The morphology of fetal gonads of spontaneous mouse hermaphrodites

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SUMMARY

The gonads of 107 spontaneous, chromosomal mosaic, day-15 fetal hermaphrodites derived from BALB/cWt strain male mice are described and photographs of seven gonads representative of the major types are presented. There were 16 ovaries, 15 testes, and 183 ovotestes. The ovotestes contained on the average more testicular tissue than ovarian, and the ovarian tissue was more frequently located at the gonad poles, particularly the cranid pole. There was no difference between left and right sides with regard to gonad type, but more pure gonads were found on the left than on the right side (21/10). Meioses were observed throughout the ovarian tissue and also in some testicular cords, particularly in the caudad pole of the gonad. Some meiotic figures contained sex vesicles.

The significance of these findings is discussed in relation to several aspects of sex determination.

INTRODUCTION

Reproduction in many invertebrates and in some fish depends on sex reversal or on fertile hermaphrodites, whereas, in higher vertebrates hermaphroditism must be considered pathological. Nevertheless, spontaneous true hermaphrodites have been described in frogs (Witschi, 1956), reptiles (Taylor, 1918; Hansen, 1943), birds (Riddle, Hollander & Schooley, 1945; Cock, 1954; Hollander, 1975), lower mammals (Hooker & Strong, 1944; Hollander, Gowen & Stadley, 1956; McFeely, Hare & Biggers, 1967; Lyon, 1969; Dunn, McEntee & Hansel, 1970; Whitten, 1975; Seldon et al. 1978), and man (Jacobs, 1969; Polani, 1970; Niekerk, 1974).

By definition hermaphrodites must possess both ovarian and testicular tissues. In eutherian mammals they could develop from embryos that contain male and female cell clones derived from separate zygotes or, the male determining function usually on the Y chromosome could have been lost in one or more clones of a male fetus. Such sex mosaics may be more common in mammalian

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embryos than previously thought, but, because there are several sampling events during development when some cells are relegated to non-embryonic structures, or later in the fetus to tissues other than the gonads, only a small proportion of these individuals exhibit hermaphroditism at puberty. In addition to these sampling events one clone, often the male, may have a selective advantage and overgrow the embryo, or there may be some uncharacterized mechanism which mitigates against hermaphrodite fetuses continuing to develop as such.

Virtually nothing is known about the course of development of spontaneous mammalian hermaphrodites, but there have been some studies of mouse fetuses derived from aggregated morulae (McLaren, Chandley & Kofman-Alfaro, 1972; Mystokowska & Tarkowski, 1970). In all hermaphrodites, conflicts must develop soon after the appearance of the genital ridge at 10 days. For example, will the germ cells proceed to enter meiosis as in the normal ovary on the 13th day (Crone, Levy & Peters, 1965), or will this be delayed until 10 or more days after birth as in the normal testis (Kofman-Alfaro & Chandley, 1970)?

Preliminary findings of Beamer, Whitten & Eicher (1978) that were based on metaphase chromosome preparations from fetal hermaphrodite mouse livers revealed these hermaphrodites to be sex chromosome mosaics \((XO/XY\) or \(XO/XY/XYY\)). The complete data and hypotheses regarding the causative cytogenetic events leading to hermaphroditism in BALB/cWt mice are presented in Eicher, Beamer, Washburn & Whitten (1979). In this report we describe the identification and the morphology of the gonads of 107 fetal hermaphrodites found among the progeny of BALB/cWt male mice in which there is a high frequency of spontaneous hermaphroditism (Whitten, 1975; Beamer et al. 1978). We also develop systems to classify the ovotestes according to the situation and proportion of the ovarian component in each gonad. These findings are discussed with respect to gonadal development, sex determination and the fate of the mixed gonads. The occurrence of meiosis and the role of the rete in the induction of this process are also considered.

