Observations on CBA-p/CBA-T6T6 mouse chimeras

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A considerable amount of information bearing upon mouse chimeras obtained experimentally by fusion of cleaving eggs has already been accumulated (Tarkowski, 1961, 1963, 1964a, b, 1965; Mintz, 1962a, b, 1964a, b, 1965a, b, 1967). Direct and indisputable proofs of the chimerism of the individuals produced were obtained by establishing that two types of pigment cells were present in the outer layer of the retina (Tarkowski, 1963, 1964a) and in the coat (Mintz, 1965a, b, 1967). The cell composition of other organs and tissues has not as yet been investigated. True hermaphroditism in some of the animals obtained was also accepted as an indirect proof of chimerism (Tarkowski, 1961, 1963, 1964b; Mintz, 1965b) but the postulate of these individuals being six-chromosome chimeras has not as yet been directly confirmed by karyological research.

The aim of the present study was to examine more exactly both the participation and distribution of the cells originating from the two eggs in the animals produced and to ascertain whether or not there was any regularity in this respect. A further aim of the study was to supply new data on the sex ratio in chimeras and to check the hypothesis put forward by Tarkowski (1961, 1963, 1964b), according to which animals resulting from the fusion of a genetically female egg with a male egg may be either hermaphrodites or phenotypically normal males. This in turn is connected with the fate of the germ cells of XX constitution in such males and whether these cells undergo normal spermatogenesis. Only karyological research on chimeras themselves and genetic research on their progeny can provide answers to these last questions.

Realization of the above aims necessitated the use of parental strains characterized by: (1) differences in pigmentation, (2) differences in karyotype and (3) presenting a possibility of discovering gametes of both genetic types by means of breeding tests. The above conditions are fully met by the CBA-T6T6/CBA-p combination used in the present study. Individuals of CBA/H-T6T6 strain, established by Dr Mary Lyon, are characterized by the presence of two very small chromosomes which makes it possible to distinguish their

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cells from cells of a normal karyotype (Ford et al. 1956; Ford, 1966a). The mice of this strain are darkly pigmented of the agouti type. The CBA-p strain (pink-eyed dilution) is characterized by the normal karyotype, light-coloured coat and slight amount of pigment in the outer layer of retina. The hybrids obtained from the cross between the individuals of these two strains display pigmentation of CBA-T6T6 agouti parents.

After this paper had been submitted for publication new evidence on the chimeric nature of mice derived from fused embryos was provided by studies of Mintz & Baker (1967) on isocitrate dehydrogenase isozymes in skeletal muscle, cardiac muscle, liver, lung, kidney and spleen.

MATERIALS AND METHODS

Spontaneously ovulating females of the inbred CBA-p and CBA-T6T6 strains mated with males of the same strain were used as donors. Dissection was carried out between the hours of 11.00–19.00 on the third day after copulation, taking the day on which the vaginal plug was found as the first. The eggs obtained were most frequently in the eight-cell stage, but some younger and some slightly older stages were also encountered.

The CBA-p and CBA-T6T6 females were killed simultaneously and the eggs washed out with Ringer solution containing 0.1% bovine plasma albumin fraction V (Armour) into two separate watchglasses. In order to remove the zonae pellucidae the eggs were first treated with pronase (Mintz, 1962c) and the thinned membranes finally removed in Ringer solution by sucking the eggs into a micropipette several times. Cultures were set up in drops of the medium elaborated by Brinster (1963, 1965) placed in Petri dishes filled with liquid paraffin (Tarkowski, 1961, 1963, 1965). One egg from one donor was first placed in each drop, then an egg from the second female was added. The two eggs were brought into contact with a glass rod, then the dish was left for 10–15 min on a warmed plate at a temperature of 37 °C. This procedure was based on the observation made by Mintz (1962a, b, 1964b) that when manipulations are carried out at physiological temperature the eggs put into contact tend to adhere strongly to each other and need not to be squeezed together by special means. After checking the fusion of the eggs the dish was placed in a desiccating chamber gassed with alveolar air and incubated at 37 °C.

Four groups of experiments can be distinguished depending on the duration of culture and on the type of transplantation performed:

I. Culture period 5–7 h, transplantation to oviduct on first day of pseudopregnancy or to uterus on third day.

II. Culture period 17–24 h, transplantation to oviduct on first day.

III. Culture period 17–30 h, transplantation to uterus on third day.

IV. Culture period 36–40 h, transplantation to uterus during morning hours of fourth day.
Mouse chimeras

The recipients were albino females of the A inbred strain, mated with vasectomized males of the same strain. All the females were kept up to the time they gave birth, which took place 20 days after mating.

After the chimeras produced had reached sexual maturity they were mated with CBA-p males or females. The sex of their progeny was determined by dissection soon after birth and the type of pigmentation of each individual recorded. After accumulating the relevant data on progeny, i.e. after a period of 6–8 months, the chimeras were killed in order to carry out histological and karyological investigations.

The animals were given an injection of water solution of Colcemid (CIBA) (0.004 mg/g of body weight) 1 h before they were killed. Karyological examination was made of bone marrow, germ cells in the testes (primary spermatocytes at diakinesis), and of the somatic tissue of the ovotestis of the only available hermaphrodite. The bone marrow was taken from both the humeral and femoral bones and the air-dried preparations made according to the procedure described by Ford (1966a). Meiotic preparations from testes were made by the method of Evans, Breckon & Ford (1964). After staining with lactic-acetic-orcein or with Giemsa the preparations were mounted in DPX. About 200 metaphase plates from the bone marrow and 100 plates from the testes of each animal were identified. Only those plates in which it proved possible to distinguish and count all 40 chromosomes in the case of mitosis and all 20 bivalents in the case of meiosis were taken into consideration. Plates in which it was impossible to count all chromosomes, even if these were most certainly CBA-T6T6 plates, as borne out by the presence of markers, were not scored.

