Cytogenetic observations on XX/XY chimaeras and a reassessment of the evidence for germ cell chimaerism in heterosexual twin cattle and marmosets

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Summary. Testicular preparations were obtained from 7 bulls, twins of freemartins, and 1 male marmoset, all proved XX/XY chimaeras. X and Y sex chromosomes were confidently identified in nearly all the 87 spermatogonia at mitotic metaphase and 1052 primary spermatocytes at diakinesis–metaphase examined: no cell was identified as containing two X chromosomes. The germ cell chimaerism previously reported in these species is therefore not confirmed. Cultures grown from presumptive somatic cells in the testes of two of the bulls yielded 248 identifiable mitotic spreads, all XY-type; cultures from the gonads of their freemartin twins yielded 442 mitotic spreads, all XX-type. Direct preparations from one freemartin gonad, however, yielded 3 XY mitotic spreads out of 18 examined. The conflicting evidence concerning germ cell chimaerism in cattle and marmosets is discussed, particularly in relation to reports of XX/XY bulls that have sired a great excess of daughters. The possibility that XX germ cells contributed to the functional spermatozoa of these bulls is not favoured by present information, but is not excluded.

Introduction

The primordial germ cells of mammals arise in the yolk sac and migrate to the gonadal ridges during early embryogenesis (Witschi, 1948; Mintz & Russell, 1957). Haemopoietic chimaerism in twin cattle has long been known (Owen, 1945) and when Ohno, Trujillo, Stenius, Christian & Teplitz (1962) found XX cells at mitosis in the testes of new born bull calves, twins of females, they suggested that germ cells might also be exchanged. Ohno & Gropp (1965) found presumptive germ cells in the blood vessels of triplet fetuses at a time when migration of germ cells was in progress, as indicated by the presence of alkaline phosphatase-positive cells in the dorsal mesentery. The fetuses were thought to be at approximately the 30th day of gestation and the finding of XX/XY chimaerism in direct preparations from liver proved that vascular connections had already been established. These observations were supported by Jost & Prépín (1966) and show that opportunity for the transport of germ cells in the circulation from one fetus to the gonads of another exists, at least in some fetal pairs. Teplitz, Moon & Basrur (1967) later claimed to have seen XX germ cells, including primary spermatocytes at diakinesis–metaphase, in the testes of three XX/XY bull calves aged 2, 10 and 11 months.

Exchange of cells between heterosexual cattle twins *in utero* has recently been placed in a new light by the discovery that ligation of blood vessels between pairs of fetuses between 37 and 45 days of gestation does not prevent the development of cellular chimaerism yet apparently suppresses the factor that normally brings about intersexual development of the female twin, as judged by observations on ovaries and Müllerian tubes at 60–61 days of gestation (Vigier, Locatelli, Prépín, du Mesnil du Buisson & Jost, 1976).

Haemopoietic chimaerism is also common in marmoset monkeys. Benirschke & Brownhill (1963) searched for germ cell chimaerism and reported finding XX spermatogonia or XX spermatocytes, or

both, in the testes of single males of three species. These observations were supported by Egozcue, Perkins & Hagemenas (1968, 1969), and in an extensive study of meiosis in marmosets of both sexes Hampton (1970) claimed to have seen XY oocytes in some females and XX spermatocytes in some males.

Our interest in the nature and fate of the germ cells in chimaeric mammals has been renewed by finding that an exceptional son of a fertile XX/XY female mouse had received his Y chromosome from his mother, implying that a Y-bearing oocyte had survived to functional maturity (Ford *et al.*, 1975). We now report observations on testicular preparations from 7 XX/XY bulls born twins to freemartins and 1 male XX/XY marmoset.

Materials and Methods

Fresh testicular material was obtained at slaughter from 7 bulls, aged 44–56 weeks, each born as a twin to a freemartin. The single male marmoset was one of a group of 6 used for a study of chimaerism in the lymphomyeloid complex (Ford, 1966a). It was obtained from a dealer and was of unknown age. It was allegedly of the species *Saguinus (Oedipomidas) oedipus* (the cotton-top marmoset) and conformed with descriptions of this species. Preparations from blood cultures established that all 8 animals were XX/XY chimaeras.