**MATERIALS AND METHODS**

Adult female mice of several strains (Beamer et al. 1978) were paired monogamously with BALB/cWt males and observed each morning between 8 a.m. and 10 a.m. for the presence of copulation plugs. The day on which the plug was found was defined as day zero of gestation. It had previously been determined that fertilization in BALB/c mice takes place on the morning of the day the plug is observed (Whitten, 1971). Thus females slaughtered on the morning of day 15 contain embryos which are about 360 h old. Almost all of the animals were killed at this time, but a few were killed on the afternoon of day 13, 14, or 17.

The pregnant dams were killed by cervical dislocation, the uteri were exposed and the number of fetuses or resorptions counted and recorded. Resorptions
were classified as early if the estimated time of death occurred before day 10. The fetuses were freed from their membranes, pinned onto a waxed dish, and their abdomens opened. Blood was washed away with saline and the gonads were observed through an operation microscope with vertical incident illumination at ×10 magnification. The gonads and attached structures were removed with watchmaker's forceps and placed in a drop of EDTA buffer (8.0 g NaCl; 0.2 g KH₂PO₄; 0.2 g KCl; 1.15 g Na₂HPO₄; 0.2 g disodium EDTA per liter distilled H₂O; pH 7.0) on a flat slide for examination under a dissecting microscope (×25) with transmitted light. The sex of each fetus was determined from the phenotype of both gonads.

Gonads were considered to be ovaries if there was no thickened capsule and the body contained only a fine grained reticular structure (Fig. 1). Gonads were considered to be testes if they had a thickened capsule with subcapsular vessels and contained within their bodies 7 to 11 distinct cords each forming a single loop (Fig. 7). If the gonad showed only a partial capsule or contained six or fewer cords and at the same time exhibited one or more zones of ovary-like structures it was recorded as an ovotestis. Animals with one or two ovotestes or with one ovary and one testis were considered hermaphrodites.

Almost all of the abnormal gonads, and a small proportion of the normal ones, were sketched and photographed using an inverted microscope. The gonads were fixed in Zenker's solution processed in the usual manner for paraffin embedding, serially sectioned at 6 μm, and stained with PAS and hematoxylin.

Embryos collected on day 13 were too small and fragile to examine effectively and differentiation had not progressed sufficiently to make clear cut diagnoses. A series of three hermaphrodites was collected on day 17 of gestation. These were harder to identify because the greater mass prevented examination of the structure effectively by transmitted light and because the cords were so convoluted that counting was not possible. The day-17 hermaphrodites are not included in the tables, or discussed further.

RESULTS

The number of hermaphrodites found and the validity of the diagnoses

Over 200 day-15 fetal hermaphrodites were found during the study of maternal factors involved in their production in the progeny of BALB/cWt males (Beamer and Whitten, in preparation). In this paper we report on the morphology of the gonads of 107 fetal hermaphrodites collected after we became proficient with the above procedures. There is no reason to expect that the sample is biased nor have we found any evidence of an effect of the strain of the dam on the morphology of the hermaphrodites. Therefore, this sample should represent all BALB/cWt derived hermaphrodites.

All of the ovotestes that were diagnosed under the dissecting microscope were confirmed by histological examination. In contrast none of the gonads
FIGURES 1-8

Gonadal preparations from 15-day fetal hermaphrodites sired by BALB/cWt males and classified as indicated in Table 2.

Figs. 1-7(a) and 8. Photographs taken through an inverted microscope of whole, unfixed, unstained gonads with their attachments.

Figs. 1-7(b)-(d). Photomicrographs of sections (6 μm) stained with PAS and hematoxylin.

Fig. 1. Left gonad (class 1, ovary) of fetus 765.5 from an SJL/J female. The septa are more marked than usual for an ovary and pachytene nuclei (dark staining and irregular) can be seen throughout the gonad. See Fig. 3 for the other gonad of this fetus.