Eyes, parts of the ovaries and testes of adults and eyes and gonads of newborn animals which died soon after birth were fixed in Bouin’s fluid for histological investigations. In the case of the hermaphrodite, the whole reproductive tract was fixed and sectioned. Sections were cut at 6 μ and stained with Ehrlich’s haematoxylin and eosin.

RESULTS

1. General remarks

Transplantation of 195 fused eggs to 48 recipients resulted in seven females (14.6 %) giving birth to a total number of 14 young, 7.2 % of the transplanted eggs. Four of the young mice failed to survive the first 5 days after birth (found dead or bitten by the mothers), one young mouse died at the age of 18 days and nine individuals survived and attained sexual maturity. These data, and Mintz’s observations also (1965a, b, 1967), prove that the viability of chimeras is not reduced and that the high mortality rate among chimeras soon after birth observed by Tarkowski (1961) could not be connected with their mosaic constitution.

In comparison with the earlier experiments made by one of us (Tarkowski, 1961) the present series of experiments is characterized by very low effectiveness, expressed both in the small number of females giving birth and in the small
number of mice born from the transplanted eggs. With the exception of a few experiments in which eggs cultured for 5–7 h were transplanted to the uterus on the third day of pseudopregnancy, at least one mouse was born in each series of experiments (see Materials and Methods). The best results, although far from satisfactory, were obtained in series II and IV. We consider the unsatisfactory conditions for culturing the eggs as the main cause of failure. After a period of 17–40 h in culture many of the eggs looked unhealthy: the degree of rounding-out was slight and the rate of mitotic divisions markedly retarded. This resulted in a low percentage of blastocysts being obtained from cultures even after a period of 36–40 h. In no case were young born when eggs not appearing completely healthy were transplanted. Poor development was most probably due not to the medium itself, but to the culture method under liquid paraffin. Evidence of this is provided by the fact that similar stages cultured in the Brinster medium in corked tubes, in which pH of the medium was constant, developed far better. It is possible that when the dish is exposed to atmospheric air, CO₂ escapes from the liquid paraffin to an extent which results in pH in the drops of medium not returning to the proper level for a long time even after the dish has been placed in the desiccating chamber. The reason for the poor results may also lie in the fact that we used eggs in the early eight-cell stage for the experiments. When older eight-cell eggs and later stages of the LAB Grey strain (Tarkowski, 1961) and the A strain (Mystkowska and Tarkowski, unpublished results) were cultured in an identical way they were found to develop completely normally.

2. Pigmentation

I. New-born animals

Co-occurrence of CBA-p and CBA-T6T6 cells was found in the outer layer of retina in three new-born mice which died soon after birth (predominance of CBA-T6T6 cells in two of the mice, and predominance of CBA-p in the third).

II. Adult animals

A. Coat colour. The coat colour of adult chimeras exhibits great variability. In different individuals both the participation and distribution of hair characteristic of CBA-p and CBA-T6T6 differs greatly (Plate 1, figs. 1–8; Plate 2, figs. 10, 11). Exact evaluation of the participation in the coat of hair of these types is practically impossible. The main difficulty consists in both types of hair occurring together nearly everywhere in the body, and, consequently, in the ‘light’ and ‘dark’ areas not being clearly demarcated. The term ‘area’ should be understood, therefore, as a region in which hair of one type predominates. It is also impossible to exclude the possibility, although the relevant investigations were not carried out here, that hairs of mixed pigmentation also occur in the fur of chimeras, which in effect would produce a generally lighter colour of coat. For the above reasons we limited ourselves to arranging the animals in
Figs. 1–8. Flat skins of adult animals. Apart from Fig. 1 and Fig. 8 which show fur of an all-pink-eyed and all-agouti animal respectively, the remaining demonstrate pigmentation mosaicism. The figures are arranged according to the increasing participation in the coat of agouti hair (CBA-T6T6). Numbers of figures correspond to numbers of particular animals used throughout the text. Reduced size.
order of increasingly dark colour of coat, i.e. of the increasing participation of agouti (CBA-T6T6) hair. The numeration of animals adopted throughout the text and in Plate 1 is in accordance with this arrangement.

Animal no. 1 is of uniform colour of CBA-p type, animals nos. 2 and 3 have a light-coloured coat with slight admixture of dark areas, animals nos. 4 and 5, coats with a generally intermediate tone, against which it is possible to distinguish lighter areas, animals nos. 6 and 7, a dark coat (although the general tone is markedly lighter than in CBA-T6T6 individuals) with lighter spots, and animals nos. 8 and 9 are of uniform colour characteristic of CBA-T6T6 mice. Of the three animals with uniform colour of coat it was only in the case of animal no. 8 that cells of the second type were found in the eyes and bone marrow (see below). In the light of these observations the possibility cannot be ruled out that single CBA-p hairs do not occur at all in this animal’s coat, or, speaking generally, that the skin was not populated by CBA-p melanocytes. In any case in this particular animal chimerism was not expressed at all in the coat, but was established after examination of the outer layer of the retina and in particular as the result of karyological examination of the bone marrow.

The size, shape and distribution of dark and light areas are different in each of the mottled chimeras and we were not able to note the existence of a definite and constant pattern of pigmentation. The animals are mottled over the whole of the body, including the tail, on which bands of light and dark pigmentation can be seen particularly clearly (Plate 2, figs. 9–11).

Plate 2

Fig. 9. Chimeras nos 5 and 7 at the age of 10 days. The mottled character of skin pigmentation and light and dark bands on the tail are already visible. Slightly reduced size.

Fig. 10. Chimera no. 3 (hermaphrodite) together with CBA-p and CBA-T6T6 animals. Mosaicism of coat pigmentation is not so well seen as in the flat skin (cf. with Fig. 3, Plate 1). Bands of differing pigmentation are, however, clearly seen on the tail. Reduced size.

