Effect of prenatal treatment with busulfan on the hypothalamo–pituitary axis, genital tract and testicular histology of prepubertal male rats

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Summary. Female Wistar rats were treated with busulfan or with solvent on Day 20 of pregnancy. Thirty male offspring of each group were killed at 38 days of age. In busulfan-treated rats, compared to controls, hypothalamic LH-RH content was decreased by 52%, whereas pituitary LH and FSH concentrations were increased by 60 and 43% respectively. Plasma LH and FSH were increased by 112 and 275% respectively. Prolactin concentrations were not changed, but plasma testosterone concentration was decreased by 48%. The total number of Leydig cells per testis was decreased by 52%, and LH binding sites per testis were decreased by 70%. The total number of Sertoli cells was decreased by 44%, while FSH binding sites per testis were decreased by 62%. Spermatogenesis was practically absent after prenatal exposure to busulfan. These data demonstrate that on Day 20 of pregnancy all the dividing cells in the fetal testes were depleted by an antimitotic treatment. The stimulation of the hypothalamo–pituitary axis could have been partly induced by the decrease in testosterone production, and by the aplasia of germ cells involving modifications of the remaining Sertoli and Leydig cells.

Introduction

Germinal cell aplasia (or Sertoli cell-only syndrome) can be experimentally induced in different ways, e.g. by fetal irradiation, heat sterilization, experimental cryptorchidism, ligation of the testicular efferent ducts, vitamin A deficiency, or treatment with an alkylating compound such as busulfan (de Kretser, Kerr, Rich, Risbridger & Dubos, 1980).

Busulfan forms cross-links in DNA in mitotic cells, and so induces chromosomal aberrations and death of cells in proliferative tissues. Treatment of pregnant rats with busulfan produces sterile male offspring (Jackson, Partington & Fox, 1962; Jackson, 1970). Busulfan treatment on Day 20 of pregnancy (Gillet & Laporte, 1973a) leaves very few germ cells in the embryonic testis. Germ cell aplasia stimulates the hypothalamo–pituitary axis (Franchimont *et al.*, 1972). The aim of this experiment was to correlate the endocrinological status of prepubertal male rats with the activity of Leydig cells and Sertoli cells in the almost total absence of germ cells. The growing male rat was chosen because its hypothalamo–pituitary axis is more sensitive to the negative feedback of testosterone than that of the adult rat (Bloch, Masken, Kragt & Ganong, 1974; Negri & Gay, 1976; Caraty, Martinat & Blanc, 1981a).

Materials and Methods

Animals. Two groups of 12 pregnant female Wistar rats were treated on Day 20 of pregnancy. In Group I, rats received one subcutaneous (s.c.) injection of busulfan, 20 mg/kg (Techni-Pharma, Monaco). In Group II, the rats received one s.c. injection of solvent (olive oil-benzyl alcohol, 9:1, v/v). Thirty male offspring from Group I and 30 from Group II were killed by decapitation at 38 days of age. Testes, epididymides, ventral prostate and seminal vesicle glands were immediately dissected out and weighed. Blood samples were collected from the neck section, centrifuged and deep frozen until radioimmunoassay for LH, FSH, prolactin and testosterone. Hypothalami and pituitaries were dissected out, homogenized in 0.1 N-HCl for hypothalami, or distilled water for pituitaries, and then frozen until radioimmunoassay for LH-RH content (hypothalami) and for gonadotrophin concentrations (pituitaries). The limits of the hypothalamic samples were defined as the optic chiasma rostrally, the hypothalamic fissures laterally and the mamillary bodies posteriorly. Each hypothalamic section was approximately 2–3 mm in depth.

