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Germ-line chimaeras can produce both strains of fowl with high efficiency after partial sterilization

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The drug busulphan is known to be cytotoxic to migrating primordial germ cells (PGCs). A technique is described in which doses of 0, 25, 50 and 250 μg busulphan in 40 μl sesame oil were injected into the yolk of White Leghorn eggs incubated for 0, 24, 48 and 72 h. The percentage survival values of these embryos showed that the older the embryo at the time of injection, the greater the survival. Increasing the dose of busulphan decreased the survival. The percentage of embryos showing abnormalities increased with higher doses of busulphan. The number of germ cells in histological sections from gonads of 16-day embryos was estimated and in embryos treated with 50 μg and 250 μg busulphan the number of germ cells was significantly less than in the controls. Eggs were injected with 50 μg busulphan at 24–30 h, and at 50–55 h the embryos received an intravascular injection of a germinal crescent cell suspension containing PGCs from Rhode Island Red embryos. Twenty hatchlings from these experiments were raised to sexual maturity. All these birds were fertile and half of the breeding groups producing offspring from the transferred germ cells at a rate of about 35% of the total. The technique would improve the efficiency of producing transgenic gametes.

Introduction

The characteristically large and yolky egg of the bird has led to considerable technical difficulties in producing transgenic offspring. The usual technique of microinjection of foreign DNA into the pronuclei of the fertilized egg is very successful in mice (Palmiter *et al.*, 1982) but the inability to localize these structures in the cytoplasm of the avian egg has meant that this approach has not yet been successful in birds (Sang and Perry, 1989). As a result, most of the work on birds has concentrated on manipulating either undifferentiated stem cells (Petitte *et al.*, 1990) or primordial germ cells (Simkiss *et al.*, 1990). These two approaches rely upon introducing foreign DNA into a cell line that will eventually produce gametes and thus transgenic offspring.

It has been shown that it is possible to introduce foreign genes into avian primordial germ cells (PGCs) by means of defective retroviruses (Savva *et al.*, 1991). These transfected cells become incorporated into the gonads. Thus, by using donor PGCs that are injected into recipient embryos, it is possible to produce offspring with chimaeric gonads (Simkiss *et al.*, 1989). Male offspring from such experiments contain the foreign DNA in their spermatozoa and produce transgenic offspring (Vick *et al.*, 1993).

The percentage of transgenic offspring produced by this method is clearly related to the ratio of the number of transfected PGCs that are injected from the donor embryo in relation to the number that are produced endogenously by the recipient embryo. Thus, providing the manipulated cells behave like normal PGCs, the ratio of transfected donor PGCs (N_D) to recipient PGCs (N_R) will determine the relative numbers of spermatozoa or ova carrying the foreign DNA. For these reasons there has been considerable interest in methods for destroying PGCs in avian embryos. Three types of approach have been adopted. The first, involving physical removal or interference with PGC migration (McCarrey and Abbott 1982), is too damaging to enable the production of normal hatchlings. The second approach using UV or X-ray irradiation (Fargeix, 1976; Reynaud, 1976) or lasers (Mims and McKinnell 1971) is difficult to apply to all the germinal crescent cells and leads to damage to the underlying tissues (Aige-Gil and Simkiss, 1991). As a result the chemical approach, using a chemosterilant such as 1,4-butanediol dimethane sulfonate (busulphan), has attracted increasing interest. (Aige-Gil and Simkiss, 1991; Hallett and Wentworth, 1991). This drug causes sterility in mammalian fetuses by destroying PGCs during their migratory phase (Hemsworth and Jackson, 1963). Similar effects have been reported for birds (Reynaud, 1977), although Swartz (1980) found that the drug was teratogenic when injected into the egg albumen. Our recent study avoided these effects by injecting busulphan into the yolk of domestic fowl eggs. Doses below 100 μg per egg produced minimal abnormalities, while inducing up to 97% sterility (Aige-Gil and Simkiss, 1991). The following experiments were therefore undertaken to establish (i) the effect of increasing doses of busulphan on the number of germ cells in the ovary and testis (ii) the incidence of abnormal embryos and the percentage survival of these embryos (iii) whether busulphan permitted donor PGCs to settle in the treated gonad and (iv) how this affected the ratio of donor and recipient germ cells in chimaeric hatchlings and their offspring.