Fig. 11. Same hermaphrodite animal as in Fig. 10, together with a CBA-T6T6 individual. Pigmentation mosaicism clearly visible both in the coat and on the tail. Reduced size.

Fig. 12. A ‘mixed’ litter composed of 3 CBA-p and 5 hybrid young born by a CBA-p female mated with the chimeric male no. 4. Reduced size.

Fig. 13. Eye-ball of chimera no. 2, viewed from internal hemisphere. Pigmentation mosaicism clearly visible macroscopically. × 10.

Figs. 14 and 15. Reproductive tract of the hermaphrodite (chimera no. 3) seen from dorsal side (Fig. 14) and from ventral side (Fig. 15). Gonads removed. Left side of the reproductive tract typically masculine, right side intersexual—presence of both Müllnerian and Wolffian ducts clearly manifests itself externally, especially in the caudal region. Seminal vesicles developed on both sides. × 1-5.

Fig. 16. Cross-section through the testis of a 5-day old chimera showing oocytes (arrows) inside sex cords. × 60.

Figs. 17 and 18. High power magnifications of fragments of testis shown in Fig. 16. Fig. 17. Typical sex cords containing growing oocytes. × 300. Fig. 18. Section through another sex cord containing an oocyte together with two typical pre-spermatogonia (arrows). × 500.
B. Pigmentation of the outer layer of the retina. The basic differences in pigmentation of CBA-p and CBA-T6T6 cells make it possible to determine mosaicism of the outer layer of the retina and of the choroid even macroscopically after taking out the eyeball (Plate 2, fig. 13). Two individuals had an outer layer of the retina composed solely of one type of cells: no. 1—CBA-p, no. 9—CBA-T6T6. In the other animals (nos. 2-8) this layer was found to possess a mosaic constitution, the proportion of the two types of cells, as in the coat, differing in different individuals. Although light- and dark-pigmented parts can be easily distinguished, it is practically impossible, even on histological sections, to assess quantitatively the participation of the two types of cells. Approximate assessment agrees satisfactorily, however, with assessment on the basis of pigmentation of coat. Except for chimera no. 8, in which only a few small groups of CBA-p cells were found against a uniform background of CBA-T6T6, in the remaining animals there is agreement between the type of pigmentation (mono-colour versus bicolour) in the outer layer of the retina and in the coat. These data indicate that assessment of chimerism of new-born mice made only on the basis of the composition of the outer layer of the retina (data from this paper and observations made by Tarkowski, 1963, 1964a) is very reliable and is a good 'indicator' of the chimerism of the whole body.

3. Sex ratio among chimeras

Data on the sex of chimeras obtained from fusion of CBA-p and CBA-T6T6 eggs, and forming the subject of the present paper, and also similar data from unpublished studies carried out by one of us (E.M.) on chimeras obtained from fusion of eggs of A strain, are given in Table 1. For purposes of comparison data from the paper of Tarkowski (1963) are also included. Marked prepon-
derance of males over hermaphrodites and females was observed in all three series of experiments. The total number of 40 chimeras is sufficiently high to permit accepting that the numerical relations observed deviate significantly from the expected ratio of 25:50:25. Combined frequency of occurrence of males and hermaphrodites fluctuates around 75%, and of females around 25%, which gives important support to Tarkowski's suggestion based on a far smaller sample (Tarkowski, 1961, 1963, 1964) that some of the males are in fact sex chimeras. Direct karyological proofs bearing out this assumption are presented below.

Table 1. Sex ratio among mouse chimeras

<table>
<thead>
<tr>
<th>Origin of chimeras</th>
<th>Total no.</th>
<th>♂♂</th>
<th>♀♀</th>
<th>♀♂</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBA-T6T6+CBA-p (this paper)</td>
<td>12</td>
<td>9</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>A+A (Mystkowska, unpublished results)</td>
<td>12</td>
<td>8</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>LAB Grey+LAB Grey and LAB Grey+LAB Grey x A2G (Tarkowski, 1963)</td>
<td>16</td>
<td>11</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>28</td>
<td>8</td>
<td>4</td>
</tr>
</tbody>
</table>

(70% 20% 10%)

Table 2. Genotype and sex of the progeny of chimeras

<table>
<thead>
<tr>
<th>No. and sex of chimera</th>
<th>Total no. of young</th>
<th>CBA-p young</th>
<th>Hybird young</th>
<th>CBA-p</th>
<th>Hybird young</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>1. ♂</td>
<td>120</td>
<td>120</td>
<td>100</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>2. ♀</td>
<td>52</td>
<td>48</td>
<td>92.3</td>
<td>4</td>
<td>7.7</td>
</tr>
<tr>
<td>3. ♀</td>
<td>— Hermaphrodite—sterile</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>4. ♂</td>
<td>168</td>
<td>42</td>
<td>25.0</td>
<td>126</td>
<td>75</td>
</tr>
<tr>
<td>5. ♂</td>
<td>190</td>
<td>64</td>
<td>33.7</td>
<td>126</td>
<td>66.3</td>
</tr>
<tr>
<td>6. ♂</td>
<td>183</td>
<td>0</td>
<td>—</td>
<td>183</td>
<td>100</td>
</tr>
<tr>
<td>7. ♂</td>
<td>76</td>
<td>76</td>
<td>100</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>8. ♂</td>
<td>148</td>
<td>0</td>
<td>—</td>
<td>148</td>
<td>100</td>
</tr>
<tr>
<td>9. ♀</td>
<td>22</td>
<td>0</td>
<td>—</td>
<td>22</td>
<td>100</td>
</tr>
<tr>
<td>Total</td>
<td>959</td>
<td>350</td>
<td>36.5</td>
<td>609</td>
<td>63.5</td>
</tr>
</tbody>
</table>

4. The progeny of chimeras

The F₁ generation obtained from crossing chimeras with CBA-p individuals may consist of either or both of two phenotypically different types of individual, i.e. CBA-p and hybrids CBA-p/CBA-T6T6 with agouti colouring. All the fertile chimeras, i.e. six males and two females, were used for cross-breeding. Data on the genotype and sex of progeny are given in Table 2.