The mitotic chromosomes of cattle (*Bos taurus*; $2n = 60$) have been figured most recently by Short *et al.* (1969) and Vigier, Prépın & Jost (1973). Popescu (1971) has given an account of meiosis. Males have 29 pairs of acrocentric autosomes, a long sub-metacentric X, and a metacentric Y about equal in length to the shortest autosomes. The chromosomes of *S. oedipus* ($2n = 46$) have been illustrated by Benirschke & Brownhill (1962). The X chromosome is sub-metacentric and not easily distinguished from a group of 9 autosomal pairs; there are therefore 20 chromosomes of this group in the female and 19 in the male. The Y chromosome is shown as a small metacentric chromosome, clearly shorter than any autosome, but in our experience may be longer and not readily distinguishable from the 6 short acrocentric autosomes.

Both testes were removed from the bulls immediately after slaughter and cut longitudinally. Small samples of tubules were taken from several positions on the cut surface to ensure good representation of the whole organ. The samples were combined in Hanks's balanced salt solution for transport to the laboratory where chromosome preparations were made by an air-drying method (Evans, Breckon & Ford, 1964) which involves teasing the tubules in a mildly hypotonic fluid as the first step. Few spermatogonia in mitosis were present in the initial preparations. More were recovered by immersing the residual mass of tubules, after teasing, in 0.25% trypsin (Difco, 1:250) in phosphate-buffered saline until the tubules appeared translucent (about 6–8 min), diluting with Hanks's solution to slow down the enzymatic action, and then aspirating gently with a Pasteur pipette. The cells are easily damaged after exposure to trypsin but it was found that damage could be lessened by reducing the period in hypotonic fluid and fixing the cells as a pellet rather than as a suspension. Testicular preparations from the marmoset were made by our standard procedure (Evans *et al.*, 1964).

The preparations were searched systematically and the chromosomes (or bivalents) in all technically satisfactory spermatogonia and spermatocytes at suitable stages were counted and examined. A few cells with less than the normal number of chromosomes or bivalents were assumed to have been damaged during preparation and were disregarded.

Preparations of gonadal tissue from two of the freemartins were made by a direct method (Ford, 1966b). Monolayer cultures were set up from gonadal explants from two of the twin pairs and harvested 3–9 days later after one sub-culture. Air-dried preparations were made by the same basic procedure. Phytohaemagglutinin-stimulated cultures of peripheral blood were set up and harvested by an adaptation of the method of Moorhead, Nowell, Mellman, Battips & Hungerford (1960).

Results

The observations on 1241 cells are summarized in Table 1. The X and Y were identified as individual chromosomes in spermatogonia and secondary spermatocytes of the bulls, and (mainly) as the sex

bivalent in the primary spermatocytes of both species. Only the Y chromosome was uniquely identifiable in spermatogonial mitotic spreads of the marmoset. In a small minority of cells of poorer technical quality X and Y were not identified with assurance but no germ cell in either species was confidently identified as XX. The X and Y chromosomes in a few primary spermatocytes of both species were present as univalents. This is a common phenomenon in mammalian species and no special note was taken of it. No hyperdiploid counts were recorded, so the results provide no evidence of non-disjunction either during spermatogonial mitosis or at first meiotic anaphase.

Table 1. Cells identified in the testes of XX/XY chimaeras of cattle and marmoset

Animal no.	Breed*		Spermatocytes							Lymphocytes	
			Spermatogonia		Primary		Secondary				
	Sire	Dam	XX	XY	XX	XY	X	Y	XX	XY	
<i>Bull twins</i>											
B1	F	F×A	0	29	0	286	—	—	231	19	
B2	A	A	0	5	0	131	0	2	112	292	
B3	A	A	0	14	0	62	—	—	95	96	
B4	Not known		—	—	0	138	—	—	196	304	
B5	Not known		—	—	0	101	—	—	219	20	
B6	Not known		0	11	0	150	52	48	176	174	
B7	Not known		0	1	0	45	—	—	224	76	
<i>Marmoset</i>											
O5			0	27	0	139	—	—	33	3	
Total cells			0	87	0	1052	52	50	1286	984	

* F = Friesian, A = Ayrshire.

