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Differences in Spermatogenesis in Cryptorchid Testes among Various Strains of Mice

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The purpose of the study reported here was to define strain differences in spermatogenesis in cryptorchid testes in mice. Mice of strains A/J, BALB/c, CBA/N, C3H/He, C57BL/6 (B6), ddY and ICR were found to be sensitive to heat stress attributable to experimentally induced cryptorchidism. In contrast, mice of strains AKR/N (AKR), MRL/MpJ-+/+ (M+) and MRL/MpJ-*lpr/lpr* (*lpr*) were resistant to heat stress. Relative increases of apoptotic cells were detected in the sensitive group, but not in the resistant group. A decrease of proliferating cell nuclear antigen-immunoreactive cells after experimentally induced cryptorchidism was observed only in the sensitive group. These results suggested that heat stress-resistant germ cells were present in MRL and AKR strains, possibly originating from the genetic background.

About 15% of human couples have reduced fertility, and in approximately half of all cases, it is due to male infertility, usually of genetic origin (6). Cryptorchidism and Y-chromosomal microdeletions are infertility problems resulting in azoospermia or oligozoospermia (14, 19). From recent investigations in humans, it is feared that exposure to environmental hormones induces abdominal cryptorchidism, suggesting the notion that cryptorchidism could be a risk factor for testicular cancer (2, 10). Additionally, it is well known that radiation therapy against cancer gives rise to the arrest of spermatogenesis (3, 13). Infertility is necessary for maintenance of genomic quality; however, it also exists as an incurable disease that induces serious damage in humans and domestic animals (10). Therefore, identification of the heat stress-resistant factor could supply new approaches to reproductive therapy for inherent cryptorchidism and radiation-induced sterility (16).

Experimentally induced cryptorchidism has been reported in many animal species, including dogs, sheep, and wallabies, as well as laboratory rodents and rabbits, for investigation of the mechanism of germ cell loss and spermatogenesis (8, 18, 23, 27, 31). Despite extensive histologic and cytologic characterization of the heat-stressed testis over the past century, little is understood about the mechanisms of temperature-induced germ cell loss (5). In mice, it is known that the seminiferous tubules exist in 12 stages, and it takes 4.5 cycles of 233.6 h for spermatogonia to mature. In this process, the premiotic spermatocytes (leptotene, zygotene, pachytene) and first-step spermatids are more sensitive to heat stress, resulting in germ cell loss due to cryptorchidism and irradiation (4, 25). Recent studies have indicated that the germ cell loss induced by heat stress is due to apoptosis, and is associated with the p53 pathway, although the relationship with the Fas pathway is still not clear (30, 32).

Mice, with about 500 inbred strains and about which we have much genetic information, are quite useful for comparative analysis of the phenotypes and diseases associated with strain differences. For example, large numbers of apoptotic cells are detected among the first metaphasic spermatocytes in seminiferous tubule stage XII in MRL/MpJ mice, but not in that in other strains (15, 17). These strain differences are caused by genetic factors that play major roles in determining the phenotypic variations observed. We recently reported that genetic mutation of *exonuclease 1* was associated with meiotic metaphase-specific apoptosis in MRL/MpJ mice (21). The purpose of the study reported here was to clarify strain differences with regard to heat stress associated with experimentally induced cryptorchidism.

Materials and Methods

Animals. Mice of strains A/J, AKR/N (AKR), BALB/c (BALB), CBA/N (CBA), C3H/He (C3H), C57BL/6 (B6), ddY, ICR, MRL/MpJ-+/+ (M+), and MRL/MpJ-*lpr/lpr* (*lpr*), aged eight to nine weeks, were studied. Specific-pathogen-free mice were purchased (Japan SLC, Inc., Hamamatsu, Japan), and were maintained under conventional conditions. Microbial monitoring for the following agents was carried out: Sendai virus, mouse hepatitis virus, ectromelia virus, *Pseudomonas aeruginosa*, *Salmonella* spp., *Pasteurella pneumotropica*, *Escherichia coli O115*, *Corynebacterium kutscheri*, *Mycoplasma* spp., Tyzzer's organism, *Syphacia* spp., *Giardia* spp., *Spironucleus* spp., *Trichomonas* spp., and *Entamoeba* spp. Mice had negative test results for all the aforementioned infective agents. The animal room was kept at 24 ± 2°C and 50 to 70% humidity under a 12-h light-dark cycle. Mice were housed in polycarbonate cages (210 × 310 × 120 mm [length by width by depth]) with sterilized wood chip bedding. A commercially formulated standard diet (MR-A2; Japan SLC, Inc.) and UV-light sterilized water were provided ad libitum. Research was conducted according to the *Guidelines for the Care and Use of Laboratory Animals of Graduate School of Veterinary Medicine of Hokkaido University*. The experimental protocol was approved by

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the Institutional Animal Care and Use Committee, School of Veterinary Medicine, Hokkaido University.