Radioimmunoassays. Pituitaries and plasma samples were analysed for FSH content using a specific radioimmunoassay kit for rat FSH (NIAMDD) and for LH content using a specific double-antibody radioimmunoassay as previously described (Viguier-Martinez, 1976). The results were expressed in terms of NIAMDD-rat-FSH-RP1 for FSH, and purified rat LH-SX-1-1 for LH. One unit of LH-SX-1-1 was equivalent to 1.58 units of NIH-LH-S11. The detection limit was 100 ng/ml for FSH and 0.6 ng/ml for LH; the intra-assay coefficients of variation were 10% for LH and FSH. Plasma levels of prolactin were measured using a specific radioimmunoassay (Martinat, Hall, Ravault & Dubois, 1979). The potency of the standard (PRL-I.N.R.A.) was about twice that of the NIAMDD-rat-PRL-RP1. The detection limit was 0.3 ng/ml and the intra-assay coefficient of variation was 10%. Plasma testosterone was measured after solvent extraction using a radioimmunoassay as previously described (Caraty, de Reviers, Martinat & Blanc, 1981b). The intra-assay coefficient of variation was 6% and the detection limit 50 pg/ml. LH-RH content was estimated by a specific radioimmunoassay (Caraty, de Reviers, Pelletier & Dubois, 1980). The intra-assay coefficient of variation was 8% and the detection limit 4 pg/ml. All samples of each hormone were processed in duplicate. The hormone concentrations of plasma samples were determined using logit-log transformation.

Testicular LH and FSH receptors. Numbers of LH and FSH receptor sites were measured from a pool of testicular membranes (10 testes per group) as the protein content per rat testis was not sufficient to perform individual assays.

Crude plasma membranes were prepared as previously described for the ram by Barenton & Pelletier (1980). Membrane proteins were measured by the method of Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin (BSA) as reference, after hydrolysis of the membranes for 1 h at 65°C in 0.1 N-NaOH. Testicular LH and FSH receptor numbers were measured as previously described (Viguier-Martinez, Hochereau-de Reviers, Barenton & Perreau, 1983). The detection limit was 0.2 fmol/mg protein for LH receptors, and 0.5 fmol/mg protein for FSH receptors; the intra-assay coefficient of variation was 8% for both assays.

Testicular histology. In each group, one testis of each rat was fixed in Bouin-Hollande solution. Histological analyses were performed on 5 animals chosen within the range of the mean testicular weight $\pm$ s.e.m. for each group. Intertubular and tubular tissues were analysed as described previously (Hochereau-de Reviers, Loir & Pelletier, 1976; Hochereau-de Reviers *et al.*, 1979). The total number of Sertoli cells per testis was corrected according to Abercrombie (1946).

Statistical methods. Hormonal levels and histological data were analysed using Student's *t* test. The level of significance is noted in each figure or table.

Results

Body weight and organ weights

Prenatal treatment with busulfan decreased testicular weight by 80% ($P < 0.001$; Text-fig. 1a). Body weight and the weights of the epididymis, ventral prostate and seminal vesicles were also

Text-fig. 1. Effect of prenatal treatment with busulfan on body weight and other characteristics in 38-day-old male rats. Values are mean $\pm$ s.e.m. for 30 rats/group (a, c) or 10 rats/group (b). ** $P < 0.01$, *** $P < 0.001$. For testicular weight, left and right testis have been weighed separately.

decreased by the treatment, but to a lesser extent: by 20% for body weight, by 42% for epididymis weight, by 45% for seminal vesicle weight and by 36% for ventral prostate weight, compared with values for control rats.

Hypothalamo-pituitary axis

Hypothalamic LH-RH content was significantly decreased in treated rats by about 52% ($P < 0.01$; Text-fig. 1b). In contrast, the pituitary content of gonadotrophin was significantly increased, LH by 60% ($P < 0.001$) and FSH by 43% ($P < 0.001$).

Plasma gonadotrophin concentrations were also significantly increased after busulfan treatment ($P < 0.001$, Text-fig. 1c), LH by 122% and FSH by 275%. Plasma testosterone concentration was significantly decreased by 48% in treated rats ($P < 0.001$). There was no significant difference in plasma prolactin concentrations between control and treated rats.

Testicular histology

The total volumes of intertubular tissue and of Leydig cells per testis decreased by about 73% compared with controls (Table 1).

The areas of Leydig cell cytoplasm were slightly, but significantly ($P < 0.01$), decreased in the treated rats, but the areas of Leydig cell nuclei were not modified by the treatment, resulting in an increased nucleus-cytoplasmic ratio compared with control rats. The total number of Leydig cells per testis was decreased by 52% in the busulfan-treated animals ($P < 0.001$).

The mean diameter of the seminiferous tubules was decreased by 33% ($P < 0.001$) and the total length of seminiferous tubules was decreased by 63% ($P < 0.001$) in treated rats as compared with control rats.