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Materials and Methods

Animals

White Leghorn birds from the line zero strain (Astrin *et al.*, 1979) were used as recipients in all these experiments. An inbred strain of Rhode Island Red birds was used as a source of donor PGCs when required. The eggs were incubated in a forced air incubator at 37.5°C and 60% relative humidity and their development staged according to the scheme of Hamburger and Hamilton (1951).

Treatments

Busulphan (1,4-butanediol dimethane sulfonate; Sigma, Poole) was suspended in sesame oil. Eggs incubated for 0, 24, 48 and 72 h were injected with doses of 0, 25, 50 or 250 μg busulphan in 40 μl sesame oil. Each dose was injected directly into the yolk through a hole in the egg (Aige-Gil and Simkiss, 1991) according to the method of Brunström and Örberg (1982). The hole in the eggshell was sealed with Micropore surgical tape (3M Health Care, Swansea) and the eggs returned to the incubator. The eggs were checked daily for viability by candling with a cold light source. Dead embryos were removed and their ages were recorded together with any visible abnormalities.

In a second experiment, doses of 0, 50 and 250 μg of busulphan in 40 μl sesame oil were injected into eggs incubated for 24–30 h. The eggs were incubated until day 16 when the embryos were killed, sexed and their gonads fixed in Bouin's fluid. They were embedded in paraffin wax, sectioned and stained with haematoxylin and eosin. The number of germ cells in five random sections of the testis or in the ovarian cortex were counted for each sample. The maximum thickness of the cortex of the ovary was also measured.

In a third experiment, a suspension of PGCs was prepared from germinal crescents of Rhode Island Red embryos at stages 5–11 of development using the method of Vick *et al.* (1993). White Leghorn embryos that had been injected with 50 μg busulphan in sesame oil at 24 h incubation were incubated to stage 15 of development and injected with approximately 1 μl of Rhode Island Red PGC suspension. This was done by removing a small square window of eggshell from over the embryo and injecting into the vasculature with fine glass pipettes attached to a micromanipulator (Singer Instrument Co. Ltd., Watchet) and a microinjector (Narishige IM-5A/B, Tokyo). Post-operative eggs were sealed with Micropore tape and incubated normally. Twenty hatchlings from these experiments were raised to sexual maturity and six cocks were crossed with 12 hens on a 1:2 basis in six breeding groups. The first 20 eggs from each group were incubated and the chicks classified as pure white (WL × WL) spotted white (RIR × WL) or brown (RIR × RIR).

Results

It is clear that the older the embryo at the time of injection of busulphan, the greater the survival (Table 1). Increasing the dose similarly decreases the number of embryos hatching. An

Table 1. Percentage survival of untreated control chick embryos† treated with 0, 25, 50 or 250 μg busulphan in 40 μl sesame oil at 0, 24, 48 or 72 h incubation

Time of treatment (h)	Dose (μg)	Percentage surviving		
		7 days	15 days	Hatch
0	0	65	59	46
	25	44	22	17
	50	40*	20*	10*
	250	30*	10*	0*
24	0	70	70	58
	25	76	40	28
	50	76	44	26
	250	63	53	11
48	0	81	68	47
	25	79	68	26
	50	75	56	44
	250	39	11	11
72	0	87*	75*	71*
	25	86	76	52
	50	93*	64*	57*
	250	76	44	24
Untreated	—	92	91	84

†$n = 16$ or more in all groups except those marked * where it was at least 10.

