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Paper

Analysis of breeding and pathology helps refine management practices of a large-scale *N′*-ethyl-*N′*-nitrosourea mouse mutagenesis programme

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Abstract

***N′*-ethyl-*N′*-nitrosourea (ENU) is a powerful germline mutagen used in conjunction with phenotype-driven screens to generate novel mouse mutants. ENU also induces genetic lesions in somatic cells and dosage requires optimization between maximum germline mutation rate versus induced sterility and tumourigenesis that compromise the welfare and fecundity of the ENU-treated males. Here, we present our experience with BALB/cAnNCrl and C57BL/6J mice in terms of the pathology induced by ENU and its impact on breeding. In both mouse strains, morbidity and mortality rises with ENU dose. In more than 75% of C57BL/6J males, morbidity and mortality were attributable to the development of malignant T-lymphoblastic lymphoma. Approximately 50% of ENU-treated BALB/cAnNCrl males develop early malignant T-lymphoblastic lymphoma, but the cohort that survives develops late-onset lung carcinoma. Within strains, the latency of these clinically important tumour(s) was not dosage-dependent, but the proportion of mice developing tumours and consequently removed from the breeding programme increased with ENU dosage. The median number of offspring per ENU-treated C57BL/6J male in standard matings with C3H/HeH females decreased with increasing dosage. The two most important underlying causes for lower male fecundity were increased infertility in the highest dosage group and reduced numbers of litters born to the remaining fertile C57BL/6J males due to a higher incidence of morbidity. These findings have allowed us to refine breeding strategy. To maximize the number of offspring from each ENU-treated male, we now rotate productive males between two cages to expose them to more females. This optimizes the number of mutation carrying offspring while reducing the number of ENU-treated males that must be generated.**

Keywords: *N′*-ethyl-*N′*-nitrosourea, mouse mutagenesis, pathology, breeding management

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Phenotype-driven screens using chemical mutagens, such as *N′*-ethyl-*N′*-nitrosourea (ENU), have provided a powerful means of investigating genetic causes of heritable phenotypes. The principles of ENU mutagenesis and the location of major mutagenesis centres worldwide have been recently reviewed.[1] Mouse ENU mutagenesis has increased the pool of mutants in many areas of biology, helped identify novel genes involved in physiological processes and disease, and assisted in assigning functions to genes.[2] The contribution of ENU mutagenesis to different areas has been the subject of an increasing number of specialist reviews, for example in host defence, allergy and autoimmunity,[3] craniofacial abnormalities,[4] male infertility[5] and behaviour.[6]

ENU is a commonly used mutagen for phenotype-driven screens in mice. As an alkylating agent its ethyl group is transferred to nucleophilic sites on each of the four nucleotides of DNA. This results in mispairing at the next round of DNA replication. ENU predominantly mutates A:T base pairs, and in mouse phenotypic screens approximately 60% of the mutations detected are A:T to T:A transitions or A:T to G:C transversions. In descending order of occurrence, ENU produces missense, nonsense and splice-site

mutations and these tend to occur in genes with longer coding sequences and higher exon numbers.[2]

The most important factors in the design of a mutagenesis experiment are the rate at which germline mutations are induced and the ability to recover healthy, fertile male mice for breeding. Several studies have been published on optimizing ENU dosage in different inbred strains of mouse and these are summarized in recent reviews.[7,8] Although the exact dose that produces the highest mutation frequency may vary from one strain to another when administered in a single dose, increasing the dose of ENU increases the mutation rate. However, this effect does not remain linear at high, single doses. Instead, the maximum efficiency is achieved when high doses are administered in a fractionated form with a short period separating each injection, typically a week.

Published data on the pathology of ENU-treated males in the context of a mutagenesis programme are generally scant. In one of the few examples, a tumour survey in retired ENU-treated (C57BL/6 × CBA) F_1 males, lung tumours, thymoma (taken as a synonym for thymic lymphoma) and liver tumours were the most common and skin, kidney, prostate and colon tumours occurred, but were less frequent.[7] In this paper, we have explored the breeding and pathology records from our experience with the mutagenesis programme at the Medical Research Council (MRC) Harwell to optimize the programme by developing breeding management practices and to help address welfare issues. This paper is a retrospective case-control study of the breeding and fate of 760 male ENU-mutagenized mice (BALB/cAnNCrl and latterly C57BL/6J) and a more detailed analysis of tumour pathology in 226 of these mice. We have focused on how ENU treatment affects lifetime fecundity in relation to ENU-induced pathology, especially clinically important tumour entities that necessitated premature culling. This information has been used to refine the breeding programme.

Materials and methods

Study design

The MRC Harwell operates phenotype-driven dominant and recessive screens. During the course of the early MRC mutagenesis projects, the inbred strain of mice used was BALB/cAnNCrl. For the latter mutagenesis projects commencing in 2004, C57BL/6J was the major strain used though smaller numbers of BALB/cAnNCrl, BALB/cByJ and CeH/HeH were also used.

Three ENU dosage regimens were used. The ENU dose was titrated in an attempt to optimize mutagenic rate in offspring as determined by heritable phenotypes. The experimental aim was to be just above the borderline of infertility as higher ENU doses increase the mutation frequency. The numbers of mice of each strain used in each dosage regimen and necropsy sampling statistics are given in Table 1. A general overview of the timeline for the programme is given in Figure 1a.

Mice, husbandry and health screening

The humane care and use of mice in the rederivation programme was conducted under the Home Office regulations. The earlier MRC Harwell mutagenesis projects were performed in a largely conventional mouse house facility; however, the ENU-treated mice were kept in individually ventilated cages (IVCs). This old low health status mouse house has now been replaced by a new high health status, mouse only, specific pathogen free (SPF) barrier facility, the Mary Lyon Centre (MLC). The new centre also has digital record-keeping that has greatly facilitated data mining.