Experimentally induced cryptorchidism. Animals were anesthetized with sodium pentobarbital (50 mg/kg of body weight, i.p.), and an abdominal incision was made. To induce unilateral cryptorchidism, the right testis was manipulated through the inguinal canal into the abdomen and sutured to the abdominal wall through the epididymal body by use of silk thread. For investigation of strain differences, all strains, the testes of which were translocated to the abdomen, were sacrificed by use of cervical dislocation at 14 days after surgery. Additionally, for comparison of postsurgical changes, strains B6, ICR, M+, and *lpr* were examined on days 7, 10, and 21 after surgery by use of the same procedure. To investigate genetic effects causing heat stress resistance, F1 progeny among the couples of either B6, M+, or AKR were produced, and similar surgery was performed on them. The removed testes were weighed and fixed overnight in either Bouin's solution or 4% paraformaldehyde solution. Four-micrometer-thick paraffin sections prepared in routine manner were treated with hematoxylin and eosin, by use of the TUNEL reaction, or with proliferation cell nuclear antigen (PCNA)-immunohistochemical stain.

Histologic analysis. For the quantitation of germ cells, the Sertoli cell index was estimated by counting the numbers of spermatogonia, early spermatocytes (preleptotene, leptotene, zygotene), late spermatocytes (pachytene, diplotene, metaphase), round spermatids, and elongated spermatids per Sertoli cell in 20 cross sections of seminiferous tubules from each cryptorchid strain. For comparison with normal spermatogenesis, the Sertoli cell index of each germ cell type was calculated for 100 cross-sections of seminiferous tubules from three testes each of B6 and ICR mice aged eight to nine weeks. The count was performed independently and repeatedly by three persons, using blinded preparations.

Staining for the TUNEL reaction. The deparaffinized sections were autoclaved for five minutes in 121°C in 2× standard saline citrate (SSC) (1× SSC = 150 m*M* NaCl, 15 m*M* sodium citrate, pH 7.0), then were rinsed with distilled water. The sections were incubated with a reaction solution including terminal transferase (Wako Purechem., Japan; 1 U/μl), 1 μ*M* digoxygenin-deoxyuridine triphosphate (DIG-dUTP; Roche Diagnostics Co., Tokyo, Japan, 200 m*M* potassium cacodylate, 25 m*M* Tris-HCl, bovine serum albumin (0.25 mg/ml), 5 m*M* $CoCl_2$ (pH 6.6), for 60 to 90 min at 37°C in a humidified chamber. After being washed in stop solution (30 m*M* sodium citrate, 300 m*M* NaCl), the sections were incubated with a blocking buffer composed of 100 m*M* Tris-HCl, 150 m*M* NaCl, and 1% blocking reagent (Roche Diagnostics Co.) for 30 min at room temperature, then with anti-DIG antiserum conjugated to alkaline phosphatase (Roche Diagnostics Co.) for two hours at room temperature. After three washes in Tris-HCl buffer, the sections were reacted with a colored substrate consisting of 4.5 μl of nitroblue tetrazolium, 3.5 μl of X-phosphate (0.75 ml of 5-bromo-chloro-3-indolyl phosphate and 50 mg of toluidinium/ml of dimethyl formamide) in one milliliter of a solution composed of 100 m*M* Tris-HCl, 100m*M* NaCl, 50 m*M* $MgCl_2$ (pH 9.5) in a dark room for 60 to 90 min at room temperature. The sections were washed in a solution of 10 m*M* Tris-HCl and 1 m*M* EDTA (pH 8.0) for five minutes, then in distilled water, and finally were mounted in glycerin-gelatin.

For estimation of apoptosis, three mice of each strain (B6, ICR, M+, *lpr*) were examined at seven, 10, 14, and 21 days after surgery. All seminiferous tubules in TUNEL-stained sections were classified into grades 0 to 4 by the number of TUNEL-positive cells. Briefly, grades 0, 1, 2, 3, and 4 indicated presence of 0, 1 to 5, 6 to 10, 11 to 15, and 16 or more TUNEL-positive cells per tubule, respectively. Grade-0, -1, -2, -3, and -4 tubules were scored 0, 1, 2, 3, and 4, respectively. The TUNEL-positive index was estimated by multiplication of each graded tubule by its score. Finally, the ratio of the TUNEL-positive index in cryptorchid testis to that of the intact testis was compared among four strains. The count was performed independently and repeatedly by three persons, using blinded preparations.