Fig. 2. Right gonad (class 2, ovotestis) of fetus 1790.2 from CXBH/By female. Ovarian components are present at each pole and contain many pachytene nuclei. There are portions of two testicular cords in the central zone and one shows a lumen.

Fig. 3. Right gonad (class 3, ovotestis) from fetus 765.5 (same as in Fig. 1) from SJL/J female. Ovarian portions are situated at both poles and contain pachytene nuclei. Meiosis can also be seen in the hilus. There is a thickened capsule and there is a testicular cord in the central zone.

Fig. 4. Right gonad (class 4, ovotestis) of fetus 1821.6 from a BALB/cWt female. The ovarian component with pachytene nuclei is situated only in the cranial pole and there are clear-cut connections (arrows) between this and the extragonadal rete. The caudal half is testicular with thickened capsule and four cords, three of which have lumina.

Fig. 5. Right gonad (class 5, ovotestis) of fetus 728.7 from SJL/J female. The ovarian portion occupies the central zone and contains many meiosis and connections (arrows to the extragonadal rete. Testicular cords and thickened capsule occupy both poles.

Fig. 6. Left gonad (class 6, ovotestes) of fetus 1787.3 from a CXBH/By female. Ovarian portion with retal connections (short arrows) and meioses in the cranial pole. Testicular portion with four cavitated cords, thickened capsule and subcapsular vessel (long arrows) penetrating between ovarian and testicular components.

Fig. 7(a). Left gonad (class 7, testis) of fetus 1804.6 from BALB/cWt female. Eight normal testicular cords. (b) Right gonad (class 7, testis) of fetus 10.9 from BALB/cWt female. Normal testicular cords but meioses visible in the most caudal cord where it is close to the Wolffian duct (arrow). (c, d) Higher magnification of area outlined in 7(b). (c) and (d) are of adjacent areas but photographed at different focuses. Sex vesicles are visible in a pachytene nucleus (short arrow) and in a leptotene nucleus (long arrow).

Fig. 8. Right gonad (class 6, ovotestes) of fetus 1823.5 from BALB/cWt female showing connection (arrow) between the extragonadal rete and the ovarian portion situated in the cranial pole. The testicular portion has 5 or 6 ballooned cords, a thickened capsule and blood vessels penetrating to the hilus.
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1a 100μm

1b 100μm

2a 100μm

2b 100μm
Spontaneous fetal mouse hermaphrodites
Spontaneous fetal mouse hermaphrodites

from normal fetuses showed any evidence of both male and female elements, but this sample was much smaller than that of the hermaphrodites. However, the one gonad that was considered doubtful proved to be hermaphroditic when sectioned. Another gonad collected subsequent to this series was diagnosed as the normal ovarian partner of an ovotestis. On section this gonad was found to contain septa organized like those in a testis but in which all of the germ cells were in the pachytene stage of meiosis. Blood sinuses could be traced from the capsule to the hilus, penetrating between the ovarian and testicular portions (Figs. 6 and 8). An accumulation of hemopoietic cells was observed in gonads of several hermaphrodites of this series, an observation that has been confirmed in fetal ovaries by Dr Hannah Peters (personal communication).

From these findings we conclude that diagnosis with the aid of a dissecting microscope is reasonably reliable but that some gonads with minimal deviations from normal may be missed. Nevertheless, the method had the advantage of being quick and the results can be recorded on photographs that can catch some of the three dimensional quality of the fresh material before any distortion from fixation occurs.