In 2 of the 5 animals analysed, germinal cells were absent in all the seminiferous tubules, and in the remaining 3, germ cells were present in 4% of the seminiferous tubules. The total number of Sertoli cells per testis in treated animals was only 44% of the total number present in controls ($P < 0.01$). The mean area of Sertoli cell nuclei was decreased by 16% in treated animals ($P < 0.001$). The Sertoli cells in the 4% of normal tubules in the busulfan-treated animals had a slightly greater area (11%, $P < 0.01$), as compared with the other tubules of the same animals, but this area was still lower (8%, $P < 0.01$) when compared to control animals. These tubules were morphologically normal, with all the steps of spermatogenesis present. However, the daily production of primary spermatocytes at 38 days of age by the few normal tubules in the treated animals was very small ($0.2 \times 10^6/\text{day}$) when compared with control animals ($46.0 \times 10^6/\text{day}$).

Gonadotrophin receptors

The binding affinities (K_a) of LH and FSH to their respective testicular receptors were not affected by the treatment (Table 2). Scatchard plots were linear, indicating only one class of

Table 1. Effect of prenatal treatment with busulfan on testicular histology of 38-day-old male rats

	Control rats	Busulfan-treated rats	Decrease	Significance
Testicular weight (mg)	644.0 $\pm$ 27.0	116.0 $\pm$ 8.0	82%	$P < 0.001$
Intertubular tissue volume (mm ³)	55.0 $\pm$ 3.0	14.8 $\pm$ 1.7	73%	$P < 0.001$
Leydig cells				
Total volume/testis (mm ³)	20.0 $\pm$ 1.2	7.4 $\pm$ 0.8	63%	$P < 0.01$
Total no./testis ($\times 10^6$)	4.4 $\pm$ 0.4	2.1 $\pm$ 0.2	52%	$P < 0.001$
Cytoplasmic area (μm^2)	71.0 $\pm$ 2.0	61.2 $\pm$ 0.9	14%	$P < 0.01$
Nuclear area (μm^2)	25.0 $\pm$ 1.3	26.2 $\pm$ 0.6	—	NS
Seminiferous tubules				
Total length (m)	11.8 $\pm$ 1.1	4.3 $\pm$ 0.2	63%	$P < 0.001$
Diameter (μm)	199.0 $\pm$ 2.7	133.0 $\pm$ 5.0	33%	$P < 0.001$
Daily production of primary spermatocytes/testis ($\times 10^6$)	46.0 $\pm$ 4.0	0.2 $\pm$ 0.1	99%	$P < 0.001$
Sertoli cells				
Total no./testis ($\times 10^6$)*	28.1 $\pm$ 3.0	15.7 $\pm$ 0.3	44%	$P < 0.01$
Nuclear area (μm^2)	52.0 $\pm$ 1.0	43.6 $\pm$ 0.9	16%	$P < 0.001$

Values are mean $\pm$ s.e.m. (5 testes per group).

* Corrected for nuclear size.

NS, not significant.

Table 2. Effect of prenatal treatment with busulfan on testicular gonadotrophin receptors* of 38-day-old male rats

	No. of LH receptor sites				No. of FSH receptor sites			
	K_a for LH receptors (10^{10} M^{-1})	fmol/mg protein	fmol/testis	Binding sites/Leydig cell	K_a for FSH receptors (10^{10} M^{-1})	fmol/mg protein	fmol/testis	Binding sites/Sertoli cell
Control rats	6.80	9.08	58.80	8045	1.60	14.50	94.20	2018
Busulfan-treated rats	5.97	29.32	17.70	5074	1.11	59.40	35.80	1373

* Numbers of LH and FSH receptor sites were measured for a pool of testicular membranes (10 testes per group).

binding sites for each hormone. The total number of LH binding sites, expressed per mg of membrane protein, was increased by 223% in treated animals as compared with controls. Conversely, the total number of LH binding sites per testis was decreased by 70%, and the number estimated for Leydig cells was decreased by about 37% in treated animals. The total number of FSH binding sites, expressed per mg membrane protein, was increased by 309% in the treated animals, corresponding to a decrease of 62% when expressed per whole testis and a reduction of about 32% when expressed per Sertoli cell.

