Genotypic effects on gonadal size in fetal mice*

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Summary. Fetal gonadal size was measured on Days 13, 16 and 19 of gestation in the C57BL/6ByEss (B) and BALB/cByEss (C) inbred strains, their two reciprocal F1 hybrids (CXB and BXC) and in the CXBD and CXBE recombinant inbred lines. At Day 13, CXB F1 fetuses, with C57 fathers and BALB mothers, had significantly larger testes and ovaries than did fetuses of the other 5 stocks. On Day 16, BALB fetuses had significantly larger testes than did C57, while at Day 19 C57 fetuses had significantly larger testes than did BALB fetuses. The CXB and BXC F1 fetuses had significantly larger testes than did mice of the two parental strains on Days 16 and 19, even though the mothers of all 4 kinds of fetus came from the same two inbred strains. C57 and BALB mice did not differ significantly in ovarian size, but had significantly smaller ovaries than did mice of the other genotypes on Days 16 and 19. CXBD mice had the largest ovaries, followed by those of the F1 hybrids. Ovarian size in CXBE mice was similar to that in the CXB hybrids. There were strong maternal effects on gonad size on Days 13 and 19 of gestation. The genes that influenced fetal testicular and ovarian growth appeared to differ from those expressed post-natally at 30 and 60 days.

Keywords: ovary; testis; fetus; inbred strains; maternal effects; mouse

Introduction

The effects of genetic and environmental factors on gonadal size have been investigated in mice known to differ in reproductive characteristics as adults. The C57BL/6 and BALB/c strains were chosen for comparison because of reproductive and behavioural differences (McGill & Manning, 1976; Shire & Whitten, 1980; Shire, 1984; Bander *et al.*, 1988) and differences in the post-natal development of gonads and other endocrine organs (Shukri & Shire, 1986; Janat & Shire, 1987; Shukri *et al.*, 1988). Gonadal development cannot be followed over time within a single animal. Such studies require replicated observations of animals of the same genotype at different ages. This is possible for mice of homozygous inbred strains and F1 hybrids, as all members of the same sex of an individual strain or F1 cross have identical genotypes. However, each individual in a segregating F2 or backcross generation has a unique genotype and cannot be pooled with others for analysis of developmental traits. The use of recombinant-inbred (RI) strains permits the analysis of the segregation patterns of such characters (Bailey, 1971, 1981). Reciprocal F1 hybrids were studied, together with two of the recombinant-inbred strains derived from crosses between C57 and BALB mice. The CXBD and CXBE RI lines were chosen because CXBD resembled the BALB strain in testis weight and androgen target organ weights at both 30 and 60 days of age while CXBE resembled C57BL at 30 days of age but was much more like BALB when 60 days old (Shukri, 1988).

Sexual differentiation of the indifferent gonad takes place around Day 11 of gestation (Mittwoch, 1973; Byskov, 1978). Mittwoch & Delhanty (1969) found that male fetal gonads in rats were consistently larger than female gonads and proposed that the Y chromosome speeded up

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development by increasing the rate of cell proliferation in the developing male gonad. Molecular cloning and hybridization experiments, using the testis-determining sequence (*Tdy*), have provided evidence that the primary switch gene for sex determination is on the Y chromosome (review in McLaren, 1988), although the X chromosome and autosomes may also be involved (Goodfellow & Darling, 1988). Hayward & Shire (1974) showed a Y-chromosome effect on testis weight in adult mice. This has been confirmed by Hunt & Mittwoch (1987), who showed that CBA/Gr fetuses had smaller testes than did BALB/cOla fetuses on Days 14, 16 and 18 of gestation.

In this study, gonadal size of mice in 6 genotypes was examined at 3 prenatal stages of development, on Days 13, 16 and 19 of gestation. Testes and ovaries of fetuses of the C57 and BALB strains, CXB and BXC F1 hybrids and CXBD and CXBE recombinant lines were measured and the observations analysed for possible chromosomal and/or parental effects. The patterns of testicular and ovarian growth were compared to investigate any genetic and environmental influences on gonadal growth common to both sexes.

