 2 & 3 that mice started on DES at the earlier age did not develop mammary tumours more rapidly than mice started at the later age. Indeed, in three out of four comparisons, mice started on DES at 11 wk of age were removed with palpable mammary tumours after a shorter time of exposure than were mice started on DES when 7 wk old. This was true for C3H/HeN mice at both DES concentrations and for C3H/HeJ mice at 320 ppb DES.

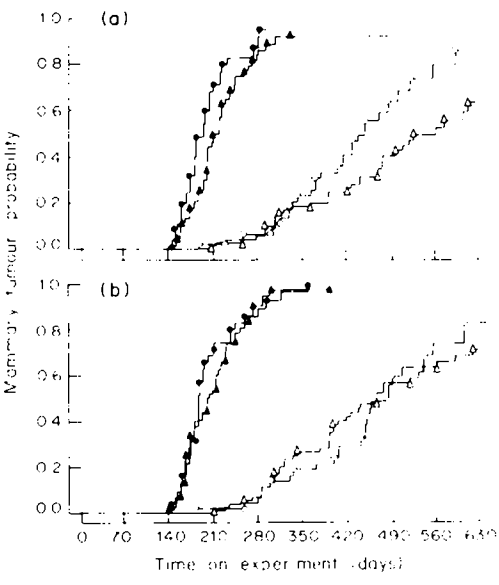

Fig. 1. Strain differences in mammary tumorigenesis. C3H/HeN or C3H/HeJ female mice were given diets containing DES continuously, beginning at (a) 7 wk of age or (b) 11 wk of age: C3H/HeN mice – 640 ppb (●), 320 ppb (▲); C3H/HeJ mice – 640 ppb (○), 320 ppb (△). Days on experiment refer to the time elapsed since weaning (21–26 days old). Mammary tumour probability is the probability of an animal being removed with a palpable, histologically verified mammary adenocarcinoma (see Experimental).

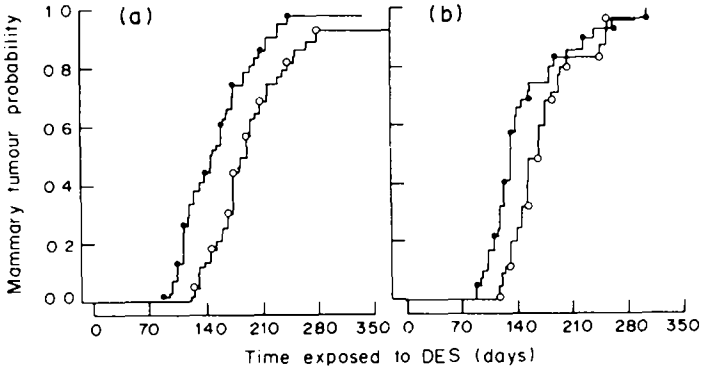

Fig. 2. Effect of age at the start of DES exposure on mammary tumorigenesis. C3H/HeN female mice were continuously exposed to (a) 320 or (b) 640 ppb DES in the diet starting at either 7 (○) or 11 (●) wk of age. The duration of exposure to DES is plotted against the probability of a mouse being removed from the experiment with a palpable, histologically verified mammary adenocarcinoma (see Experimental).

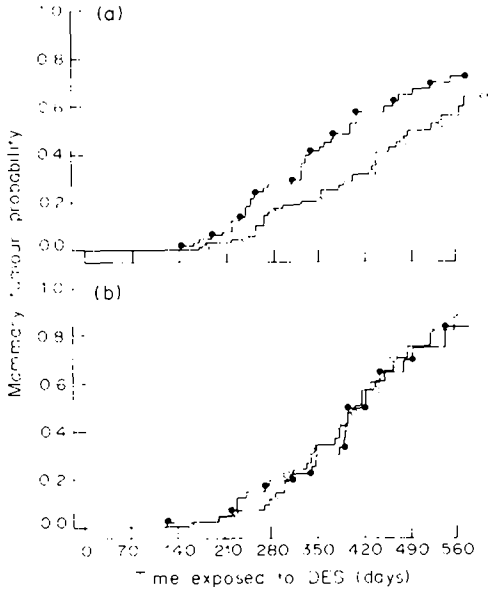

Fig. 3. Effect of age at the start of DES exposure on mammary tumorigenesis. C3H/HeJ female mice were continuously exposed to (a) 320 or (b) 640 ppb DES in the diet, starting at 7 (○) or 11 (●) wk of age. The duration of exposure to DES is plotted against the probability of a mouse being removed from the experiment with a palpable, histologically verified mammary adenocarcinoma (see Experimental).