Immunostaining with PCNA. The sections of testes fixed with 4% paraformaldehyde were deparaffinized and incubated with methanol containing 0.15% H_2O_2 for 30 min at room temperature. After being washed with distilled water, the sections were incubated with a solution composed of 100 m*M* Tris-HCl, 150 m*M* NaCl and 1% blocking reagent (Roche Diagnostics Co.) for 30 min at room temperature, then with the anti-PCNA monoclonal antibody Ab-1 (1:400 dilution; Oncogene Research, Cambridge, Mass.) at 4°C overnight. After 3 washes with 0.01*M* phosphate-buffered saline (PBS; 1*M* PBS = 0.8*M* $Na_2HPO_4 \cdot 12H_2O$, 0.2*M* $NaH_2PO_4 \cdot 2H_2O$, 14.5*M* NaCl), the sections were incubated with a biotinylated anti-mouse Ig (IgM+IgG+IgA, H+L)-goat $F(ab)_2$ fragment, then with avidin-biotin-complex (VECTASTAIN, Vector Laboratories Inc., Burlingame, Calif.) for 60 min each at room temperature. After three washes with 0.01*M* PBS, the sections were incubated with a visualizing solution composed of 0.05*M* Tris-HCl (pH 7.6), and 0.2 mg of diaminobenzidine tetrahydrochloride/ml containing 1% H_2O_2 for five minutes at room temperature.

Statistical analysis. Data are presented as arithmetic mean ± SEM. Statistical analysis was done by use of analysis of variance and a post-hoc test (Fischer's protected least significant difference [PLSD]) to examine significant differences among three or more groups. The significance of the difference between two groups was determined by use of Student's *t* test. The *P* values were assessed from the difference between the two proportions, and $P < 0.05$ was considered statistically significant.

Results

Changes in testis weight after experimentally induced cryptorchidism. Body weight, absolute weight of the cryptorchid and intact testes, and ratio of the weight of the cryptorchid testes to that of the intact testis at 14 days after surgery are shown in Table 1. Absolute testis weights were not significantly different from the reference data (Japan SLC Inc.), although those of the cryptorchid testis decreased more or less in all strains. In strains A/J, BALB, C3H, CBA, B6, ddY, and ICR at 14 days after experimentally induced cryptorchidism, the weight of cryptorchid testes was decreased to less than half that of intact testes. On the other hand, the weight of cryptorchid testis of strains AKR, M+ and *lpr* was approximately 70% of that of the intact testis. We tentatively called the former seven strains the heat-stress-sensitive group, and the latter three strains the resistant group. The differences for these values were significant between two groups, as determined by use of Fischer's PLSD, with a 95% confidence interval.

To examine the changes in the testis weight after cryptorchidism, four strains (B6 and ICR from the sensitive group and M+ and

Table 1. Body weights, absolute weights of cryptorchid and intact testes, and ratios of the weight of the cryptorchid testis to that of the intact testis at two weeks after experimentally induced cryptorchidism in 10 mouse strains

Strain	n	Body weight (g)	Cryptorchid testis weight (mg)	Intact testis weight (mg)	Cryptorchid testis/intact testis
A/J	5	25.56 ± 1.74	37.60 ± 3.38	100.40 ± 4.36	0.38 ± 0.05
C3H	3	25.03 ± 0.31	33.33 ± 2.05	87.33 ± 3.30	0.38 ± 0.02
B6	9	26.64 ± 2.50	38.89 ± 9.96	97.00 ± 13.46	0.40 ± 0.09
ddY	4	42.00 ± 2.12	48.25 ± 2.95	114.75 ± 3.49	0.42 ± 0.02
BALB	5	23.16 ± 0.52	39.40 ± 6.02	92.40 ± 4.41	0.42 ± 0.05
ICR	7	40.54 ± 1.27	56.57 ± 16.80	122.57 ± 11.07	0.46 ± 0.12
CBA	6	26.94 ± 0.78	29.67 ± 4.99	63.50 ± 1.98	0.47 ± 0.09
AKR	5	26.55 ± 0.81	40.00 ± 10.18	59.20 ± 3.06	0.67 ± 0.16*
M+	19	38.61 ± 2.67	70.37 ± 10.14	103.31 ± 9.46	0.68 ± 0.09*
lpr	7	38.97 ± 1.18	68.60 ± 3.98	90.20 ± 6.68	0.77 ± 0.08*

*$P < 0.05$, compared with mice of the heat stress-sensitive group (A/J, C3H, B6, ddY, BALB, ICR and CBA).
Data are expressed as mean ± SEM.