The normal sex bivalent at diakinesis–metaphase in both species is usually identifiable at first glance as a straight or slightly curved, rod-like structure with a subterminal constriction, representing the longer X associated terminally with the shorter Y (Pl. 1, Figs 1 and 3). However, it is not infrequently curved back on itself (Pl. 1, Fig. 2) and may give a ring-like appearance. Our experience has shown that this can sometimes lead to initial misidentification as a symmetrical autosomal bivalent.

Eighteen technically acceptable mitotic spreads were found in the direct preparations from one of the freemartin gonads: 3 clearly contained an X and a Y chromosome and the remainder were normal XX cells. However, the general appearance of the chromosomes was very similar in the XX and XY spreads and quite different from the spermatogonial chromosomes we have observed in any other mammalian species.

The 248 mitotic spreads in preparations from the cultures of ovarian tissue were all XX; the 442 from the testicular cultures were all XY.

Discussion

Our failure to find XX germ cells in 87 spermatogonia and 1052 primary spermatocytes of 7 bulls and 1 marmoset, all proved XX/XY chimaeras, accords with that of Short *et al.* (1969) who studied testicular preparations from an XX/XY bull, but does not agree with most previous reports (see 'Introduction'). However, we find that none of the published illustrations of alleged XX spermatocytes is convincing. All include a body which could be an XY bivalent curved back on itself in the manner we have described above to give a spurious suggestion of symmetry. The XX mitotic spreads and karyotypes illustrated by Ohno *et al.* (1962) and Benirschke & Brownhill (1963), on the other hand, are decisive, though we are not satisfied that they were necessarily from spermatogonia. The clusters of spermatogonia in mitosis characteristic of the spermatogenic wave are liable to be broken up by methods involving teasing to release free cells, but may persist in squash preparations of single

tubules: the identification of XX cells in such a cluster would constitute effective proof that they were indeed spermatogonia.

Weiss & Hoffmann (1969) reported the presence of small numbers of mitotic cells of contrary type in cultures from the gonads of heterosexual twin cattle up to 3 months old, but not older. Their assumption that these were germ cells has been questioned by Tarkowski (1970) and Vigier *et al.* (1973).

The remaining evidence from gonadal cultures is conflicting. Kanagawa, Muramoto, Kawata & Ishikawa (1965a) and P. R. Wilkes, I. B. Munro & W. V. S. Wijeratne (unpublished) have reported up to 8% mitotic cells of contrary type. Others could find mitotic cells only of the corresponding type, XX from freemartin gonads and XY from the testes of their bull twins (Goodfellow, Strong & Stewart, 1965; Kanagawa, Kawata, Ishikawa, Muramoto & Ohno, 1965b; Dunn, Kenney, Stone & Bendel, 1968a; Short *et al.*, 1969; Kieffer & Sorensen, 1971; Vigier *et al.*, 1973). Our own observations agree with the reports of the latter group. The reason for this disparity is not understood. Age of the animals at the time the cultures were set up cannot be solely responsible, since Vigier *et al.* (1973) obtained a negative result from three fetuses aged 60–70 days, whereas Wilkes and his colleagues claim positive cultures from 5 out of 8 freemartins approximately 2 years old. Differences in the detail of the culture methods might be involved, immigrant cells being favoured in some circumstances, suppressed in others.

Our observation of a small proportion of XY cells in direct preparations from gonadal tissue of a 10-month-old freemartin is at present unconfirmed but is nevertheless indirect support for those who claim to have found XY cells in cultures of freemartin gonads. Obvious suggestions are that they were cells derived from the XY component of the lymphomyeloid complex that had been transported in the blood (see Barnes & Kruschov, 1968) and that they could have arrived subsequent to birth.

Chimaeras that are presumed to have arisen naturally by vascular exchange *in utero* between fetuses of different sex have also been recorded in man (Race & Sanger, 1975) and in sheep, goat, horse and pig (Marcum, 1974, review). Nothing, however, appears to be known about the status of the germ cells of such animals. The germ cells of artificially constructed chimaeras in the mouse, on the other hand, have been studied extensively. The weight to be placed on the evidence of analogy these studies provide rests on the strong case that can now be made for uniformity of sex chromosome activity in both somatic and germ cells of mammals generally.