General description of the hermaphrodite gonads

The gonads varied from normal ovaries to complete testes and almost all of the possible combinations between left and right were found. The distribution of the elements in the individual gonads also varied. Photographs of whole preparations and histological sections are presented in Figs. 1–8. In Figs. 1–6
Table 1. The number of hermaphrodite gonads classified according to the location of the ovarian tissue

<table>
<thead>
<tr>
<th>Position of ovarian tissue</th>
<th>Number of gonads observed</th>
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</thead>
<tbody>
<tr>
<td>Craniad pole only</td>
<td>79</td>
</tr>
<tr>
<td>Caudad pole only</td>
<td>8</td>
</tr>
<tr>
<td>Both poles, excluding whole ovaries</td>
<td>81</td>
</tr>
<tr>
<td>Equatorial only</td>
<td>6</td>
</tr>
<tr>
<td>Whole ovaries</td>
<td>16</td>
</tr>
<tr>
<td>Whole testes</td>
<td>15</td>
</tr>
<tr>
<td>Unclassified</td>
<td>9</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>214</strong></td>
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</table>

The whole preparation and the histological section are from the same gonad and demonstrate very good agreement between the methods. This made it possible to classify the gonads using the photographs of the fresh material without elaborate three-dimensional reconstructions, but the histological material was referred to in cases of doubt.

Many of the testicular cords in the ovotestes appeared to be irregular and stunted, suggesting disturbed growth and differentiation. Spaces were evident between the cords and cavitation of the peripheral loops gave the impression of premature tubules (Fig. 4 and 6).

Classification according to the position of the ovarian tissue

In this series of hermaphrodites the ovarian components of the gonads were observed most frequently at the poles, especially the craniad pole (Figs. 1-4, 6 and 8). Only rarely did it occur in the body of the gonad, as shown in Fig. 5. The distribution according to position is shown in Table 1 from which it is clear that over 90% of the ovotestes had ovarian tissue at the craniad pole. In the two fetal chimeric ovotestes described by Mystkowska & Tarkowski (1970) and McLaren et al. (1972) the ovarian portions were also found in the craniad poles.

Observations on the gonadal and extragonadal rete

In Fig. 8 there is a clear connection between the ovarian portion of the gonad and the extragonadal rete. This connection was confirmed by histological examination using the PAS positive nature of the rete cells to identify them. Similar rete connections can be seen in Figs. 4 and 6. In these three examples the rete connects to the ovarian component at the craniad pole of the gonad. However, in Fig. 5(a) the rete penetrates the central portion of the gonad just where the ovarian portion is placed and this finding was confirmed histologically. There is no evidence in the figures of rete connections with ovarian components in the
Spontaneous fetal mouse hermaphrodites

Table 2. Classification of hermaphrodite gonads according to the proportion of ovarian tissue.

<table>
<thead>
<tr>
<th>Class</th>
<th>Criteria</th>
<th>Number of gonads</th>
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<td></td>
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<td>Right</td>
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<tr>
<td>1</td>
<td>Completely ovarian as in Fig. 1</td>
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<tr>
<td>2</td>
<td>80-90% ovarian tissue as in Fig. 2</td>
<td>13</td>
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<td>3</td>
<td>60-79% ovarian tissue as in Fig. 3</td>
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<td>4</td>
<td>40-59% ovarian tissue as in Fig. 4</td>
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<td>5</td>
<td>20-39% ovarian tissue as in Fig. 5</td>
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<tr>
<td>6</td>
<td>1-19% ovarian tissue as in Fig. 6</td>
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<td>7</td>
<td>Completely testicular as in Fig. 7</td>
<td>4</td>
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<tr>
<td>Total</td>
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<td>107</td>
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</table>

Mean 40

Mean 4.45 ± 0.16 4.38 ± 0.17 4.42 ± 0.12

caudad pole. However, in vivo the main duct of the mesonephros, i.e. the Wolffian duct, is often applied to this area. The Wolffian duct is clearly seen in Fig. 7 but has been displaced during dissection.

The occurrence of meiosis in hermaphrodites

As expected and as seen in Figs. 1-6, pachytene stages of meiosis are common throughout the ovaries and ovarian portions of the ovotestes. Preleptotene condensation also was observed in many testicular cords particularly those near the poles. In the central cords meiosis was seen more frequently near the hilus than in the periphery of the loops. (Fig. 3b). Leptotene and pachytene stages of meiosis were also seen in the cord at the caudad pole, particularly the last cord next to the Wolffian duct of an apparently normal testis as seen in Fig. 7b-d.