BALB/cAnNCrl males were purchased from Charles River and imported as 4–6-week-old mice. They were protected from exposure to infectious agents (see below) by housing them in IVCs (Charles River Laboratories UK, Margate, UK) in a 12 h light/dark (07:00–19:00 light phase) cycle at 19–23°C and 45–65% relative humidity on grade 6 sawdust bedding (Datesand Ltd, Manchester, UK). All equipment was autoclaved before use. Husbandry and ENU treatments were performed in a class II biological safety cabinet. Mice were fed rat and mouse No. 3 breeding diet (Special Diets Services, Witham, Essex, UK) and provided with chlorinated water *ad libitum*.

Sentinels were challenged by infectious agents through airborne exposure. Live mice were submitted to an outside laboratory (Harlan UK Ltd Technical Services Department, Loughborough, UK) for microbiological testing based on FELASA recommendations.[9] Health screening in the pre-existing conventional colony (before rederivation of colonies into the MLC) showed the presence of mouse hepatitis virus (judged by histology to be enteropathic strains), mouse adenovirus type 2, Theiler's murine encephalomyelitis virus, the intestinal flagellates *Spironucleus muris* and *Tritrichomonas muris*, pinworms, *Helicobacter* spp. and *Pasteurella pneumotropica*.[10]

In some of the more recent ENU mutagenesis projects, male C57BL/6J (Jackson Laboratories, Bar Harbor, Maine,

Table 1 Mutagenesis programme population sizes in this study

	BALB/cAnNCrl			C57BL/6J		
Regimen (mg/kg)	2 × 100	3 × 90	3 × 100*	2 × 100	3 × 90	3 × 100
Total G_0 mice	326	100	48	49	135	102
Found dead	22	12	0	3	10	19
Culled sick	171	50	40†	20	74	72†
(Full necropsy)	(74)	(32)	(1)	(17)	(49)	(53)

*Because this dosage was not tolerated by BALB/cAnNCrl mice this was discontinued

†Includes mice that were culled after losing 20% body weight acutely post-treatment and excluded for further analysis (36 BALB/cAnNCrl and 1 C57BL/6J)

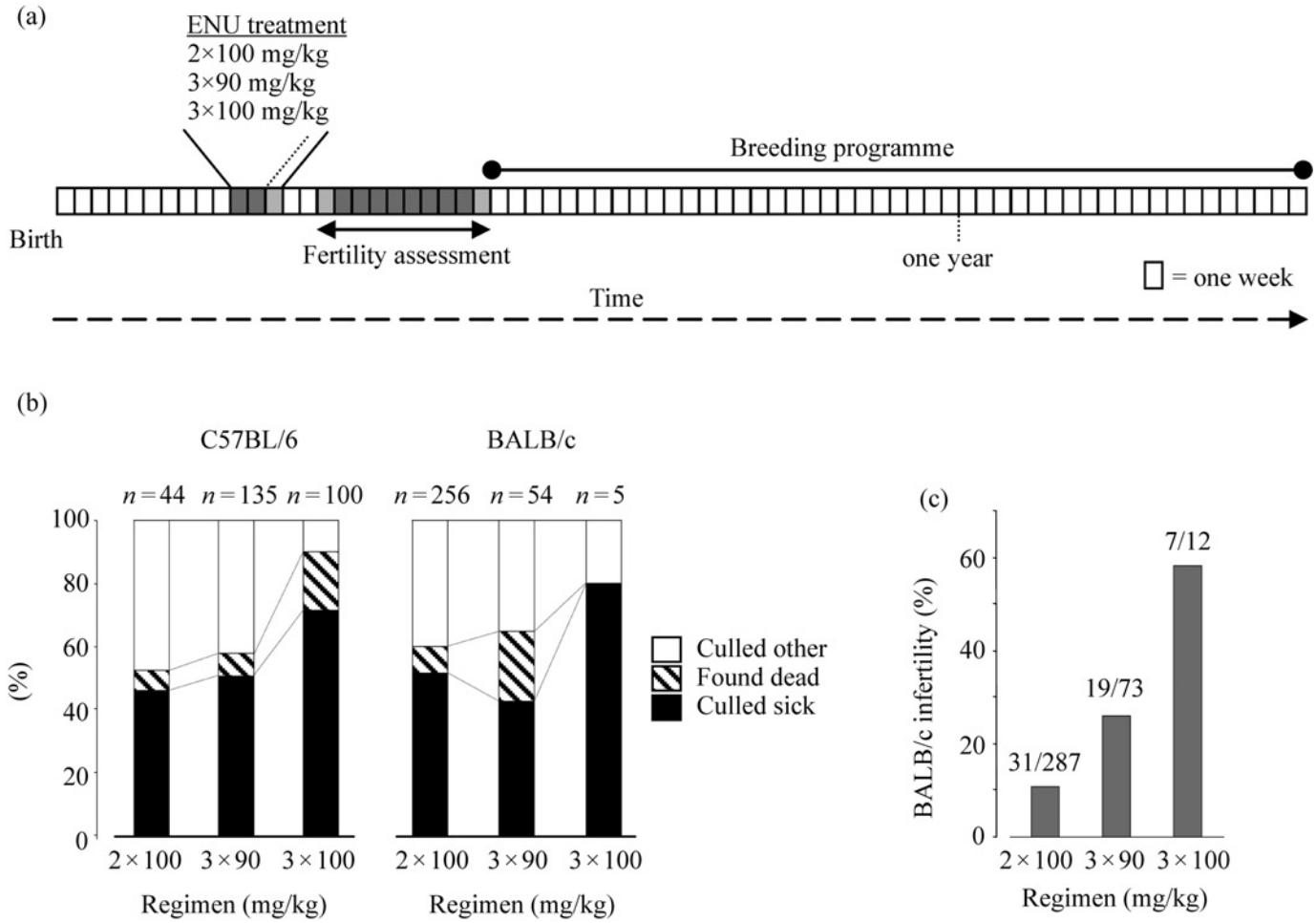

Figure 1 (a) Major timeline events for G_0 ENU-treated male mice used for the phenotype-driven screens. Starting at 10 weeks each mouse was injected intraperitoneally with ENU at weekly intervals. The regimens employed were two doses of 100 mg/kg at one-week intervals, three weekly doses of 90 mg/kg or three weekly doses of 100 mg/kg. (b) Categories of males lost to the breeding programme for C57BL/6J and BALB/cAnNCrl strains. The number of mice culled sick or found dead increases with ENU dose. (c) In BALB/cAnNCrl mice the highest dosage regimen was poorly tolerated and resulted in a high percentage of mice failing to regain fertility. These were excluded from analysis in (b) for clarity. A similar effect is seen in C57BL/6J mice which were analysed in more detail (Figure 3)

USA) mice were used from a SPF colony established in the MLC rederived by embryo transfer as described.[10] Mouse husbandry in the MLC was essentially the same as in the pre-existing mouse facilities except that the mice were housed in Techniplast IVCs (Techniplast UK Ltd, Kettering, UK) and the diet was irradiated.