Four aspects of this activity allow general statements. (1) The Y chromosome carries the primary genetic determinant(s) of maleness (see reviews by Ford, 1970; Mittwoch, 1973). It has recently been suggested that the H-Y antigen may represent the immunological expression of the agent concerned (Wachtel, Ohno, Koo & Boyse, 1975; Ohno, Christian, Wachtel & Koo, 1976). (2) One of the two X chromosomes is genetically inactive in the female soma (Lyon, 1972, review). (3) The single X chromosome in the germ cells of normal XY males is genetically inactivated at an early point in spermatogenesis (Lifschytz & Lindsley, 1972; Lifschytz, 1972). (4) XX and XXY males are sterile and lack germ cells at maturity though they are present in fetal life and in prepubertal human males (Short, 1972, review; de la Chapelle, 1972). Presumptively the second (active?) X chromosome disturbs metabolism in XX and XXY germ cells when they are present in a testicular environment and leads to their elimination.

Two further statements are supported at present by more limited evidence. (5) Both X chromosomes are genetically active in mouse oocytes (Epstein, 1969) and human oocytes (Gartler, Liskay, Campbell, Sparkes & Grant, 1972). (6) XO germ cells, though not fully efficient, may survive in testis and ovary (Short, 1972, review). XO oocytes in the human female normally have all disappeared by the time of birth or shortly thereafter though there are 4 cases on record of fertile, apparently non-mosaic 45,X females (see Lajborek-Czyz, 1976). Sterile, 'streak' gonads typically occupy the place of ovaries in 45,X adults (Carr, Haggard & Hart, 1968). The XO mouse is, by contrast, normally fertile though its supply of oocytes is exhausted long before those of its XX litter mates (Lyon & Hawker, 1973). This particular difference between species may therefore be regarded as one of degree rather than of fundamental behaviour.

A final statement rests on heterogeneous evidence. (7) Genetic activity of the sex chromosomes in germ cells is predicated by certain mosaic conditions that imply a strong selective advantage of one

PLATE 1

Fig. 1. Bull primary spermatocyte at metaphase with a normal XY bivalent (arrowed). Toluidine blue, $\times 1234$.

Fig. 2. Bull primary spermatocyte at diakinesis. The XY bivalent (arrowed) is folded back on itself. Toluidine blue, $\times 1234$.

Fig. 3. Marmoset primary spermatocyte at diakinesis showing a normal XY bivalent (arrowed). Lactic-acetic-orcein, $\times 1234$.

(Facing p. 28)

genotype over another. The spermatocytes of human XYY males are almost invariably XY (Melnick, Thompson, Rucci, Vanasek & Hayes, 1969; Hultén, 1970; Evans, Ford, Chaganti, Blank & Hunter, 1970); the XYY germ cell in the mouse testis is apparently favoured over the XO germ cell (Evans, Ford & Searle, 1969); in some 40,XX,Sxr/+ male mice active spermatogenesis takes place in some tubules but involves (presumptively XO) germ cells with 39 chromosomes (Lyon, 1974); males of the species *Microtus oregoni* have an XY soma and OY germ cells, females have an XO soma and XX germ cells (Ohno, Jainchill & Stenius, 1963; Ohno, 1964); in the marsupial family Peramelidae the soma is, in varying degree, XO in both sexes though the germ cells of ovary and testis retain their normal XX and XY constitutions (Hayman & Martin, 1974, review).

Considering now the artificial chimaeric mice, obtained by manipulation of preimplantation embryos, the elimination of germ cells from the testes of XX males mentioned in (4) above suggests that XX germ cells lying side-by-side with XY germ cells in the testes of XX/XY males would be lost before sexual maturity if not before birth. Direct observations agree: XX germ cells have not been identified either at meiosis or mitosis in the testes of adult XX/XY males although over 1000 spermatogonia and spermatocytes have been examined in preparations from 11 different animals (Mystkowska & Tarkowski, 1968; Ford *et al.*, 1975). These observations are complemented by studies which indicate that XX germ cells in the testes of XX/XY chimaeras enter meiosis during fetal life but are rapidly eliminated (see McLaren, 1972).