Among eight fertile chimeras three individuals (nos. 2, 4, 5) produced progeny of both types (Plate 2, fig. 12), which indisputably proves the mixed character of
the germ cell population. In all these three animals both types of cells were also found in the somatic tissues. The progeny of the other chimeras was of one type, either pink-eyed or agouti. One type of progeny is not surprising in the case of animals nos. 1 and 9, in which only one type of cell was found in both coat and outer layer of the retina, and also in the bone marrow. It is however interesting that three males (nos. 6–8), which were undoubtedly chimeras, produced gametes of one type only. It appears significant that of these three males both the individuals karyologically examined proved to be sex-chromosome mosaics (see below).

Among the offspring of male chimeras, no instance was seen where progeny of a particular phenotype were exclusively female (Table 2). This indicates that, in every case, the chimera component (or components) giving rise to these progeny must have been of XY constitution.

5. Karyological investigations

A. Bone marrow. The participation of CBA-p and CBA-T6T6 cells in bone marrow, like the participation of the two types of pigment cells in the coat and in the eyes, differs greatly in different individuals (Table 3). Among the three animals with uniform colour of coat, in two (nos. 1, 9) only cells of the same type as in the coat were found in the bone marrow, and in one (no. 8) about 20% of CBA-p cells were found despite the fact that the coat was uniformly agouti and only very few CBA-p cells occurred in the eyes. Assessment of chimerism made on the basis of coat colour only is therefore not completely reliable. On the other hand similarities and differences between different chimeras defined on the basis of coat pigmentation and composition of the bone marrow

<table>
<thead>
<tr>
<th>No. and sex of chimera</th>
<th>Total no. of cells</th>
<th>CBA-p</th>
<th>CBA-T6T6</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>1. ♂</td>
<td>200</td>
<td>200</td>
<td>100.0</td>
</tr>
<tr>
<td>2. ♀</td>
<td>201</td>
<td>188</td>
<td>93.5</td>
</tr>
<tr>
<td>3. ♂</td>
<td>203</td>
<td>195</td>
<td>96.1</td>
</tr>
<tr>
<td>4. ♂</td>
<td>201</td>
<td>113</td>
<td>56.2</td>
</tr>
<tr>
<td>5. ♂</td>
<td>208</td>
<td>109</td>
<td>52.4</td>
</tr>
<tr>
<td>6. ♂</td>
<td>200</td>
<td>102</td>
<td>51.0</td>
</tr>
<tr>
<td>7. ♂</td>
<td>Not investigated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8. ♂</td>
<td>201</td>
<td>39</td>
<td>19.4</td>
</tr>
<tr>
<td>9. ♀</td>
<td>200</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>Total</td>
<td>1614</td>
<td>946</td>
<td>58.6</td>
</tr>
</tbody>
</table>
Mouse chimeras

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do not always coincide. The difference in pigmentation between chimeras nos. 3 and 4 (Plate 1, figs. 3, 4) is not as great as the difference in the composition of bone marrow (respectively 96.1 and 56.2% of CBA-p cells). The difference in colour between chimeras nos. 4 and 6 is however distinct (Plate 1, figs. 4, 6), while the percentages of the two types of cells in the bone marrow are very similar (Table 3).

Up to the present time, the assumption that some chimeras are sex-chromosome mosaics has not been directly proved, even in relation to true hermaphrodites. On the basis of the studies made by Stich & Hsu (1960; see also Ford, 1966b), which demonstrated the possibility of defining genetic sex of mouse cells

Table 4. Genetic sex of somatic cells of chimeras

<table>
<thead>
<tr>
<th>No. and sex of chimera</th>
<th>Type of cells</th>
<th>Total no. of cells</th>
<th>Result of identification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Certain</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>XX cells</td>
</tr>
<tr>
<td>2. ♀</td>
<td>CBA-p</td>
<td>20</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>CBA-T6T6</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>3. ♂</td>
<td>CBA-p</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>CBA-T6T6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. ♂</td>
<td>CBA-p</td>
<td>20</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>CBA-T6T6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8. ♂</td>
<td>CBA-p</td>
<td>20</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>CBA-T6T6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>XY cells</td>
</tr>
<tr>
<td>2. ♀</td>
<td>CBA-p</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CBA-T6T6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. ♂</td>
<td>CBA-p</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CBA-T6T6</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>6. ♂</td>
<td>CBA-p</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CBA-T6T6</td>
<td>20</td>
<td>12</td>
</tr>
<tr>
<td>8. ♂</td>
<td>CBA-p</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CBA-T6T6</td>
<td>20</td>
<td>15</td>
</tr>
</tbody>
</table>

karyologically, chromosome preparations from the following chimeras were analysed from this aspect: no. 2 (female), no. 3 (hermaphrodite), and nos. 6 and 8 (males) (Table 4). There was no question of sex chromosome mosaicism in the remaining chimeras: nos. 1 and 9, one type of cells in the bone marrow; nos. 4 and 5, both types of germ cells of XY constitution. Chimera no. 7 was not karyologically examined. In the case of chimeras nos. 2, 3, 6 and 8, 20 CBA-p and 20 CBA-T6T6 metaphase plates were examined (only 10 CBA-T6T6 plates were found in the case of the hermaphrodite).