Discussion

As previously shown in older male rats (Hall & Gomes, 1973), prenatal exposure to busulfan induces a decrease in plasma concentrations of testosterone. The significant decrease in hypothalamic LH-RH content may be a consequence of decreased negative feed-back by testosterone, stimulating the rate of discharge of hypothalamic LH-RH more than the rate of synthesis. Such an increase in LH-RH release has been previously observed after castration of prepubertal (38 days) and adult (121 days) rats (Payne, Kelch, Murolo & Kerlan, 1977; Badger, Wilcox, Meyer, Bell & Cicero, 1978). A low level of negative feed-back of testosterone, an increase in LH-RH concentration in pituitary portal blood (Eskay, Mical & Porter, 1977) and perhaps an increase in pituitary sensitivity to LH-RH could explain the large increase in pituitary and plasma gonadotrophins. This response was greater in our animals than that observed in adult rats by Gomes, Hall, Jain & Boots (1973). This was to be expected because, as has been shown previously by many authors, the hypothalamo-pituitary axis of the young rat is more sensitive to inhibition by testosterone than is that of the adult rat (Bloch *et al.*, 1974; Negri & Gay, 1976; Caraty *et al.*, 1981a). Plasma prolactin concentration was not affected by prenatal exposure to busulfan, indicating that neither testosterone level nor Sertoli and germ cell depletion affect prolactin secretion.

A drastic decrease in the weight of the testis and seminal vesicles has been previously observed by Hall & Gomes (1973) and a similar decrease in the weight of the testis and epididymis by Gillet & Laporte (1973a, b). The low concentration of testosterone probably accounts for this retarded development of the accessory sex organs.

Intertubular tissue volume was reduced by 73%, and this decrease was greater than the decrease in the total volume of Leydig cells, which was about 63%. This difference probably reflects a deterioration in the lymphatic and blood vessel supply to the intertubular tissue. While the total number of Leydig cells was affected by the treatment, the cytoplasmic and nuclear areas of the Leydig cells hardly differed from those of control rats. The large decrease in testicular LH receptors, expressed as fmol/testis, may be related to the decrease in the total number of Leydig cells per testis. When expressed as fmol/Leydig cell, the number of testicular LH receptors was reduced by only 37%. These results are comparable to those observed after X-irradiation of the male fetus (Rich & de Kretser, 1979) and may be due to the high plasma LH level inducing a down-regulation (Haour & Saez, 1977). The observed decrease in testosterone secretion may result mainly from the decrease in the total number of Leydig cells, and only partly from the decrease in the number of LH receptors per Leydig cell.

A significant decrease in the total length of seminiferous tubules per testis, and in their diameter, was induced by busulfan treatment, as previously observed by other authors (Gillet & Laporte, 1973a; Hall & Gomes, 1973). The daily production of primary spermatocytes, estimated on the few normal seminiferous tubules remaining after treatment, was practically zero, and the total population of Sertoli cells was decreased by 44% in busulfan-treated rats. The nuclear size of the Sertoli cells, which is related to their functional activity (Vendrelly, Guerillot & Dalage, 1972), was reduced in Sertoli cell-only tubules, while it tended to be normal in the few normal tubules of treated rats. This suggests an active interaction between germ cells and Sertoli cell differentiation. The decrease in number of FSH receptors per testis (62%) was greater than that of Sertoli cells per

testis (44%), but not comparable to that of germ cells (about 99%). This suggests that FSH receptors are mainly located on Sertoli cells.

In the rat fetus, the maximum proliferation of Sertoli cells occurs on Day 20 of pregnancy (Orth, 1982), while the Leydig cell population is formed later, between 25 and 60 days of age (Bergh & Damber, 1978). Busulfan treatment could act first by a direct effect, the destruction of a large proportion of the dividing cells *in utero* (germ cells, Sertoli cells and precursors of Leydig cells) resulting in a lower number of somatic and germ cells at birth. Our results suggest that the depletion in germ cells induced by busulfan impairs the development and functional activity of the remaining Sertoli and Leydig cells.

Sterilization induced by busulfan treatment has consequences comparable with those induced by cryptorchidism: elevation of plasma gonadotrophin concentration (Gomes & Jain, 1976) and a decrease in the number of LH and FSH receptors (Hagenäs, Ritzén, Svensson, Hansson & Purvis, 1978). However, the plasma concentration of testosterone remained normal in cryptorchid rats (Schanbacher, 1980). This suggests that treatment with busulfan during gestation, or X-irradiation of the fetus (Rich & de Kretser, 1977), has a more dramatic effect than cryptorchidism on the structure and activity of the rat testis.

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