The artificial chimaeric XX/XY mice are effectively equivalent to the rare XX/XY chimaeras in cattle that originate at or shortly after syngamy, either by dispermic fertilization of an ovum with two female pronuclei or by fusion of two preimplantation embryos (McFeely, Hare & Biggers, 1967; Dunn, Kenney & Lein, 1968b; Rieck, 1973, 1976). Chimaerism in such animals is widespread through all the tissues of the body and they have been variously termed 'primary' chimaeras (Tarkowski, 1970), 'whole-body' chimaeras (Benirschke, 1972), 'autonomous' chimaeras (Rieck, 1973) and 'dispermic' chimaeras (Race & Sanger, 1975). By analogy with the mouse (see Ford *et al.*, 1975), primary chimaeras in cattle would be expected to range in phenotype from normal fertile males, through sterile males, intersexes, and sterile females to normal fertile females. All investigated so far have been intersexes (Rieck, 1976), but this may only reflect choice of material for study and the identification of members of the two extreme classes would depend on fortuitous discovery. Mäkinen (1974) reported a fertile XX/XY bull, born a singleton, which we think may have been a primary chimaera. If germ cells of type contrary to the phenotype are found not to survive in the gonads of fertile primary XX/XY chimaeras (as in the artificial male XX/XY mouse chimaeras), despite normal migration within a single individual during fetal life, survival in secondary XX/XY chimaeras after transport in the circulation from one fetus to another would be very unlikely indeed.

A small proportion of the artificial XX/XY chimaeric mice develop into fertile females. Indirect evidence for a functional Y-bearing oocyte in one such chimaera (Ford *et al.*, 1975) has recently been supported by the finding of an oocyte at diakinesis in a direct preparation from the ovaries of another (E. P. Evans, unpublished observation). Burgoyne & Biggers (1976), however, present experimental evidence favouring the probability that ova derived from XY oocytes would be deficient in products of the single X chromosome and give embryos that would have a reduced probability of survival in competition with embryos ultimately derived from XX oocytes.

Hampton (1970) claimed two probable XY oocytes in preparations from 2 of 12 female marmosets (not otherwise examined for chimaerism) and illustrates both of them. We believe the bodies she thought were XY bivalents could have been autosomal bivalents with single terminal or sub-terminal chiasmata. Should XY oocytes nevertheless occur in chimaeric female marmosets and be capable of maturing into functional ova, a modified secondary sex ratio with an increased proportion of males would be expected (Benirschke & Brownhill, 1963). There are records, from 9 different colonies, of 1505 males to 1236 females at birth or weaning (Hampton, 1970; Gengozian, 1971; and unpublished data of J. Ingram, B. M. Levy, I. R. Phillips and P. Purton, T. B. Poole, and J. Turton). The 9 sets of data, though drawn from 4 different species, are homogeneous ($\chi^2_8 = 7.44$, $P \sim 0.5$), and when combined give a ratio of 121.8 ± 2.11 males to 100 females. The deviation from a 1:1 ratio is highly significant and in the direction expected if the functional oocyte populations included some that were XY. A first direct test shows no significant difference between the sex ratios of progenies

of XX/XY chimaeric females and XX (or XX/XX) females (Gengozian, 1971). The numbers of offspring, however, were not large enough to exclude the possibility of a substantial real difference.