Table 2. Percentage of day 16 embryos† showing developmental abnormalities after they were injected with doses of 0, 25, 50 or 250 μg busulphan in 40 μl sesame oil at 0, 48 or 72 h incubation

Time of injection (h)	Percentage of embryos showing abnormalities Busulphan (μg)			
	0	25	50	250
0	2	6	20*	60*
48	0	0	19	50
72	0*	0	0*	16*

†$n = 16$ or more in all groups except those marked * where it was at least 10.

Table 3. Numbers of germ cells per mm^2 in male and female gonads of day 16 embryos treated with doses of 0, 25, 50 and 250 μg busulphan in 40 μl sesame oil at 24–30 h incubation

Sex	Busulphan dose (μg)			
	0	25	50	250
Females	1104 ± 67	721 ± 52	384 ± 32	41 ± 9
Males	465 ± 33	298 ± 22	170 ± 11	53 ± 11

Values are means ± SEM and all are significantly different at $P < 0.001$.

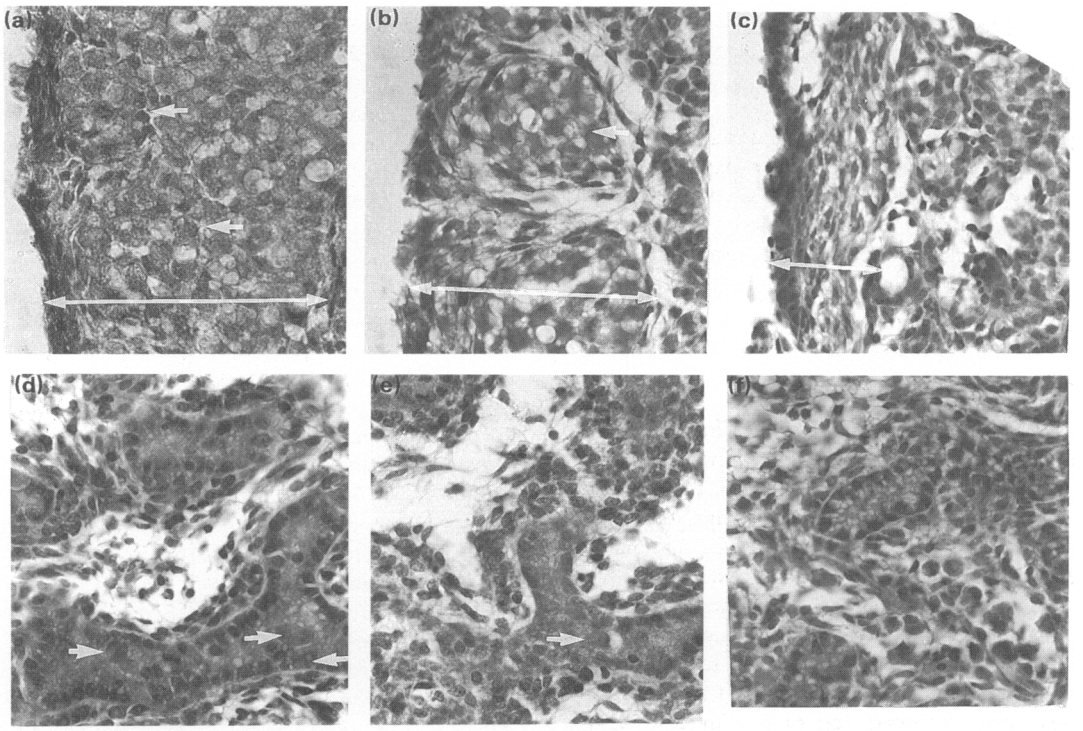

Fig. 1. Light micrographs of transverse sections of the left ovary of day 16 embryos; (a) controls, (b) treated with 50 μg busulphan at 24–30 h incubation, (c) treated with 250 μg busulphan at 24–30 h incubation. Note how the cortex is reduced in thickness (double headed line) and the clusters of primordial germ cells are reduced in size (arrows) following busulphan treatment. Transverse sections of testes are also shown for (d) control embryos, (e) testes after 50 μg busulphan and (f) after 250 μg busulphan was injected at 24–30 h incubation. Note how the busulphan reduces the number of seminiferous cords and decreases the number of germ cells within them (arrows). The overall size of the busulphan treated testes was smaller than those of controls. × 450