The microbiological screening programme in the MLC involves sending mice from sentinel cages (one per 56 cages) that are challenged with samples of dirty bedding from separate columns in each rack on an eight-week rotation as previously described.[11] Health screening has shown that the SPF status described above has been maintained since the facility opened more than two years ago.

ENU treatment and mouse breeding

As the BALB/cAnNCrl in the earlier mutagenesis projects were imported at approximately six weeks of age, they were assigned an estimated birth date. Records of exact birth dates were available for the C57BL/6J and BALB/cAnNCrl males bred in the MLC. At approximately 10–12 weeks of age (target 10 weeks), ENU doses were administered intraperitoneally at weekly intervals: 3 × 90 mg/kg or 2 × 100 mg/kg in BALB/cAnNCrl males and 3 × 90 mg/kg, 2 × 100 mg/kg or 3 × 100 mg/kg in C57BL/6J males (Figure 1a). These 'generation zero' ENU-treated males are referred to hereafter as G_0 males to distinguish them from their first generation offspring hereafter termed as G_1 mice.

Successful ENU treatment causes a period of sterility of approximately eight weeks starting 3–4 weeks post-treatment[12] reflecting exposure of pre-spermatid stages and significant germcell death. For the first three weeks, post-injection progeny may arise from spermatozoa that were in the later stages of development when exposed to ENU. Therefore, ENU-treated males were test-bred (one male and one female) with similarly aged females from week 4 to week 11 after the last dose. The females from these matings were culled at 11 weeks and examined at postmortem for the presence of fetuses. The males were judged to have regained fertility very early (see below) if a

pregnancy was detected and the males were subsequently withdrawn from the programme.

At 12 weeks after ENU treatment, males were then set up for matings with two C3H/HeH females with an aim to produce 50–60 offspring. A target of 50–60 offspring was chosen to avoid the theoretical possibility of producing multiple identical mutations in offspring due to the oligoclonality of ENU-mutagenized spermatogonial stem cells that repopulate the testis following ENU treatment. ENU treatment can result in poor fertility in G_0 males (see below), so the number of G_1 offspring produced in each mating was carefully monitored. If an ENU-treated male did not produce offspring in two months, or was a poor breeder (i.e. had one litter but had not produced a litter for 3 months), the decision on whether to continue with the mating was made based on the number of active matings in the breeding colony as a whole and the availability of new replacement ENU-mutagenized G_0 males. In a later pilot study to increase male productivity, some ENU-treated males were rotated between two cages each with two C3H/HeH females.

Approximations of the efficacy of ENU treatment were made at regular intervals as the number of heritable phenotypes per G_0 male. This is used for guidance in programme management meeting requirements for the various screens. Because of the differences in the screen protocols, like-for-like inter-group comparisons cannot be made for the output of the various screens and are, therefore, refractory to rigorous statistical analysis. Furthermore, the various heritable mutations are usually in various stages of mapping again complicating rigorous analysis.

Case definitions for analysis of breeding and pathology

To analyse the breeding and pathology data for ENU-treated males, mice were categorized as those 'found dead' (i.e. sudden unexpected death without premonitory signs), 'culled sick', 'culled regained fertility too early' or 'culled did not regain fertility'. The remaining category 'culled other' consisted of mice that were culled because they were poor breeders or those that had reached their required offspring target (50 progeny for each G_0 male), males that were injured by fighting or were priapistic. The case definition of culled sick included both neoplastic causes and some non-neoplastic causes of morbidity, although this latter category comprised $<5\%$ of cases categorized as 'culled sick'. Cases that were excluded from analysis were males with acute post-treatment toxicity ($>20\%$ weight loss was considered as grounds for culling).

Welfare assessments, necropsy and histology

ENU-treated mice were examined every day at 08:00 h and 14:30 h and those which were found ill (clinical signs most often noted were rapid respirations, hunched, piloerect) were culled immediately by an overdose of barbiturate administered intraperitoneally (Pentoject, Animalcare, York, UK). They were weighed, then necropsied and gross pathology findings were recorded. Histological analysis and tissues examined were as previously described.[11] Later, testes and sternebrae were included as additional tissues. Testes were collected and fixed in Bouins solution for 8 h. Bones were decalcified in Formical (Decal Corp, Congers, NY, USA) for 48 h. Tissues were embedded in wax and 4 μm sections were prepared. Sections were stained with haematoxylin & eosin (H&E).

To compare the incidence of lymphoma in ENU-treated C57BL/6J males with spontaneous rates in untreated males, 28 one-year-old retired breeders from the same stock colonies were euthanized and examined at necropsy for gross tumour burden. The mediastinum including the thymus lobes were fixed and examined histologically.