Sex vesicles were seen in gonocytes in meiosis. Two sex vesicles are shown in Fig 7b-d, one in a cell in the leptotene and one in a cell at the pachytene stage of meiosis. These findings clearly show that some male cells enter meiosis at the time characteristic for meiosis in an ovary.

Classification of the gonads according to the proportion of ovarian tissue and the question of laterality

As mentioned above, the proportion of ovarian and testicular tissue in the gonads varied between the two possible extremes. Because this sample contained 214 gonads it was possible to classify them into seven classes with significant numbers in each class. Table 2 gives the criteria used for this classification and the numbers of gonads that fall into each class for the left and right sides. The distribution is skewed towards gonads with a low ovarian content.

Table 2 also gives the mean class numbers for both left and right gonads;
Table 3. The number of hermaphrodite fetuses arranged according to the difference between the classification of both gonads and the mean gonadal score

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<th>Mean gonad score</th>
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<th>4-0</th>
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<td>17</td>
<td>16</td>
<td>9</td>
<td>10</td>
<td>107</td>
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</table>

* The numbers in parentheses represent the classes used to calculate the mean score.
† Fetuses with mainly ovarian gonads. Those included in the box in the upper left would probably develop into sex mosaics with female phenotype.
‡ Fetuses with gonads of equal mixture. These and those unboxed will probably continue to develop as hermaphrodites.
§ Fetuses with mainly testicular gonads. Those in the box in the upper right would probably develop into sex mosaics with male phenotype.
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there was no significant difference between the means or their variances. However, there were ten ovaries and eleven testes on the left side but only six and four, respectively on the right. This gives a \( \chi^2 \) of 4.56 which is significant \((P < 0.05)\). A similar excess of pure gonads on the left side \((17:6)\) has been reported by Niekerk (1974) for human hermaphrodites. These findings suggest that the pool of cells from which the left gonad develops is smaller but it does not indicate which cells in the pool are the most critical in gonad development.

The distribution of the fetuses according to the classifications of both their gonads is given in Table 3. This shows that for the great majority both gonads are alike, so that in 73 of the hermaphrodites there is one class or less difference between gonads. In a further 18 there is a difference of two classes. In only 16 animals is the difference as great as three classes and in nine of these one gonad is distinctly ovarian while the other is testicular. In no case was a normal ovary accompanied by a normal testis. The table also emphasizes the greater number of testicular gonads (67) than ovarian (32) or balanced gonads (8).

DISCUSSION

In their series of 1439 inbred BALB/cWt day-15 fetuses (Beamer et al. 1978), 43 (3.0%) hermaphrodites were found, whereas only 14 (0.4%) were found amongst the 3310 young of our colony at weaning. Thus about 87% of the hermaphrodites ‘disappear’ during late fetal or postnatal development but there is no evidence of selective mortality during this period. We are confident that few if any overt hermaphrodites are missed at weaning because we examined them carefully. We have conducted extensive breeding experiments and have maintained a breeding colony of 60 pairs during the last decade, and have rarely encountered sterile animals and we would expect most hermaphrodites to be infertile. However, a few males with one small gonad have been observed and these could be sex chromosome mosaics. It seems probable that many of the gonadal defects observed in day-15 fetuses are sequestered during subsequent development. Thus deficiencies in the numbers of cords could be made up by greater coiling or the formation of additional branches of the remaining cords. Correction could develop towards male or female phenotypes, but the former may be more common.

We examine all BALB/cWt progeny at weaning and laparotomies are performed on suspected hermaphrodites. Most (65%) contain what appear to be an ovary and a testis whereas only 5% possess two ovotestes. Half of the remainder (15%) contain a normal ovary and an ovotestis and an equal number with testis and an ovotestis. These results resemble those summarized earlier by Whitten (1975) but are quite different from the fetal data.