Table 4. Numbers of hatchlings of various phenotypes in the first 20 hatchlings produced by crossing six male and 12 female germ-line chimaeras in six breeding groups

	Phenotypes of offspring-1		
Group	White	White and spots	Brown
1	20	0	0
2	20	0	0
3	20	0	0
4	17	3 (15%)	0
5	15	5 (25%)	0
6	11	9 (45%)	0

White hatchlings are typical WL × WL; white birds with spots are RIR × WL and go brown after three months; brown birds are RIR × RIR.

injection of sesame oil on its own caused some decrease in survival compared with survival values for untreated embryos.

The percentage of embryos showing developmental abnormalities increased with the dose of busulphan (Table 2). The most common abnormalities observed were abnormal limb buds in the form of pointed distal ends, haematomas and eye abnormalities, including microphthalmia. Embryos exposed to busulphan after 48 and 72 h incubation showed fewer teratogenic effects than younger specimens. All the control embryos that survived beyond day 5 were normal in appearance.

An analysis of the number of germ cells in histological sections of gonads of day 16 embryos (Table 3) showed significant differences ($P < 0.001$) in the effects of dose of busulphan. The thickness of the ovarian cortex was 86.0 ± 4.2 μm in control embryos, 54.3 ± 4.4 μm after 50 μg and 31.9 ± 1.4 μm after 250 μg busulphan (Fig. 1). The decrease in cortex thickness corresponds to an equivalent decline in the numbers of germ cells per unit area. In male embryos the testicular cords were reduced in size, contained few germ cells and were surrounded with extensive interstitial tissue as the dose of the drug was increased (Fig. 1).

The numbers of white, spotted and brown offspring from the busulphan White Leghorn–Rhode Island Red chimaeras are shown (Table 4).

Discussion

The stroma of the vertebrate gonad is derived from cells of the coelomic epithelium in which the primordial germ cells settle early in development. The interaction of these two quite separate cell lines has been studied in considerable detail. According to Dubois (1969) the germinal epithelium secretes a chemoattractant that causes the primordial germ cells to leave the vascular system and settle in the coelomic epithelium.

Experiments by Kuwana *et al.* (1986) demonstrated this effect in culture and provide at least a partial explanation for the entry of the primordial germ cells into the definitive gonad tissue. Once within this epithelium there is a release from the mitotic block that appears to prevent primordial germ cells from dividing (Donovan *et al.*, 1986), but experimental manipulations of these cell populations led Fargeix (1969) to propose that there was also autoregulation of these numbers. It is generally agreed that the sex of the germinal epithelium dictates whether the primordial germ cells will develop into spermatozoa or ova (Hajji *et al.*, 1988). Thus germ cell settlement, their division, population size and differentiation all appear to depend upon an intimate interaction with the germinal epithelium.

The drug busulphan is a cytotoxic chemical that has been used extensively to treat myeloid leukaemia (Dunn, 1974). It also appears to affect stem cells and exerts a strong influence on migrating PGCs. Its sterilizing ability is well documented (Merchant, 1975; Reynaud, 1977), but when applied via the egg albumen it is also teratogenic. In this work small doses were injected into the yolk in a lipophilic solution. The drug then probably reaches the embryo via the subembryonic fluid which increases in volume until day 7 of incubation when it occupies about 25% of the total egg volume (Simkiss 1980). This rapid dilution of a small amount of the drug, which has a half-life of 10 h in the plasma and which is applied to a rapidly growing embryo probably results in a short pulse of busulphan which avoids the major teratological effects reported by Reynaud (1977) and Hallett and Wentworth (1991). Any teratological effects decrease with age of embryo at the time of application and large numbers of apparently normal hatchlings were obtained in our experiments by using doses of no more than 50 μg per egg.