Despite the fact that the best plates were chosen not all of them were suitable
for sex identification. The result of identification of each plate was allocated to one of three categories: certain, probable, unidentifiable (Table 4). Despite these difficulties the results are completely unambiguous. The female no. 2 is not a sex-chromosome mosaic: both types of cells are genetically female. The hermaphrodite (no. 3) is a sex-chromosome mosaic: the CBA-p cells are genetically female, the CBA-T6T6 genetically male. The ‘maleness’ of CBA-T6T6 cells is beyond doubt on account of the presence of XY bivalent in the primary spermatocytes (see below and Plate 3, fig. 25). This result, although anticipated, constitutes in relation to hermaphrodite chimeras the first direct proof of their sex-chromosome mosaicism. The most interesting fact, however, is that both males no. 6 and 8 were also found to be sex-chromosome mosaics (Plate 3, figs. 19–24). The suggestion put forward by Tarkowski (1961, 1963, 1964b, 1965) that some sex-chromosome mosaics may develop into phenotypically normal males has thus been proved.

**Table 5. Percentages of CBA-p and CBA-T6T6 primary spermatocytes (diakinesis) in the testes of chimeras**

| No. and sex of chimera | Total no. of cells | Type of cells | CBA-p | | CBA-T6T6 | |
|------------------------|-------------------|---------------|-------|---------|-------|
|                         |                   |               | No.  | %       | No.   | %     |
| 1.  ♂                   | 101               |               | 101  | 100.0   | 0     | —     |
| 3.  ♀                   | 103               |               | 0    | —       | 103   | 100.0 |
| 4.  ♂                   | 103               |               | 30   | 29.1    | 73    | 70.9  |
| 5.  ♀                   | 100               |               | 35   | 35.0    | 65    | 65.0  |
| 6.  ♂                   | 102               |               | 0    | —       | 102   | 100.0 |
| 7.  ♂                   | Not investigated  |               | —    | —       | —     | —     |
| 8.  ♀                   | 100               |               | 0    | —       | 100   | 100.0 |

B. Testes. Karyological investigations were made only of germ cells and were aimed at defining the presence and percentage of the two types of primary spermatocytes at diakinesis (Table 5). Co-occurrence of CBA-p and CBA-T6T6 spermatocytes was found only in males nos. 4 and 5, that is, only in those which produced progeny of both types. Ratios between CBA-p and CBA-T6T6 spermatocytes correspond almost exactly to the ratios between CBA-p individuals and hybrids in the progeny (cf. Tables 2 and 5). This indicates that at least from the stage of the first reduction division neither type of germ cells is ‘privileged’ in either the period of spermatogenesis or in the process of fertilization and that ratios at the diakinesis stage define the ratios among the fully formed spermatozoa.

In the case of male no. 1, the chimerism of which remains open to doubt, only CBA-p spermatocytes were found. It is a striking fact, however, that only one type of spermatocyte (CBA-T6T6) is found in males nos. 6 and 8 and in the
testis of the hermaphrodite, the chimerism of which is undoubted. All three individuals proved to be sex-chromosome mosaics, and all exhibit complete absence among primary spermatocytes of genetically female cells, even when the latter are very numerous in the somatic tissues (cf. Table 5 and 3).

C. Ovotestis of the hermaphrodite. Squash preparations from part of the hermaphrodite's ovotestis (chimera no. 3) were intended to supply data on the composition of somatic tissue. Although the number of metaphase plates suitable for analysis was small (12) the presence of both types of cells was established beyond doubt (CBA-p-10, CBA-T6T6-2). As in the case of bone marrow, the predominance of genetically female CBA-p cells is very marked.

6. Reproductive system

A. Hermaphrodite individual. The only hermaphrodite (chimera no. 3) was externally similar to a male in which the testes had not descended. In the description of the reproductive system given below, we have emphasized only the basic features of its structure, without going into greater anatomical detail. The left side of the system is typically male (Plate 2, figs. 14, 15). Complete spermatogenesis had taken place in the testis, despite the fact that it was situated in the body cavity. Spermatozoa were found in the histological and chromosomal preparations from the testis and in the histological sections from the epididymis. The right side of the system included an ovotesticular gonad and dual efferent ducts (Plate 2, fig. 15). Proximally Müllerian and Wolffian ducts are enveloped in a common muscular layer, distally each duct has its own muscular layer but they remain connected by connective tissue. The general character of the ovotestis is more similar to an ovary than to a testis. The greater part of the gonad is occupied by ovarian follicles at different stages of development, some of them fully formed Graafian follicles with either healthy or degenerated oocytes. The remainder of the gonad is occupied by very altered testicular tissue composed of poorly developed and completely sterile seminiferous tubules. Seminal vesicles were formed on both sides. The vesicle situated on the male side is, as regards size and histological differentiation, slightly better developed than its counterpart.

B. Other individuals. Histological examination of parts of the testes and ovaries of the other adult chimeras did not reveal any anomalies. The testes of two new-born mice were also examined and while in one animal they were found to be completely normal, in the other, which was 5 days old, typical oocytes in the growth phase were found within the sex cords of one of the testes (Plate 2, figs. 16–18). The oocytes are grouped in a small part of the testis, while in the remaining part and in the whole of the second testis the germ cells in the sex cords are represented only by typical pre-spermatogonia. A few pre-spermatogonia also co-occur with oocytes within the same or adjacent sex-cords (Plate 2, fig. 18). The somatic tissue of the whole gonad is typically testicular and there is nothing which justifies treating it as an ovotestis.
DISCUSSION

The two basic questions emerging during studies on chimeras are concerned with: (1) the effectiveness of the operation itself, that is, the degree of certainty that the individual formed from the fusion of two eggs is in fact a mosaic, and (2) whether or not there is any regularity in the participation and distribution of cells of the two types in different organs and tissues of such animals. The information so far accumulated proves that the integration of two cleaving eggs into one entity and formation of one apparently normal blastocyst does not guarantee the formation of a chimeric individual from such a blastocyst, although it does lead to this in the majority of cases. On the basis of the three criteria employed in the present investigations—differences in pigmentation of melanocytes and cells of the outer layer of the retina, genetic differences expressed by different pigmentation of progeny (gametes), and differences in the karyotype (bone marrow, primary spermatocytes)—it was found that two of the nine adult individuals were formed from one type of cell only and were probably not chimeras at all. Although the entodermal organs were not examined it would appear very improbable that cells derived from the second egg occurred in derivatives of this germ layer when they were completely absent in ecto- and mesodermal derivatives.