Tissue array manufacture and immunohistochemistry

Tumours were cored in duplicate (2×600 μm cores) from donor formalin-fixed paraffin-embedded tissue blocks into recipient paraffin blocks using an automated Beecher arrayer (Beecher Instruments, Sun Prairie, WI, USA) according to manufacturer's protocols. After tempering the block overnight, 5 μm sections were cut onto APES (3-aminopropyltriethoxysilane)-coated slides for immunohistochemical staining. Additional sections were stained by H&E and assessed for adequate tumour representation. All immunohistochemistry was performed on the Ventana Discovery automated stainer (Ventana, Tucson, AZ, USA). For CD3 immunostaining, sections were pretreated with alkaline protease 0.03 U/mL for 10 min at 37°C, then heat-treated with cell conditioning buffer (Tris/EDTA/Borate pH 8.5; Sigma-Aldrich, Gillingham, UK) for 16 min at 95°C. Primary antibody (CD3, SP7 clone, rabbit monoclonal antibody, Vector Laboratories, Peterborough, UK) was used at a dilution of 1:200 for 20 min and followed by washing and biotinylated secondary antibody staining for 8 min. Streptavidin-peroxidase conjugates were applied for 16 min and visualized using 3,3-diaminobenzidine tetrahydrochloride with copper enhancement. All incubations were performed at 37°C. For B220 immunostaining, sections were heat-treated with cell conditioning buffer (Tris/EDTA/Borate pH 8.5) for 28 min at 95°C. Primary antibody (B220 rat polyclonal antibody, R&D systems, Abingdon, UK) at a dilution of 1:100 and staining and subsequent treatment was similar to CD3 immunostaining. All incubations were performed at 37°C. Immunohistochemical staining was assessed by a histopathologist on a semi-quantitative basis according to percentage of cell stained and intensity of staining, i.e. negative, weakly positive, moderately positive or strongly positive. An additional assessment of the percentage of total tumour cells positive for each marker was made.

Statistical analysis

Dosage effects on tumour latencies were analysed by the analysis of variance (ANOVA) across multiple groups or by a two-sample *t*-test. Analyses of G_1 offspring produced per G_0 male were made using the non-parametric equivalent, the Kruskal-Wallis test or by a two-sample Wilcoxon's rank test. Contingency analysis was performed on

lymphoma incidences using the Fisher's exact test. In all analytical methods $P < 0.05$ was considered statistically significant.

Results

ENU-induced pathology

The higher ENU dosage regimens (3 × 90 mg/kg and 3 × 100 mg/kg) were associated with an increasing percentage of C57BL/6J and BALB/cAnNCrl males that were culled due to illness ('culled sick') or were found dead without premonitory signs (Figure 1b). However, in BALB/cAnNCrl males there was an increasing dose-dependent proportion of mice that never regained fertility (Figure 1c). Therefore, this regimen was discontinued prior to initiating the pathology studies. In the earlier mutagenesis projects conducted in the old mouse house, some batches of ENU-mutagenized BALB/cAnNCrl males were culled because of acute 20% weight loss after ENU treatment (Table 1), but this has not recurred under SPF conditions.[10]

Morbidity and mortality in the vast majority (>95%) of the G_0 mice we studied were due to the development of neoplasia. A few individuals had conditions such as hydronephrosis, bite wounds or priapism (categorized as 'culled other' for subsequent analysis). However, even these mice were usually found to have some additional occult tumour burden, usually lung adenomas. By far, the most common tumours in both the BALB/cAnNCrl and C57BL/6J strains were multisystemic lymphomas and lung tumours (see below, Table 2). Small benign lung adenomas were commonly found in ENU-treated C57BL/6J and BALB/cAnNCrl mice of all ages, but by gross and histological assessment these were not sufficiently large or numerous enough to be classified as a primary cause of morbidity or mortality. In contrast, large lung carcinomas were likely to be clinically significant in BALB/cAnNCrl mice, as clinical signs could not be attributed to multisystemic lymphoma (only three of 42 of these mice had multisystemic lymphoma). Some morbidity in C57BL/6J mice was associated with intestinal tumours causing a degree of bowel obstruction, and the incidence of such tumours appeared to increase with ENU dosage. The incidence of intestinal tumours appeared lower in BALB/cAnNCrl males. In contrast, renal neoplasia was a common, clinically non-significant finding in both BALB/cAnNCrl and C57BL/6J males. Fewer than 7% of mice had neoplastic lesions in other tissue origins, for instance there were five myeloid leukaemias and two oligodendrogliomas (data not shown). Other pathological findings included a spectrum of endothelial lesions in the spleen (7–27% in both strains; the most common was angiectasis, then haemangioma, whereas haemangiosarcoma was rare) and liver (biliary) cysts (9–21% incidence in both strains).

A subset of lymphomas from C57BL/6J ($n = 33$) and BALB/cAnNCrl ($n = 32$) mice were further analysed by pathological examination of tumour morphology and immunohistochemistry of tumour samples on tissue arrays. Tumours were assessed for positivity for a pan-T-cell marker, CD3, and a pan-B-cell marker, B220. One hundred percent of tumours that were immunophenotyped in this manner ($n = 65$) were found to be positive for CD3 and negative for B220, and all were cytologically blastic, predominantly lymphoblastic, typical of T-lymphoblastic lymphoma. Most T-lymphoblastic lymphoma cases were presented as a space occupying (>5 mm) mediastinal mass with intrathoracic direct spread to epicardium, tracheobronchial lymph node, pleura, perivascular and peribronchiolar sites in lung, diaphragm and intercostal muscle. A large proportion had extrathoracic involvement (in approximate descending order), liver > spleen > kidney > lymph node > meninges > adrenal; 49% ($n = 99$) of the C57BL/6J males had additional sternebral bone marrow involvement by lymphoma.