Materials and Methods

The highly inbred progenitor strains BALB/cByEss (C) and C57BL/ByEss (B) were used in all experiments. The reciprocal F1 hybrids are referred to as CXB and BXC, with the strain of the mother given first. The RI lines CXBD/ByEss and CXBE/ByEss were also used. All mice were kept under constant environmental conditions (Shukri *et al.*, 1988).

For the production of fetuses, 1 male and 1 female of a particular genotype were placed together in a cage between 16:00 and 19:00 h. The following morning vaginas were examined for the presence of copulation plugs. The day of appearance of the copulation plug was designated as Day 1 of pregnancy.

Pregnant females were killed by halothane anaesthesia (May & Baker Ltd, Dagenham, UK) on Days 13, 16 and 19 of gestation. For each female the 2 uterine horns (and the fetuses) were removed and immediately placed in Petri dishes containing ample amounts of phosphate-buffered saline, pH 7.4. Each fetus was separated from its uterine horn, the trophoblast was removed and the fetus was dissected under a Nikon binocular microscope. The fetal sex was determined (except on Day 13) by the size, shape and position of the gonads (Schlegel *et al.*, 1967). The gonads were removed with fine forceps and placed in vials containing 15 ml 10% formol saline and 2 ml 5% water-soluble eosin. The use of an isotonic fixative minimizes the shrinkage during fixation, and the direct measurement of the fixed tissue avoids further shrinkage during dehydration and embedding. The volume of each gonad was determined from measurements of its size in three dimensions perpendicular to each other. The measurements were made, in mm, using a projection microscope fitted with an eyepiece micrometer (Dynascope: Vision Engineering Ltd, Send, Surrey, UK). The image was viewed at $\times 3$ magnification (confirmed by the use of a calibrated microscope slide) and the two adjustable measuring lines were placed tangent to the curved surface of the image of the gonads using the calibrated micrometer adjustment. The distance was read and corrected to the absolute length (or breadth or depth) of the gonad by dividing by the magnification factor. The volume was calculated from the formula for an ellipsoid, using half the length, half the breadth and half the depth as the three radii. Ten separate measurements of the volume of a single testis had a coefficient of variation of 2%.

The gonadal sex of Day-13 fetuses is not readily identifiable in whole gonads and so 8 μ m cryostat sections stained with haematoxylin and eosin were prepared. The thickening of testicular capsule and the appearance of seminiferous tubules (Beamer *et al.*, 1978; Whitten *et al.*, 1979; Taketo & Koide, 1981) provided the basis for sexing Day-13 gonads. In the absence of these structures, the gonads were designated as ovaries.

The statistical analyses were carried out on the total volume of the left and right gonads of each mouse. They included *t* tests and one- and two-way analyses of variance. The Newman-Keuls test was used to identify pairs of samples that differed (Snedecor & Cochran, 1980).

Results

Figure 1 shows the mean gonadal volumes for male and female fetuses of the 6 genotypes studied at 3 developmental ages. One-way analyses of variance showed that testes grew significantly ($P < 0.001$) in all strains as gestation proceeded from Day 13 through Day 16 to Day 19. The growth in testis size was most dramatic in C57 mice ($F_{2,60} = 629$) and least in the CXB F1 ($F_{2,60} = 188$). Testis size differed significantly between the 6 genotypes at all 3 ages (Table 1).

On Day 13 of gestation BXC F1 fetuses had significantly larger testes ($P < 0.05$, Newman-Keuls test) than did fetuses of the other strains, whereas there were no significant differences in

Fig. 1. The mean volumes (+s.e.) of ovaries and testes in fetal mice at 13, 16 and 19 days of gestation. The six stocks shown are C57BL (B), BALB/c (C), CXBD (D), CXBE (E), and the two reciprocal F1 hybrids (CXB and BXC). The numbers of mice are given at the top of each column. Means sharing the same superscript do not differ significantly by the Newman-Keuls test.