In a previous study, Aige-Gil and Simkiss (1990) treated 48 h embryos with 100 μg busulphan in sesame oil and reduced the numbers of germ cells in day 6 embryos to approximately 5% of control values. Histological examination of the gonads of day 16 embryos treated with 50 μg busulphan at 24–30 h shows that in these tissues the germ cell population has been reduced to about a third of control values. This result suggests that there may be some autoregulation of germ cell numbers as has been suggested previously by Fargeix (1976). Despite this, the cortex of the ovary was reduced in thickness in direct proportion to increasing dose and the germ cells formed progressively smaller clusters. In the males the testicular cords of busulphan-treated animals were reduced in number and contained fewer germ cells. Similar histological effects were noted by Reynaud (1977, 1981). It would appear, therefore, that a low dose of busulphan should increase the ratio of donor to endogenous PGCs i.e. (N_D:N_R) at least threefold in chimaeric birds.

It has recently been shown that transgenic birds can be produced by introducing foreign DNA into the PGCs that are used to make germ-line chimaeras (Vick *et al.*, 1993) so that the use of busulphan could increase the efficiency of this procedure. Before that can be done, however, it is necessary to show that the pulse of busulphan that is used to destroy the endogenous PGCs has disappeared before the donor PGCs are introduced. Clearly the time of the injections and the dose used are critical if they are not to destroy the donor PGCs as well as the endogenous cells. It is also clear that the germinal ridge that receives the PGCs must not be damaged (Reynaud, 1981). Obviously if the sterilizing effect of busulphan was mediated by blocking the settlement of the PGCs in the definitive gonad then both endogenous and donor cells would be excluded.

The experiments using PGCs from Rhode Island Red and White Leghorn birds produced chimaeras that enabled these possibilities to be investigated. These results show that all these birds were fertile, unlike the sterile ones produced by Reynaud (1981) who used much larger doses of busulphan. Of the six breeding groups set up, three produced only White Leghorn offspring, indicating either that none of the Rhode Island Red PGCs had been successfully transferred or that they had not been successful in producing fertilized eggs. The other three groups produced an average of 72% White Leghorn (WL × WL) and 28% Rhode Island Red (RIR × WL) crosses. For any individual parent that suggests an average of 14% Rhode Island Red gametes or presumably one donor PGC for every six recipient PGCs. Our previous work in breeding from chimaeras that had not been partially sterilized with busulphan gave success rates of less than 4% (Vick *et al.*, 1993). It is concluded, therefore, that when used in small doses and at the right time busulphan will partially sterilize a chick embryo and increase the relative number of donor PGCs in the germ-line chimaera. In the study reported here both the histological effects and the breeding experiments suggest a roughly threefold reduction in the number of endogenous PGCs in the treated animals.

The results reported in these experiments are in complete contrast to those obtained by Petitte *et al.* (1991). They produced 59 birds which they had attempted to make into germ-line chimaeras by transferring PGCs from White Leghorn embryos into Barred Plymouth Rock recipients. They raised 3117 offspring from these birds without finding any evidence for the effective transfer of primordial germ cells into a germ-line chimaera, whereas by our technique we would have expected to produce 436 positives from these numbers. This difference in results is probably due to a number of technical differences. First, Pettite *et al.* used bloodborne PGCs, whereas much better samples of these cells are obtained from germinal crescent preparations. Second, there must be some doubt about their experimental manipulations, both in terms of the age of the embryos they used and the sites and effectiveness of their injections. We attribute our 50% failure rate to these types of difficulty, although it is equally likely that transfer of PGCs between the sexes may account for these results.

The results from these experiments indicate that a large increase in germ-line chimaerism can be achieved by the use of a partial chemosterilant such as busulphan. This technique may be of considerable value in increasing the efficiency of producing transgenic birds by transferring transformed PGCs (Vick *et al.*, 1993) and in understanding the interactions between these cells and the germinal ridge.

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