Temporal pattern of tumour development in relation to the breeding programme

The potential impact of tumourigenesis on the breeding programme was evaluated by plotting the date of necropsy or sudden unexpected death after ENU treatment. This latency for the four most common tumours is presented in Figure 2. In cohorts of BALB/cAnNCrl mice, there was an early cluster of lymphomas followed by a later cluster of lung carcinomas. In contrast, in C57BL/6J mice morbidity

Table 2 Major tumour incidences in G_0 mice culled sick

	BALB/cAnNCrl		C57BL/6J		
Regimen (mg/kg)	**2 × 100**	**3 × 90**	**2 × 100**	**3 × 90**	**3 × 100**
Lymphoma	27% (20)*	69% (22)†	82% (14)*	76% (37)†	100% (53)
Lung					
Adenoma	51% (38)	62% (20)	47% (8)	18% (9)	47% (25)
Carcinoma	45% (33)	28% (9)	–	–	–
Kidney					
Adenoma	28% (21)	25% (8)	–	14% (7)	6% (3)
Carcinoma	4% (3)	3% (1)	–	–	–
Intestines					
Adenoma	1% (1)	3% (1)	6% (1)	12% (6)	26% (14)
Carcinoma	1% (1)	–	6% (1)	6% (3)	–
Total (*n*)	74	32	17	49	53

The figure in the body of the table represents percentage incidence and in parentheses are the number of case observed of the total (*n*)
*The incidence of lymphoma in 2 × 100 mg/kg dosage regimen is significantly lower in BALB/cAnNCrl males ($P < 0.0001$) (one-tailed Fisher's exact test)
†Incidence of lymphoma is not significant at 3 × 90 mg/kg ($P = 0.34$) (one-tailed Fisher's exact test)

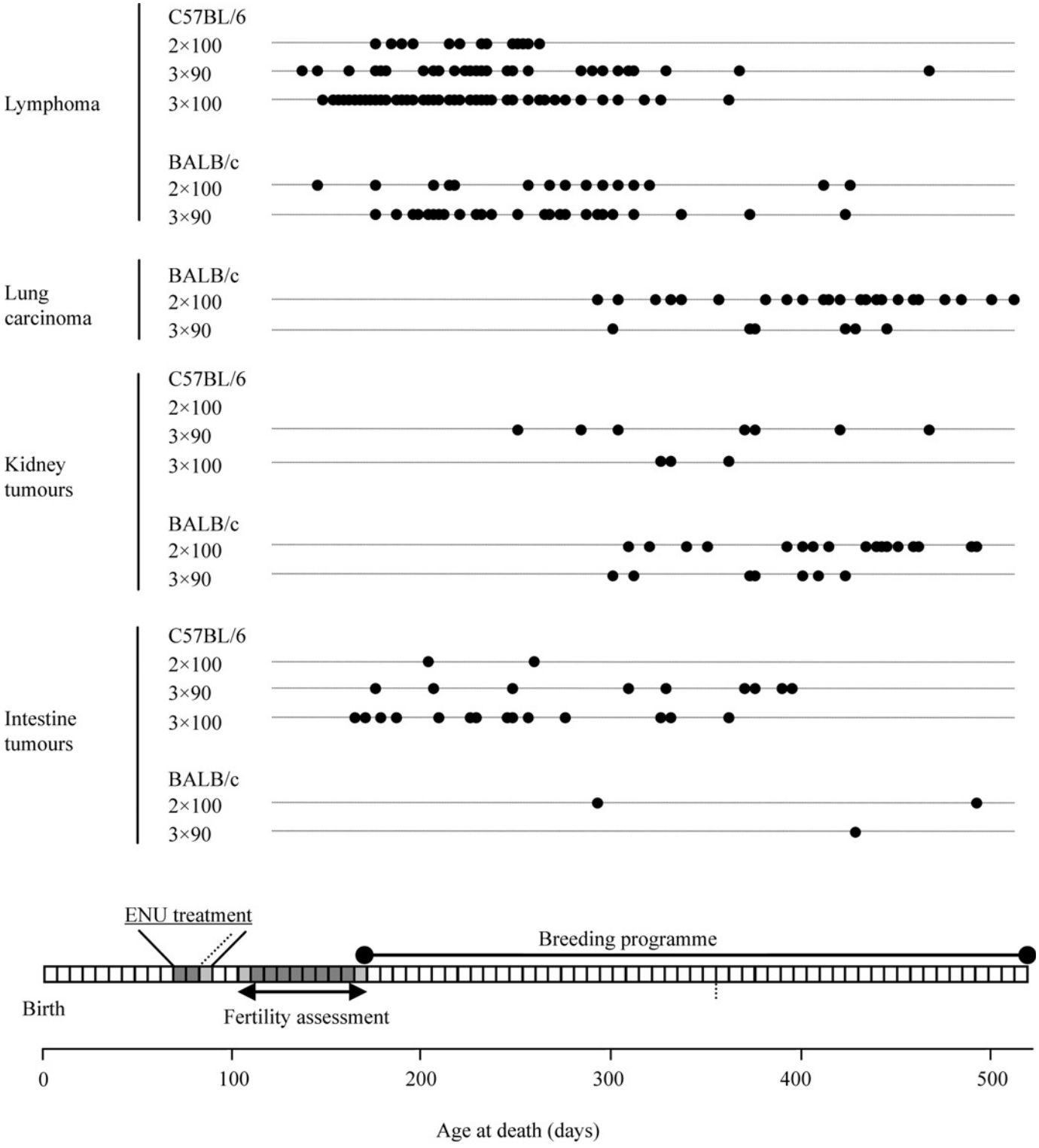

Figure 2 Age-related incidence of the four most common tissue neoplasms found at necropsy. In C57BL/6J mice, morbidity is almost exclusively due to early-onset lymphomas, though some mice develop early-onset intestinal neoplasms and late-onset renal tumours. Early-onset lymphomas are also seen in BALB/cAnNCrl mice, but these mice also develop late-onset lung carcinomas (not seen in C57BL/6J mice) and renal tumours. Note: in this series of C57BL/6J males did not develop lung carcinoma

was almost exclusively due to lymphoma early in the breeding programme. In both strains, the peak of lymphoma incidence was well within the breeding period for these mice (Figure 2) significantly curtailing the number of G_0 mice that would produce a target of 50 progeny (see below in this section). The significantly later onset of lung carcinoma in BALB/cAnNCrl mice suggests that these lesions were far less likely to impact on the breeding programme as many more of these mice would have lived long enough to reach their pup limit.