It seems likely that the proportion and distribution of the male and female elements will determine whether a particular fetus will continue to develop as a hermaphrodite. In the present series it is suggested that those fetuses shown in
the left-hand box in Table 3 will develop as phenotypic females and those in the right-hand box as phenotypic males. Of the remainder, the two in the first row should exhibit bilateral ovotestes and the eight in rows five and six should become lateral hermaphrodites. The fates of the rest are more conjectural, but we have already postulated how 80% of missing hermaphrodites develop.

In 1976 Byskov & Saxen demonstrated that the ovarian rete induced meiosis in male germ cells *in vitro* and concluded that it produced a meiosis-inducing substance. Later Byskov (1978) observed meiosis in germ cells excluded from the testicular cords but near the connecting or extragonadal rete. She then postulated that even the male rete could induce meiosis but male germ cells were protected from meiosis induction when they were aggregated into cords. If this is the correct interpretation then such protection could be membrane mediated and it is tempting to speculate that both the clumping into cords and the protection from meiosis induction derive from the \( H-Y \) antigen (Ohno, 1976).

The meiosis-inducing substance could cause \( XY \) cells in a class 2 ovotestis to enter meiosis and possibly function as fertile ova, as suggested by the report of Evans, Ford & Lyon (1977). Such a possibility would support the conclusion of Evans *et al.* (1977) 'that the sex of the germ cell is not an autonomous property but is determined by the nature of the gonad in which it finds itself'. Thus, the use of meiosis at 14 days of gestation as a marker for female germ cells (McLaren *et al.* 1972) may not be valid. It is also difficult to understand the failure of these latter investigators to find sex vesicles or the pattern of incorporation of thymidine characteristic of \( XY \) cells in any of the 164 meiotic cells, unless by chance all of the cells they examined were female.

Beamer *et al.* (1978) postulated and Eicher *et al.* (1979) fully developed hypotheses of \( Y \) chromosome non-disjunction as the cause of the hermaphrodites, sex chromosome mosaics, and low sex ratio in BALB/cWt derived mice. In the livers of BALB/cWt fetal hermaphrodites the overall ratio of \( XO:XY \) cells was 2:1 and when \( XXY \) clones were present, they were a distinct minority. The excess of female \( XO \) cells contrasts with our findings that the hermaphrodite gonads were more testicular than ovarian, and suggest that \( XY \) cells have a more significant impact on gonad morphology than \( XO \) cells. A similar conclusion that \( XY \) cells have an advantage over \( XX \) cells was reached by Mullen & Whitten (1971) because of the very high per cent of aggregation chimeras derived from embryos of balanced genotypes. These results suggest that whenever the proportion of \( XY \) cells in a sex mosaic is greater than one third, perhaps 40%, the individual will develop into a male phenotype. The proportion of male cells may be critical in forming a lattice throughout the gonad. In a recent theoretical study, Whitten (1978) has shown that both major and minor components of a three-dimensional mosaic of two components form interlocking lattices with few if any isolated cells, even when the minor component comprises as little as one fourth of the total cells. Thus at 40%, \( XY \) cells could be linked throughout the gonad.
In conclusion, we have described a model of true hermaphroditism in BALB/cWt-derived mice. The incidence of hermaphroditism is sufficiently high and consistent to permit studies on the development of the gonads, accessory structures, and endocrine functions. These mice should also provide a valuable model for chromosome non-disjunction and, perhaps, also for clone selection.

We wish to acknowledge the excellent technical assistance of Stetson Carter, Lois Maltais and Anne Cleary, and the competent preparation of the manuscript by Barbara Dillon. This work was supported by NIH grants HD 04083, AM 17947 and BRS 05545 from Division of Research Resources to the Jackson Laboratory. The Jackson Laboratory is fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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(Received 23 October 1978, revised 7 March 1979)