Additional information on this subject is supplied by data on the composition of the outer layer of the retina in new-born mice (present study and Tarkowski, 1964a), and the coat colour of adults (Mintz, 1967). As the composition of outer layer of the retina is a very sensitive criterion of chimerism (see Results section 2) these data are reliable and the same trust may be placed in them as in the far more comprehensive data on adult individuals. Tarkowski (1964a) found that among nine new-born mice seven had mosaic composition of the outer layer of the retina and two a homogeneous composition. Frequency of occurrence of non-chimeric individuals is therefore similar in the two series of experiments (two out of 12 and two out of nine) and in general is about 20%. Mintz (1967) in analysing the coat colour of 109 chimeras obtained from fusing eggs in several different genetical combinations, found only 41 individuals with two-colour pigmentation. Undoubtedly some of the individuals, which on the ground of colour of coat should be defined as non-chimeras, are in fact mosaics on account of the mixed composition of other tissues. This possibility is demonstrated by chimera no. 8, described in this paper, which despite its uniform colour characteristic of CBA-T6T6 has as much as 20% of CBA-p cells in the bone marrow. Mintz (1967) also refers to the fact that some of the single-colour individuals displayed mosaicism of the internal tissues. Nevertheless, it seems to us that frequency of occurrence of mosaic individuals, or at least individuals characterised by pigmentation mosaicism, is higher in our present experiments and earlier experiments by one of us (A.K.T.) than in Mintz's experiments. The difference in results is perhaps due to the degree of genetic difference between
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the eggs used, a small degree in the first case, but considerably greater in the second. The occurrence of non-chimeric individuals will not appear so surprising if we bear in mind that the animals described in this study represent, with regard to degree of chimerism, a whole range of variation from one extreme to another. It seems to us that when there is great genetic similarity of the two eggs, as was the case in our experiments, every quantitative combination between the two components may be expected in the animals produced. This, however, must not necessarily be so in the case of other genetic combinations, when even a slight difference in proliferative capacities of the two types of cells can change original quantitative proportions and result in dominance of one component. Secondary quantitative shifts are also to be expected in the postnatal period, especially with regard to the composition of the hematopoietic tissue. Thus evaluation of the degree of chimerism of an adult individual on the basis of the composition of the bone marrow may prove less reliable than that made on the basis of pigmentation.

The observations made in the present study do not indicate that there is any regularity in distribution of pigment cells in the body of CBA-p/CBA-T6T6 chimeras. The light and dark areas in the coat of these animals usually have mixed composition of hair, are irregular in outline, variable in size, and their distribution does not exhibit any definite and constant pattern. Contrary to our observations Mintz (1965a, b, 1967) concludes that the chimeras she obtained are characterized by a definite pattern of distribution of melanocytes, consisting in alternate arrangement of a definite number (17) of transverse stripes. Although on theoretical grounds discussed elsewhere (Tarkowski, 1963) the occurrence of alternate transverse bands is to be expected, and in some of our mottled animals such bands could in fact be discerned soon after the pigmentation started to appear (Plate 2, fig. 9), we have not found confirmation in our material of the existence of a definite and constant pattern such as described by Mintz. We found distinct stripes in CBA-p/CBA-T6T6 chimeras only on the tail but both their number and breadth varied greatly.

One of the problems which can be fruitfully investigated by means of chimeras is the background of sex differentiation in mammals and the role of sex-chromosome mosaicism in the development of sexual disorders. Chimeras represent an excellent model of sex-chromosome mosaics, although the type of their mosaicism (XX/XY) is relatively rarely encountered in nature. As sex-chromosome mosaics should form the group most numerous represented among chimeras (50 %) and hermaphrodite individuals occur relatively rarely, the suggestion was made (Tarkowski, 1961, 1963, 1964b) that sex-chromosome mosaics may develop not only into hermaphrodites but also into normal males. Numerical data presented in Table 1 show that this second possibility is the one far more frequently found. The present study supplied direct karyological proofs in support of this supposition, by demonstrating sex-chromosome mosaicism in two phenotypically normal and fertile males. A hermaphrodite
was also shown, as was expected, to be a sex-chromosome mosaic. Among six individuals which were undoubted chimeras and were karyologically examined one was found to be an XX/XX mosaic (female), three XX/XY mosaics (two males, one hermaphrodite), and two XY/XY mosaics (males). If it is possible to draw any conclusions from such a small sample the frequencies observed may be considered as agreeing with the frequencies envisaged. The question as to why some sex-chromosome mosaics develop into normal males, and others into hermaphrodites, remains unsolved. It is probably the degree of mixing of the two types of cells in the chimera's gonads which plays the principal part here: the more thorough the mixing, the smaller the chances of ovotesticular gonads, and in consequence of a hermaphrodite individual forming. These problems have already been extensively discussed in previous papers (Tarkowski, 1963, 1964b).

The rest of the discussion will be devoted solely to the fate and behaviour of germ cells in chimeras in general, and in sex-chromosome mosaics in particular, to which issue the present study furnishes new data. Two main questions involved are: (1) to what degree is the chimerism of somatic tissues reflected in the co-occurrence of germ cells of both types in the gonads?; and (2) what is the fate of XX germ cells in sex-chromosome mosaics developing into males? It might be anticipated that such males should produce two types of spermatozoön and in consequence one of the types of progeny (in the case of genetic differences affecting, for instance, pigmentation) will be represented by females only. This assumption requires the additional supposition that XX germ cells can undergo spermatogenesis and form spermatozoa.