Although the incidence of lymphoma may be influenced by ENU dosage, the latency was not significantly different between the dosage groups for either strain. For C57BL/6J mice, the mean age for lymphoma development on each dosage regimen were 218 ± 8, 234 ± 11 and 214 ± 7 days (mean $\pm$ SEM) for the 2×100 ($n = 14$), 3×90 ($n = 37$) and 3×100 ($n = 53$) mg/kg, respectively ($P = 0.21$; ANOVA). For BALB/cAnNCrl mice, the mean latency for lymphoma development on each regimen were 264 ± 15 and 255 ± 10 days (mean $\pm$ SEM) for the 2×100 ($n = 21$) and 3×90 ($n = 30$) mg/kg, respectively ($P = 0.62$; ANOVA). In addition, there was no difference in the latency of lung carcinoma development in BALB/cAnNCrl mice for each dosage regimen; these were 408 ± 11 and 374 ± 19 days for the 2×100 ($n = 29$) and 3×90 ($n = 8$) mg/kg, respectively ($P = 0.54$; ANOVA).

In contrast to early morbidity associated with high incidence of thymic lymphoma in ENU-treated males, a cohort of 28 retired untreated C57BL/6J breeding males experienced no morbidity or sudden deaths between six months and 12 months and, at necropsy, none had any macroscopic tumour burdens or histological evidence of thymic lymphoma.

The effect of ENU dosage regimen on breeding potential in C57BL/6J males

ENU administration causes a temporary period of infertility in C57BL/6J males and also has the potential to cause permanent infertility. ENU also induces neoplasia that may impact the breeding potential of G_0 mice either by premature removal (e.g. infertility, morbidity, sudden death, etc.) or subclinical neoplasia may reduce male productivity in the terminal stages.

Each of these possible contributory factors was assessed (Figure 3) for the C57BL/6J mice. The most dramatic perturbation of fecundity was evident in an apparently dose-dependent increase in the number of G_0 males that failed to produce any offspring. This was possibly related to the cumulative dose for each regimen, and in the highest regimen of 3×100 mg/kg results in 40% of the males failing to produce any offspring at all (Figure 3a). This was not due to a dose-dependent increase in the time from the end of ENU dosage to first litter, as the time to first litter was comparable for each of the three regimens. These were 121 ± 3.1, 123 ± 2.8 and 126 ± 2.7 days (mean $\pm$ SEM) for the dosage regimens 2×100 ($n = 21$), 3×90 ($n = 78$) and 3×100 ($n = 68$) mg/kg, respectively ($P = 0.58$; ANOVA). However, the development of neoplasia during the breeding programme clearly resulted in greater losses (Figure 3c) with only approximately 12% of males treated with higher dosage regimens alive at one year compared with approximately 40% at lower dosages. This resulted in an altered breeding profile (Figure 3b) as a consequence of dose-dependent lower pup production. Pup production decreased with increasing ENU dosage (including those which produced no offspring); for each group the median pup production was 29.5, 10 and 5 pups per G_0 male for the dosage regimens 2×100 ($n = 22$), 3×90 ($n = 119$) and 3×100 ($n = 113$) mg/kg, respectively ($P = 0.0013$; Kruskal-Wallis test). The corresponding means were 28.6, 20.2 and 12.1 pups per G_0 male.

The most important contributory factor to the reduction in fecundity of males that did breed was a reduction in the number of litters produced (Figure 3d) due to increased incidence of morbidity in the highest ENU dosage cohort. The average litter size (Figure 3e) and frequency of litter production (Figure 3f) were not altered. We assessed the effect of subclinical lymphoma in C57BL/6J on male productivity (assuming that C3H/HeH female fertility was not impaired) and found C57BL/6J males showed the same pattern of siring litters up to the time that they were 'culled sick' in all dosage regimens (data not shown). Histological assessment of testes in C57BL/6J males culled sick ($n = 76$) found that there was mild focal seminiferous tubule atrophy in only 5% of cases indicating that repopulation of the testes was complete and histologically unremarkable in the vast majority of cases by the time of postmortem examination.

Effect on productivity of providing extra females to fertile ENU-treated C57BL/6J males

In view of the limited breeding lifespan of ENU-treated males in the 3×100 mg/kg dosage group, a pilot study was undertaken to determine the effect of providing more female mates. Once males had sired their first litter(s), they were then rotated between this cage and a second cage with two C3H/HeH females. The median number of litters increased from three to seven ($P = 0.0007$; Wilcoxon's test) and the median number of offspring from 13 to 30 ($P = 0.0008$; Wilcoxon's test) ($n = 65$ for two female mates, $n = 33$ for four female mates).

Discussion

We present here the findings from more than five years data on the effects of ENU dosage regimens on the breeding performance and incidence of tumour pathology in BALB/cAnNCrl and C57BL/6J mice mutagenized in the ENU mouse mutagenesis programmes at MRC Harwell. We go on to describe what influence the findings of these studies have had on our management practices.

The management of large-scale mutagenesis programmes, such as those at Harwell, are driven by phenotype demand from the various screens that are undertaken. These are subject to regular review and management practices alter to meet these demands. The value of reviewing breeding and pathology, such as with the current study, is to provide insight into the fate of the G_0 males in different dosage regimens and the impact on production of G_1 progeny. As it is known that ENU dosing increases the mutation rate linearly in the dose range we used,[13] the optimal dose regimen is the highest possible that does not reduce fertility or welfare of the mice to an unacceptable extent.