Table 1. The F values, degrees of freedom (d.f.) and probabilities for one-way analyses of variance of the effects of stock on testicular and ovarian volume of mice at three developmental ages

Day of gestation	Testis volume			Ovary volume		
	F	d.f.	P	F	d.f.	P
13	3.5	5,64	<0.01	7	5,67	<0.001
16	18	5,87	<0.001	14	5,94	<0.001
19	22	5,244	<0.001	42	5,258	<0.001

testis among C57, BALB, CXBD, CXBE and CXB F1 fetuses. On Day 16 BALB mice had significantly larger testes than did C57 mice, whereas on Day 19 the difference was reversed (Fig. 1). On Day 16 of gestation the testes of CXB F1 fetuses were the largest, but they were not significantly different from the testes of the reciprocal BXC F1 mice. Both F1 hybrids had significantly larger testes than did mice of the other strains (Fig. 1). On Day 19 the pattern was reversed and BXC F1 fetuses had significantly larger testes than did CXB F1 mice. Except on Day 13, the testes of CXBD fetuses were consistently larger than those of CXBE mice. CXBD fetal testes were significantly larger than those of both C57 and BALB on Day 16 and those of BALB on Day 19. CXBE fetuses, on the other hand, had significantly larger testes than C57 only on Day 16 and significantly larger testes than BALB only on Day 19.

Ovarian size also differed at all three developmental ages. It increased significantly ($P < 0.001$) through Days 13, 16 and 19 of gestation in all strains (Fig. 1). Growth in ovarian size was most marked in CXBD mice ($F_{2,76} = 225$) and least in BALB females ($F_{2,66} = 83$). On Day 13 of gestation the ovaries of BXC F1 hybrids were significantly larger ($P < 0.05$) than those of any of the other genotypes, which did not differ amongst themselves. The two parental strains did not differ significantly at any age (Fig. 1). CXBD fetuses had significantly larger ovaries at Days 16 and 19 (Fig. 1). On these days both F1 hybrids had ovaries that were intermediate in size between those of CXBD and those of the parental strains, but on Days 16 and 19 the ovaries of the BXC fetuses had once more become significantly larger than those of the reciprocal hybrid fetuses. The ovarian development of CXBE mice was similar to that of the CXB hybrids.

Discussion

The fetal gonads of the two progenitor strains differed significantly in size, both on Day 16, when C57 testes were smaller ($F_{1,29} = 5.4$, $P < 0.05$), and on Day 19 when C57 testes were larger ($F_{1,80} = 44$, $P < 0.001$). A similar reversal of the rank order of genotypes for testis size between Day 19 of gestation and adulthood was found in a comparison of C57BL/Fa and CBA/FaCam by Stewart (1983). Although the differences between the C57 and BALB strains were not significantly different, the means show a similar pattern for ovarian size.

The observed strain differences may be due to direct effects of nuclear or cytoplasmic factors on the cells of the developing gonad, or to indirect effects of differences in the maternal uterine environment, or to combination of both. Observations on reciprocal crosses between the strains can help to identify the sources of the difference. Such crosses keep the same maternal environments as found in the progenitor strains but analyse their effects on fetuses of a different genotype. For F1 female fetuses the genotypes of the reciprocal crosses are identical for all chromosomal genes whilst reciprocal F1 male fetuses differ in both the X and Y chromosomes. The F1 fetuses had larger gonads than did fetuses of either progenitor strain at Days 16 and 19. Even at Day 12 the ovaries and testes of the BXC F1 fetuses were significantly larger than those produced by other matings. There were significant differences between reciprocal F1 fetuses at Day 19, with BXC mice having larger testes and ovaries. In contrast, testis size was greater in CXB fetuses at Day 16. At both ages, however, the rank order of the strains and F1 hybrids was the same, with the ranking within the F1 hybrids being the same as the relative ranking of their maternal strain. Ovarian size at Day 19 also showed the same matroclinous pattern. The matroclinous pattern of differences in the males could be caused by X-linked genes but cannot be caused by differences in Y-linked genes, which are known to contribute to differences in post-natal testis size in some cases (Hayward & Shire, 1974; Jutley & Stewart, 1984; Hunt & Mittwoch, 1987). However, reciprocal F1 females do not differ in X-linked genes, but still showed a difference in ovarian size. A maternal influence on gonadal development might well affect fetuses of both sexes similarly. Such differences could affect the uterine environment in which the fetuses develop or could be present in the developing fetus from before the time of implantation. Post-implantation uterine effects could be distinguished from preimplantation effects caused by cytoplasmic factors such as mitochondria (Ferris *et al.*, 1982) or