Analysis of observations made of nine adult animals leads to the following conclusions. Only one type of germ cell, that represented by somatic cells, was found to occur in male no. 1 and female no. 9. As these individuals were most probably not chimeras at all, this result is understandable and they can be eliminated from further deliberations. Three undoubted chimeras (no. 2, female; nos. 4 and 5, males) produced progeny of two types. As can be inferred from the frequency of occurrence of CBA-p and hybrid animals in progeny produced by female no. 2, in this particular animal the proportion of each type of cell in the germinal tissue and in the bone marrow was nearly identical (cf. Tables 2 and 3). In the case of the two males there is no such far-reaching agreement, as with about 50% of CBA-p cells in the bone marrow only 25-0 and 33-7% of the spermatozoa are of this genotype. Evidence that the absence of complete agreement is primary and not secondary is provided by the fact that the proportion of each type of cell in the germinal tissue is the same among spermatozoa and primary spermatocytes (cf. Tables 2 and 5). In any case in those three chimeras about which it was additionally known that they were not sex-chromosome mosaics, the degree of chimerism in the germinal tissue agrees with, or at least does not markedly differ from, the degree of somatic chimerism defined on the basis of the composition of bone marrow and, in as far as it is possible to decide on account of the lack of exact quantitative criteria, of the pigmentation of the
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Under these circumstances the finding of only one type of offspring fathered by males nos. 6, 7 and 8 is surprising. The possibility cannot of course be ruled out that in these particular males primordial germ cells of one type only differentiated during embryogenesis, and that as a result the population of germ cells was originally homogeneous. The observations presented below show, however, that this explanation, although the simplest, is highly improbable. (1) Males nos. 6 and 8 proved to be sex-chromosomes mosaic and the spermatozoa they produced originated from the genetically male component only. (2) In male no. 6 in which CBA-T6T6 germ cells only were detected, genetically female CBA-p cells occurred in the bone marrow to as much as 51% and were relatively numerous in the coat and in the outer layer of the retina. (3) Male no. 7, which was unfortunately not karyologically examined, was characterised by distinct predominance of CBA-T6T6 pigment cells but fathered progeny of CBA-p type only. The occurrence in progeny of both females and males shows in addition that spermatozoa were produced from XY germ cells. (4) Only CBA-T6T6 primary spermatocytes of XY constitution were found in the testis of the hermaphrodite (chimera no. 3), whereas only 3.9% of cells of this type occurred in bone marrow and there was a relatively small number of them in the coat and outer layer of the retina. (5) The chimerism of the germinal tissue can easily be demonstrated if, as was the case in these investigations, genetic and karyological markers are available. When there are no such markers, the possible co-occurrence of spermatozoa originating from XX and XY cells may be expressed only in a shift in sex-ratio among progeny in favour of females. The progeny of three males obtained from fusing eggs of A strain (Mystkowska, unpublished results) were analysed from this aspect, but no significant deviations from the 1:1 ratio were found (42♂♂-50♀♀; 35♂♂-41♀♀; 20♂♂-20♀♀). These observations are limited in value as there is no absolute certainty that all these males were chimeras, or that any one of them was a sex-chromosome mosaic (lack of karyological investigations), but when accumulating all available evidence this also is of some value and strengthens the argument as a whole. All the above observations treated jointly very convincingly suggest that in the case of male chimeras which are sex-chromosome mosaics (and also in the testes of hermaphrodites) formation of spermatozoa from XX cells is never attained, and, what is more, that these cells do not even reach the diakinesis stage.

The above view is significantly at variance with the well-documented statement that in the case of vertebrates, or at least in the infra-mammalian vertebrates, the type of gametogenesis (male versus female) of germ cells does not depend on their genetic sex but on the type of somatic differentiation of the gonads, and that in the case of sex-reversal, formation of functional gametes takes place (see Burns, 1961; Turner & Asakawa, 1964, for references).

Although there is nothing which would a priori indicate that mammals differ in this respect, relevant investigations are scarce and inconclusive. Among the observations made on this problem the following would appear to be most
important: (1) The presence and proliferation of 2A-XX cells, most probably germ cells, in the testes of new-born bulls from twin pregnancies with a free-martin (Ohno et al. 1962). It is not known, however, what becomes of these cells later, as the meiotic stages in this type of adult male have not been investigated. Serological examination of 17 offspring of one such male (see discussion in Stone et al. 1960) did not supply proof that it produced spermatozoa derived from the germ cells of the female co-twin. (2) Occurrence of mitotic metaphase plates with XX constitution and plates of the first reduction division without the characteristic XY bivalent in the testes of adult male marmosets born from heterosexual twin pregnancies and exhibiting sex-chromosome mosaicism in the bone marrow (Benirschke & Brownhill, 1963). (3) Presence of germ cells at different stages of meiotic prophase and of secondary spermatocytes in the sex-cords of the masculinized parts of mouse ovaries (Turner & Asakawa, 1964). The conclusions drawn in this study unfortunately are based on histological investigations only. (4) Evidence that the course of meiosis of XY germ cells is not genetically determined is provided by studies of Burns (1956, 1961), who found that in the transformed testes of an opossum they enter into growth phase on completion of the meiotic prophase and are not subject to the first reduction division.

The above observations would seem to indicate that in mammals, as in other vertebrates, the type of gametogenesis is regulated by the somatic sex of the gonads and that XX germ cells can exist in the testes not only as spermatogonia but even reach the stage of at least secondary spermatocytes. Contrary to expectations based on the above arguments our observations indicate that in the case of mouse chimeras which are sex-chromosome mosaics spermatogenesis of XX germ cells does not take place. At the present time we are not able to state when and in which way elimination of XX germ cells is brought about. The metaphase plates of mitotic divisions, presumably of spermatogonial origin, were extremely rarely encountered in preparations made from testes (0 to 5 in particular cases) and therefore the failure to find 2A-XX cells cannot be taken as proof of the absence of spermatogonia of this constitution. The possibility cannot therefore be excluded that XX spermatogonia are present and are subject to normal gonial divisions and that only primary spermatocytes in the period of the meiotic prophase are subject to elimination. As our karyological studies were concerned only with the diakinesis stage, we can only state that XX germ cells do not attain the first reduction division.