Much of the published ENU literature focuses on mechanisms of mutagenesis, mutation rates and specific mouse

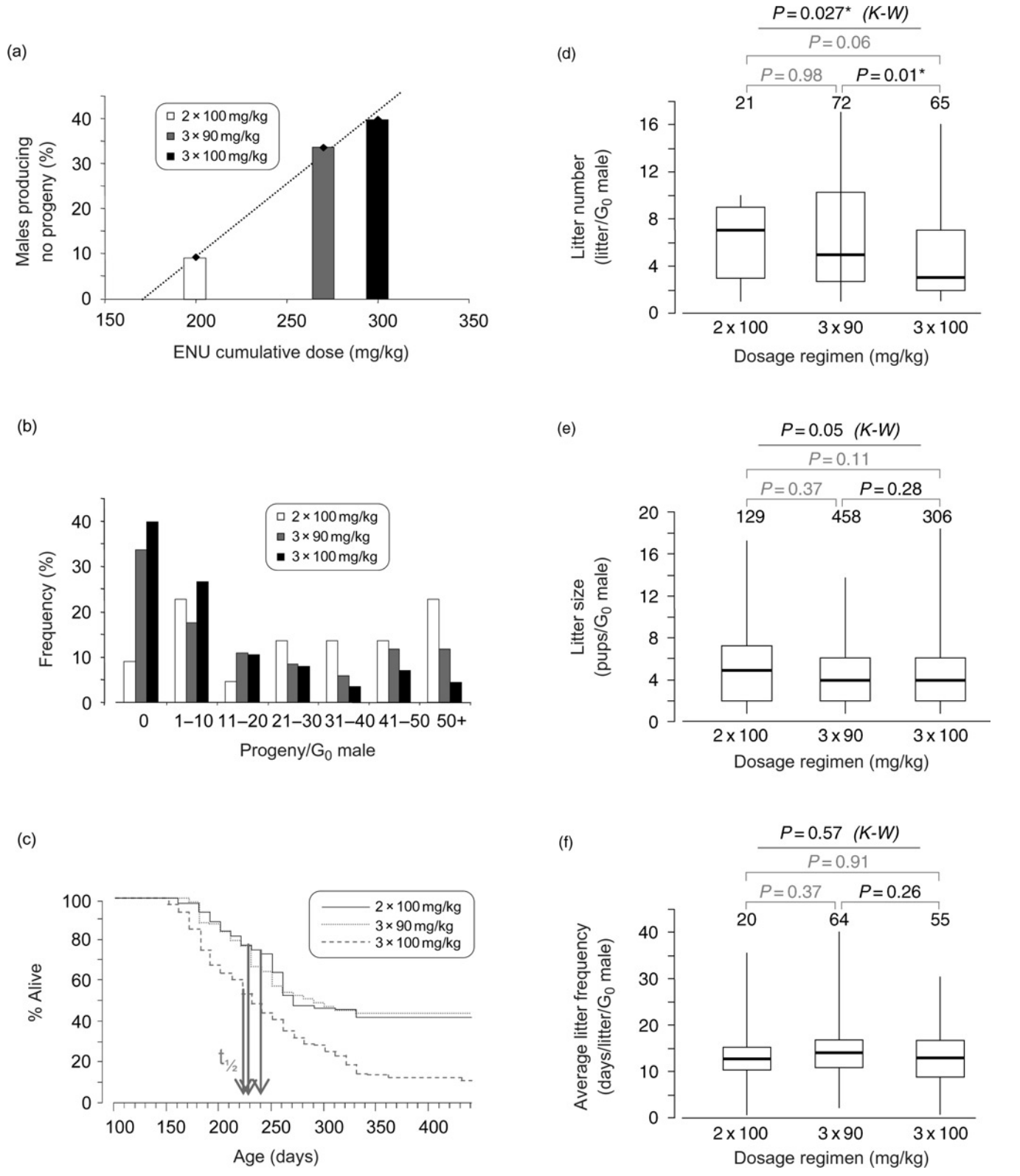

Figure 3 Decreased reproductive potential of ENU-treated C57BL/6J mice. ENU treatment decreases the reproductive potential by three possible mechanisms, impairment of fertility, premature death and a terminal decline in productivity. (a) Increasing dosage regimen, or more precisely increasing cumulative dose, results in more males rendered permanently infertile. (b) Loss of reproductive potential through premature death is manifested in fewer males producing the set target of 50 progeny. (c) Survival analysis demonstrates that in mice treated with 3 × 100 mg/kg loss from the breeding programme is through higher tumour incidence rather than significantly decreased tumour latency; $t_{\frac{1}{2}}$ is comparable for dosage regimens. (d–f) Pup litter statistics for G_0 males that produced offspring. Decreased productivity in mice treated with the highest regimen is through fewer litters (d) and not from decreased litter size (e) or littering frequency (f). Box and whisker plots for each dosage regimen have a horizontal bar for the median values within a rectangle representing the 25th and 75th percentiles; the whisker represents the minimum and maximum values. Numbers above each box and whisker plot indicate sample sizes. K-W = Kruskal-Wallis test for non-parametric comparison across all three regimens. Two-sample analyses were performed using the Wilcoxon's rank test. *Indicates statistical significance

models generated.[2] There is relatively little information regarding colony management practices and productivity in relation to welfare and pathology. The issue of productivity of males following ENU treatment is of considerable importance in ENU mutagenesis programmes because of the need to generate sufficient numbers of G_0 offspring for different phenotypic screens. Others have assessed mortality in the sterile period after exposure to ENU, and regained fertility but primarily with the view to optimize ENU dosage regimen for each strain.[7,8] Initial studies in small cohorts identified 3×100 mg/kg as an optimal regimen for both C57BL/6J and BALB/cJ strains with regained fertility rates of 6/10 (60%) and 5/6 (83%) in males for each strain, respectively.[8] Although the BALB/c is a different substrain from the one in our study, the C57BL/6J strain is comparable. In our larger sample size, we can confirm a relationship between cumulative dose and infertility rates suggested in smaller cohorts of these strains. Indeed, a more recent study of comparable population size, but in C3HeB/FeJ mice,[14] shows a similar finding to ours and is also in agreement with previously published rates in smaller cohorts of this inbred strain.[8] We were particularly interested in our study to extend this analysis and to explore the components of lifetime fecundity: regained fertility and lifetime productivity.