differential genomic imprinting (Surani *et al.*, 1988) by blastocyst transfer experiments. The maternal effect would seem likely to be gonad-specific rather than acting on the growth rate of the whole fetus. Snow *et al.* (1981) showed that fetal weight at 16 days of gestation reflected only the fetal genotype and that the uterine environment neither depressed the growth of large embryos nor enhanced that of small ones. Furthermore, Riska *et al.* (1984) found that prenatal maternal effects on body weight were, in general, small.

The recombinant-inbred lines represent new combinations of the inherited factors affecting both the rate of growth of the gonadal cells and the components of the maternal environment to which the developing mice are exposed. The gonads of the CXBD mice are consistently larger than those of their progenitor strains, and are similar in size to those of at least one of the F1 hybrids. The mice of the recombinant line are, however, homozygous at all (or very nearly all: Shukri *et al.*, 1988) loci. This suggests that the heterosis found in the F1 hybrids was not the result of increased heterozygosity but was more probably due to complementation for suboptimal alleles at several different loci (Falconer, 1981). Testicular development in the CXBE recombinant line was not significantly different from the pattern found for the C57 progenitor strain. Analysis of further recombinant lines would show whether it was possible to recover a genotype with consistently low growth rates, which would resemble C57 on Day 16 and BALB on Day 19. If, however, the pattern of growth rates is determined by a single causal step, rather than being made up of two separable growth stages, then such a recombinant phenotype could not occur.

Ovarian development in the CXBD and CXBE mice showed similarities to testicular development. CXBD ovaries were consistently larger than those of CXBE fetuses, and were as large as those of BXC F1 fetuses at Day 19 and larger than them at Day 16. Ovarian development in CXBE mice, as with testicular development, again resembled that in C57 mice. This pattern suggests that some at least of the factors affecting gonadal development may be common to both sexes. Correlations between testicular and ovarian size were significant ($r_s = 0.77$, $P = 0.05$) at both Day 16 and Day 19. One common factor might be gonad-specific as has been suggested for post-natal gonadal development (Land, 1973; Land & Carr, 1979), even though the two kinds of gonads are developing very different structures during the later stages of fetal development. Another factor might be the rate of overall fetal growth, although this would not account for the relative rankings of ovary and testis size in both CXBD and CXBE. Nor would it account for the large difference between the C57 and BALB strains in testis size at Day 19.

After birth, C57 mice have small testes (Shire & Bartke, 1972; Shukri, 1988) and BALB mice large ones. At 30 days of age there appeared to be a major effect of H-2 genotype of testicular weight, with the recombinant lines falling into two classes, closely resembling the phenotypes of the two progenitor strains (Shukri & Shire, 1986). At 60 and 120 days, however, other genes have begun to be expressed and none of the recombinant lines had testes as small as those of C57 mice. CXBD mice had larger testes than did CXBE mice at 30 days after birth, thus resembling the situation found prenatally at Day 19 of gestation. C57 females also had small ovaries as adults (Davies, 1987) but at 30 days their ovaries were larger than those of BALB (A. Hamad, personal communication). F1 females showed a matroclinous effect at both ages. However, CXBD females had relatively small ovaries while CXBE adults had relatively large ones at both ages. A significant reversal in the rank order of the two progenitor strains therefore occurred in testicular and ovarian development, but later in females than in males. Overall, different sets of genes appear to be being expressed during gonadal development and growth, from Day 16 of gestation through to at least 120 days after birth.

Future investigations planned to analyse further the strain differences described in this paper include embryo transfer experiments, measurements on the full set of CXB recombinant inbred lines and analyses of reciprocal F2 fetuses.

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