There is one observation which may be relevant to this matter. In a chimeric new-born animal (undoubted chimerism—mosaic composition of the outer layer of the retina) which died on the 5th day after birth, oocytes in the growth phase were found in one of two completely normally formed testes. In respect of stage and size they correspond to oocytes encountered in the ovaries of females of similar age. This is the first case of this kind, as up to the present descriptions have been given only of the occurrence of oocytes in the seminiferous tubules in
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ovotestis of a rat (Bradbury & Bunge, 1958) and in the testicular part of ovo-testes of mouse chimeras (Tarkowski, 1964b). Although we are concerned with ovotesticular gonads in the above two cases, the fact that some germ cells begin meiosis of the female type in the male part of the gonad is astonishing and at variance with the concept that there is a strict dependence between the type of gametogenesis and the character of the gonadal tissue (see Tarkowski, 1964b for a more detailed discussion). The case described in the present study is not therefore so exceptional as would appear at first glance, especially as it was found in a chimera which probably was a sex-chromosome mosaic. It is possible that the composition of the somatic tissue of the testis of this individual was chimeric and that despite histogenesis towards formation of a testis, the co-existence of XX somatic cells changed to a certain extent the conditions prevailing in the embryonic gonad during the period when meiotic prophase began and made it possible for some germ cells to enter upon this route. The alternative explanation is that these are XX germ cells and that the type of meiosis of such cells is under genetical rather than environmental control. As long as the genetic sex of these particular cells remains unknown and until other similar observations are made, far-reaching speculations would be premature. This problem is the object of further investigations in our laboratory.

Observations on chimeras suggest that some of the spontaneously arising sex-chromosome mosaics in mammals also develop into phenotypically normal and fertile males. If in addition our suggestion that XX germ cells are incapable of undergoing spermatogenesis was confirmed it would imply that the mosaicism of such individuals would refer only to somatic tissues and could not be discovered by means of genetic research.

SUMMARY

1. Of the 14 new-born mice obtained by fusion of CBA-p and CBA-T6T6 eggs, nine survived and attained maturity. The sex-ratio in the group of nine adult individuals and three new-born mice in which sex could be identified was 9♂, 1♀, 2♀.

2. Observations on the pigmentation of the coat and of the outer layer of the retina, karyological examination of the bone marrow and genetic investigations revealed chimerism in only seven of the nine adult individuals.

3. Participation in the coat of hair of the pigmentation characteristic of the agouti and pink-eyed component differs greatly in different individuals, and varies from one to the other extreme. Light and dark areas are not clearly demarcated and have mixed hair composition; no definite and constant pattern of pigmentation could be discerned.

4. Participation of the two types of cells in the coat and in the bone marrow is most often similar, but was not found to be completely parallel.

5. Karyological investigations revealed sex-chromosome mosaicism in two fertile chimeric males and in a true hermaphrodite.
6. The somatic tissue of the ovotestis of a hermaphrodite was found to consist of both genetically female and genetically male cells.

7. Chimeras which were not sex-chromosome mosaics produced two types of gametes, CBA-p and CBA-T6T6, in proportions not significantly different from the degree of mosaicism of the somatic tissues.

8. Males and a hermaphrodite which were sex-chromosome mosaics produced spermatozoa corresponding to the genotype of the genetically male cells only. No XX primary spermatocytes at diakinesis were found in the testes of these animals. All the observations lead to the suggestion that XX germ cells in sex-chromosome chimeras are incapable of passing through spermatogenesis and that they are eliminated before the first reduction division is attained.

9. Oocytes in the growth phase, in addition to pre-spermatogonia, were found in the sex cords of one testis of a 5-day-old chimeric mouse which had an otherwise completely normal male genital system.

RÉSUMÉ

Observations sur des chimeres de souris CBA-p|CBA-T6T6

1. Sur 14 souris nouveau-nées obtenues par fusion d’œufs CBA-p et CBA-T6T6, neuf ont survécu et ont atteint la maturité. La sex-ratio chez les neuf individus adultes et les trois nouveau-nés chez lesquels on a pu identifier le sexe était la suivante: 9 ♂♂, 1 ♀, 2 ♀♀.

2. Des observations sur la pigmentation du pelage et de la couche externe de la rétine, l’examin caryologique de la moelle osseuse et des recherches génétiques ont révélé le chimérisme dans sept seulement des neuf adultes.

3. La participation, dans la livrée, de pelage ayant la pigmentation caractéristique des composants agouti et œil-rose, diffère beaucoup selon les individus, de l’un à l’autre extrême. Les zones claires et sombres ne sont pas clairement délimitées et ont un pelage mixte; on n’a pu distinger de type de pigmentation défini et constant.

4. La participation des deux types de cellules dans la fourrure et la moëlle osseuse est le plus souvent semblable, mais n’est pas absolument parallèle.


7. Les chimères qui n’étaient pas des mosaïques sexuelles chromosomiques ont produit deux types de gamètes, CBA-p et CBA-T6T6, dans des proportions qui ne différaient pas significativement du degré de mosaïcisme des tissus somatiques.

8. Les mâles et un hermaphrodite qui étaient des mosaïques chromosomiques sexuelles, ont produit des spermatozoïdes du seul type qui se caractérisait par des cellules génétiquement mâles. On n’a pas trouvé de spermatocytes XX à la
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diacinèse dans les testicules de ces animaux. Toutes les observations conduisent à supposer que des gonocytes XX, dans les chimères chromosomiques sexuelles, sont incapables de subir la spermatogénèse et qu’ils sont éliminés avant que soit atteinte la première division réductionnelle.

9. Outre les pré-spermatogonies, on a trouvé des oocytes en phase de croissance dans les cordons sexuels d’un testicule d’une souris-chimère de cinq jours qui avait d’autre part un appareil génital mâle complètement normal.

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