DISCUSSION AND CONCLUSIONS

It is clear from this study that MMTV-positive C3H/HeN female mice develop mammary adenocarcinomas in response to DES at a much faster rate than do C3H/HeJ female mice. Other isolated studies have given evidence that these two strains have different rates and incidences of mammary tumour development. Heston, Deringer & Dunn (1956) reported a mammary tumour incidence of about 95–100% in untreated MMTV-positive C3H females with an average time-to-tumour of about 8 months in breeders and 11 months in untreated virgins. On the other hand, untreated C3H/HeJ breeding female mice have been reported to have about a 60% incidence of mammary tumours and an average time-to-tumour of 11 months, whereas virgins had an approximate 30% incidence, and a 20-month average for time-to-tumour (Richardson, 1973). No other study could be found in the literature in which these two strains have been simultaneously evaluated with respect to either spontaneous or induced mammary tumorigenesis. However, differences in the immunological system have been demonstrated for these two strains (Tagliabue, Ruco, McCoy *et al.* 1978), and it is well established that immune competence can have a marked effect upon carcinogenesis (Anisimov & Turusov, 1981).

Age at the start of dosing, in the present experiment, did not have an effect on time-to-tumour that could account for differences that have been noted between strains in earlier experiments carried out at this laboratory (Greenman & Highman, 1982; Highman *et al.* 1980). From those two studies, one might have predicted that animals started on DES at a somewhat older age would have developed tumours much more slowly than those started earlier. This clearly was not true for either strain in the present study. Indeed, for both strains at 320 ppb DES and for C3H/HeN mice at 640 ppb DES mammary tumours developed after a shorter duration of exposure in mice started on treatment at 11 wk than in those started at 7 wk of age. This observation suggests that between 7 and 11 wk of age both strains of mice are refractory to DES treatment. A similar period during which SLN mice are apparently resistant to the effect of prolactin on mammary tumour development has been reported (Nagasawa, 1983). Furthermore, this finding is reminiscent of observations made on the induction of mammary adenocarcinomas in female rats by 7,12-dimethylbenz[*a*]anthracene (DMBA; Janss & Hadaway, 1977; Meranze, Gruenstein & Shimkin, 1969). In rats sensitivity to tumour induction is highly age dependent, the precise age dependence being strain specific; for example, in studies with DMBA Sprague-Dawley rats were more sensitive to tumour induction at 50 than at 80 days of age whereas Long-Evans rats were insensitive at 50 days but sensitive at 80 days of age (Janss &

Hadaway, 1977). Rates of DNA synthesis or mammary gland growth were found to be higher during sensitive periods than during insensitive periods in both rat strains and in SLN mice (Janss & Hadaway, 1977; Nagasawa, 1983). Whether similar differences in the rate of growth or DNA synthesis are associated with differences in sensitivity to DES in C3H mice needs to be examined. Since prolactin is known to be of primary importance in mammary tumorigenesis in the mouse (Welsch & Nagasawa, 1977) and DES has been shown to increase the level of circulating prolactin (Sinha, Selby, Lewis & Vanderlaan, 1972) the refractory period noted in the present study could be explained either by a failure of DES to increase circulating prolactin or a failure of the mammary gland to respond to prolactin during the refractory period. Possible age differences in MMTV titre could also be a factor that merits investigation.

In conclusion, age at the start of treatment with DES influences mammary tumour development in C3H:HeN and C3H HeJ female mice. However, the strain of mouse is a much more important variable in determining the rate and incidence of mammary tumour development.

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