One good example of detailed analysis of fecundity in chemically mutagenized males is that by Russell *et al.*[12] but this focuses on the immediate post-treatment effects of ENU on spermatocytes. Male productivity was measured directly in (101/R1xC3H/R1) F1 males immediately after ENU treatment with 50 mg/kg, 100 mg/kg or 250 mg/kg, in seven, sequential one-week matings. This experiment showed a decrease in average litter size with time, providing an approximate indication of lethality in G_1 offspring, whereas decrease in litter number per male may represent morbidity and fewer copulations, germcell killing and/or lethality.[12] The highest ENU dose 250 mg/kg reduced the number of litters. Both ENU and another chemical mutagen MNU (*N*′-methyl-*N*′-nitrosourea also studied in this work) have mutagenic effects on stem-cell spermatogonia and elicit very high mutation rates in differentiating spermatogonia when measured by specific-locus assays, hence the mutagenic effects seen from early matings in the seven-week post-treatment period.

In a similar way, we have examined litter frequency, size and number of ENU-treated males after they have regained fertility. Using these indices coupled with the results of the pathology analysis, we can also show loss from the breeding programme through morbidity and premature death. The biggest impact of ENU on the fecundity or lifetime breeding potential of G_0 males was the adverse effect of cumulative ENU dose, increasing the number of males being rendered permanently infertile. Subsequently, the loss from the breeding programme through morbidity and premature death has a greater effect than a modest decline in productivity during subclinical stages of lymphoma. This is supported by data from those males that did breed indicating that while neither average litter size nor littering frequency were affected, the number of litters per G_0 male was significantly decreased in the highest dosage regimen.

The types of ENU-related pathology are as follows. In the earlier mutagenesis experiments, acute post-treatment toxicity with ENU treatment resulting in 20% weight loss was a problem when BALB/cAnNCrl mice were exposed to infection as a result of IVC barrier breakdown in an otherwise low health status mouse. ENU is a stem-cell mutagen and affects haematopoietic stem cells causing immunosuppression.[7] In our low health status conventional mouse colony this appeared to predispose to MHV infection. This complication of acute post-treatment toxicity has been overcome by rederivation of the stocks and performing ENU treatments in SPF conditions in the MLC.[10] The major pathology was tumourigenesis and in our analysis we found that in both BALB/cAnNCrl and C57BL/6J strains, ENU-treated males had a very high incidence of malignant T-lymphoblastic lymphoma. In addition, in BALB/cAnNCrl lung carcinoma was also important. The case for lymphoma being induced by ENU is clear-cut because the very high incidence (>75% of those culled sick) at a relatively young age is considerably greater than background incidence (9–34%) in untreated BALB/cAnNCrl and C57BL/6J mice on two-year ageing studies.[15] Furthermore, our survey of 28 retired untreated C57BL/6J breeding males found no morbidity or sudden death between six months and 12 months and no thymic lymphomas.

The incidence of other less common tumours can be age-related and we are cautious not to over-interpret the differences between strains because early lymphomas abrogate the development of a wider tumour spectrum. Furthermore, it is possible that low health status of the conventional BALB/cAnNCrl mice in the earlier mutagenesis experiments has had some effect on the development of tumours. In both BALB/cAnNCrl and C57BL/6J, the peak of lymphoma incidence within the breeding period produces a significant decrease in productivity. Lymphoma incidence in BALB/cAnNCrl and C57BL/6J mice 'culled sick' was similar at the 3×90 mg/kg dosage (no data are available for comparison with the 3×100 mg/kg dosage), but in the 2×100 mg/kg dosage group, the incidence of lymphoma in BALB/cAnNCrl was lower than in C57BL/6J mice (27% vs. 82%, $P < 0.0001$, Fisher's exact test; Table 2). Whether this represents a strain-specific lower dosage threshold for ENU-induced lymphoma in C57BL/6J mice is unclear. The incidence of spontaneous lymphoma BALB/cAnNCrl and C57BL/6J mice is approximately the same.[15] The putative mechanism of ENU-induced lymphomagenesis may be associated with perturbation of genes in DNA repair and tumour suppressor pathways,[16–19] and it will be interesting to see if these mechanisms play a role in strain-specific susceptibility. Irrespective of the ENU dosage regimen used, the majority of ENU-treated mice were culled on welfare grounds before being retired from the breeding programme.

In recognition of the early onset of lymphoma in ENU-treated males, and as part of the continual monitoring of our practices, we have implemented a major refinement to our current mutagenesis programme. Once G_0 males have sired a litter, we now rotate them to a second cage that holds two C3H/HeH females. The rationale is to

maximize productivity within a limited breeding lifetime. In C57BL/6J males treated with 3×100 mg/kg ENU this is, on average, 88 days between the birth of the first litter(s) (126 ± 3 days) and lymphoma-associated death (214 ± 7 days). This refinement in breeding strategy has increased productivity by more than two-fold.

This retrospective case-control study of breeding and pathology in an ongoing ENU mutagenesis programme does not attempt to address the question of efficiency of mutagenesis for different dosage regimens using standard measures, such as a specific-locus test[12] or the number of molecularly characterized mutations per mutagenized gamete. An assessment can be made after several more years' work to identify all the mutated genes underlying the phenotypes revealed in the screens. Truly comparative estimates of ENU dosage and mutagenesis efficiency will have to take into account the diverse and changing collection of dominant and recessive phenotypic screens across all Harwell mutagenesis projects. Under these circumstances, the question of cost/benefit analysis cannot be completely resolved because an ENU dosage that might have a high impact on animal welfare (cost) might be very efficient at producing mutant pedigrees (high scientific benefit). The closest we can come to reconcile this problem is to use published data as a guideline for the highest tolerable ENU dose to produce the greatest mutagenesis efficiency. The question then becomes how to maximize the productivity of ENU-injected males because one major outcome of the highest dose will be increased sterility. The measurement and analysis of breeding success and the pathology outcomes then becomes the key to identifying timelines to breed as many offspring in the shortest period.

In conclusion, to our knowledge, this is the first large-scale analysis of deleterious effects of ENU dosage regimens on the breeding performance, pattern and incidence of tumour pathology in an ENU mouse mutagenesis programme. This has provided new insights into lifetime productivity and allowed us to refine our breeding strategy to improve breeding productivity while minimizing the numbers of males that are treated with ENU.

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