Three XX/XY bulls, twins of freemartins, are reported to have sired a marked excess of daughters. Dunn *et al.* (1968a) stated that the progeny of one such bull consisted of 34 daughters and 14 sons, a highly significant deviation from a 1:1 ratio ($\chi^2_1 = 8.33$, $P < 0.01$). Lojda (1972) reported a ratio of 100 daughters to 68 sons in the progeny of another bull. The actual numbers were not mentioned but comparison with the combined progenies of other bulls at the same A.I. centre gave $\chi^2_1 = 6.91$ ($P < 0.01$). The third bull is stated to have sired 116 daughters and only 32 sons (de Giovanni, Popescu & Succi, 1976). These reports would find an immediate explanation if, contrary to the general evidence, XX germ cells not only succeeded in reaching and entering the future testes during fetal life, but survived in the adult to produce functional spermatozoa. But this could only be reconciled with our observations, and the fact that XY/XY bulls have transmitted red cell antigens to progeny only from one of the two cell lines (Stone, Berman, Tyler & Irwin, 1960; W. J. Thomas & D. S. Ross, unpublished results), if such survival were a highly labile property, occurring in some but not the majority of XX/XY bulls.

Some XX/XY bulls, twins of freemartins, are sterile (Gerneke, 1969) or of reduced fertility (Dunn *et al.*, 1968a; Stafford, 1972). The exceptional bull of de Giovanni and her colleagues was also of somewhat reduced fertility (a 60–90 day non-return rate of 48.9 in one period compared with a mean of 62 for other bulls at the same A.I. centre). However, even if all the excess failure of pregnancy (13.2%) had been due to selective death of male fetuses it would not account for the deviation from a 1:1 sex ratio (which would have required selective death of 36.2% of male fetuses). The aberrant sex ratio is therefore independent of lowered fertility.

The reports of biased sex-ratio just discussed are anecdotal. One systematic investigation (Milk Marketing Board, 1970) of 8 Friesian bulls, twins of freemartins, provided no evidence of heterogeneity in the sex ratio of their progenies ($\chi^2_2 = 3.26$, $P \sim 0.8$). Nevertheless, a disturbance of the sex ratio of the magnitude reported by de Giovanni and her colleagues certainly cannot be dismissed as an extreme result of random sampling though the less extreme cases of Dunn *et al.* and Lojda might be explained in this way. The likelihood of getting such a gross disproportion as 116:32 (or one more extreme) by chance when the expectation is 1:1 is vanishingly small (0.35×10^{-23}).

Mutational explanations of a grossly unequal sex ratio would have no evident relationship to chimaerism and are therefore effectively excluded, except for the unique case, even if an event early in development that produced a gonosomal mosaic were postulated. A mutation on the X chromosome of the types at present known in mammals might reduce (through incompletely penetrant dominant lethality), but not raise, the proportion of daughters. Mutations on the Y chromosome that affected adversely its meiotic transmission, its mitotic efficiency in cleavage divisions, the maturation of Y-bearing spermatids into functional spermatozoa, or the capacity of Y-bearing spermatozoa to traverse the genital tract of the cow, are all formal possibilities. All would increase the proportion of daughters but they are without precedent in the cytogenetic literature, so far as we are aware. Further formal possibilities remain, though none appears to be really plausible.

Recording errors have also been suggested as a possible explanation of aberrant sex ratios (D. L. Pollock, personal communication). However, any bias that may arise in this way should not operate differentially between one bull and another unless the semen from individual bulls is used preferentially for the insemination of particular herds.

If participation of XX germ cells in spermatogenesis is the explanation for the great excess of daughters of de Giovanni's bull, the estimate of their frequency would be 84/148 (57%). Proof of functional spermatozoa derived from XX germ cells, however, would still depend on the demonstration of transmission to progeny of a genetic or chromosomal marker that could only have come from the XX component. A new study of the inheritance of red-cell antigens by the progenies of the exceptional bulls may offer the best opportunity. It is hoped that any future investigation will include randomly selected chimaeric and control sires and that particular attention will be given to the possibilities of error in recording the sexes of their offspring.

We conclude that in certain female secondary XX/XY chimaeras (marmosets) the survival of some XY germ cells to become functional oocytes leading to an excess of male offspring is not

excluded by present evidence. In male secondary XX/XY chimaeras, were it not for the distorted sex ratios in the progenies of some bulls, the weight of the evidence would favour at most short survival of contrary-type germ cells in the gonads, if they reach them at all. But if there is only a remote possibility that the deviations from normal sex ratio have a true biological basis, and that the mechanism responsible could be understood and exploited, the potential economic value of bulls in dairy breeds that sire an excess of heifer calves should fully justify further investigation of the problem.

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