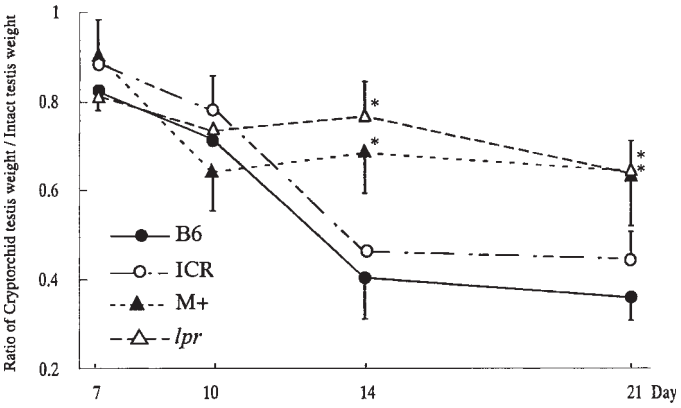

Figure 1. Change in testis weight ratios (cryptorchid testis/the intact side) from seven to 21 days after experimentally induced cryptorchidism comparing B6, ICR, M+, and *lpr* mouse strains. Values are mean ± SEM. *Significant difference compared with testes of the heat stress-sensitive group ($P < 0.05$).

lpr from the resistant group) were chosen and their testis weights were compared from seven to 21 days after surgery (Fig. 1). During the experimental period, body weight and absolute and relative weights of the intact testes for each strain were not significantly changed. The decrease in the testis weight ratio was similar until 10 days in the four strains, but then the decrease continued in B6 and ICR mice until 14 days, whereas in M+ and *lpr* mice, the decrease plateaued after 10 days.

Histologically, in both groups, nuclear pyknosis and cellular shrinkage in parallel with germ cell loss were observed at seven and 10 days after surgery, and multinucleated giant cells also were detected in some tubules. On day 14 after cryptorchidism, germ cell loss continued in the sensitive group, resulting in the appearance of only spermatogonia and Sertoli cells in the seminiferous tubules at 21 days. In contrast, in resistant groups, many pachytene spermatocytes and round spermatids were still being distributed after 14 days; however, elongated spermatids were not observed throughout the sections examined. These results indicated that the strain difference was manifested between 10 and 14 days after cryptorchidism was established (Fig. 2).

Germ cell loss. The details of this strain difference were examined by comparison of germ cell numbers with the Sertoli cell index (Fig. 3). During normal spermatogenesis, the seminiferous tubules exist in stages I to XII, with morphologic and topographic presentation of germ cells; however, since in the cryptorchid testes, it was difficult to distinguish each stage, germ cells were counted at random in fields of 20 seminiferous tubules. In the testes of B6 and ICR mice used as controls, germ cells were counted at random in the fields of 100 seminiferous tubules, because of the existence of spermatogenetic stages. The index of spermatogonia in each strain, about 0.2 to 0.4, was not significantly different, compared with that in controls. Values of 1.0 to 1.6 were detected for early spermatocytes except in B6 mice, in which the value was markedly reduced to about 0.1. In late spermatocytes, values of 1.6 to 2.1, similar to the 2.3 to 2.4 values in controls, were observed in AKR, M+, and *lpr* mice, whereas values were 0.2 to 0.8 in the other strains. None or only a few round spermatids were distributed in cryptorchid testes, except in strains AKR, M+, and in *lpr* mice, in which values were about 0.8 to 2.4. In controls, there were many round and elongated spermatids, the values of which were about 3.6 to 5.0.

Apoptosis and cell proliferating activity. To examine the relationship between germ cell loss and apoptosis caused by cryptorchidism, TUNEL reaction staining was performed for the sensitive (B6 and ICR) and resistant (M+ and *lpr*) groups from seven to 21 days after surgery (Fig. 4). Since it is known that, in the testis of two MRL strains, a high frequency of metaphase-specific apoptosis is observed in the seminiferous tubules at stage XII, even in the intact testis, high rates of apoptosis were observed, compared with those in B6 and ICR strains. Cryptorchidism in the sensitive group increased apoptosis occupying a seminiferous tubule 3.0- to 4.3-fold at seven days, then 4.7- to 5.9-fold at 10 days, whereas in the resistant group, marked changes were not observed. These results indicated that germ cell loss after cryptorchidism in the sensitive group, but not in the resistant group, was due to the increase of apoptosis. Furthermore, significant differences were not observed between M+- and Fas-deficient mice, *lpr*, suggesting that the Fas-pathway was not involved in apoptosis after cryptorchidism.

For investigation of cell cycle activity, PCNA expression on the nuclei of the proliferating cells was immunohistochemically determined. At 10 days after surgery in B6 mice, the relative number of the PCNA-positive cells occupying a seminiferous tubule in the cryptorchid testis, compared with that in the intact testis, was decreased to 83.1 ± 10.2%, whereas the figure was 97.9 ± 3.2% in M+ mice. From these results, it was suggested that the germ cells of the resistant group maintained higher proliferative activity after cryptorchidism than did those of the sensitive group.

Discussion

For several decades, experimentally induced cryptorchidism has been performed in many kinds of animals to clarify the mechanisms of spermatogenesis. However, the studies have been focused on gene expression associated with the early phase of heat stress during spermatogenesis, especially within 24 h after

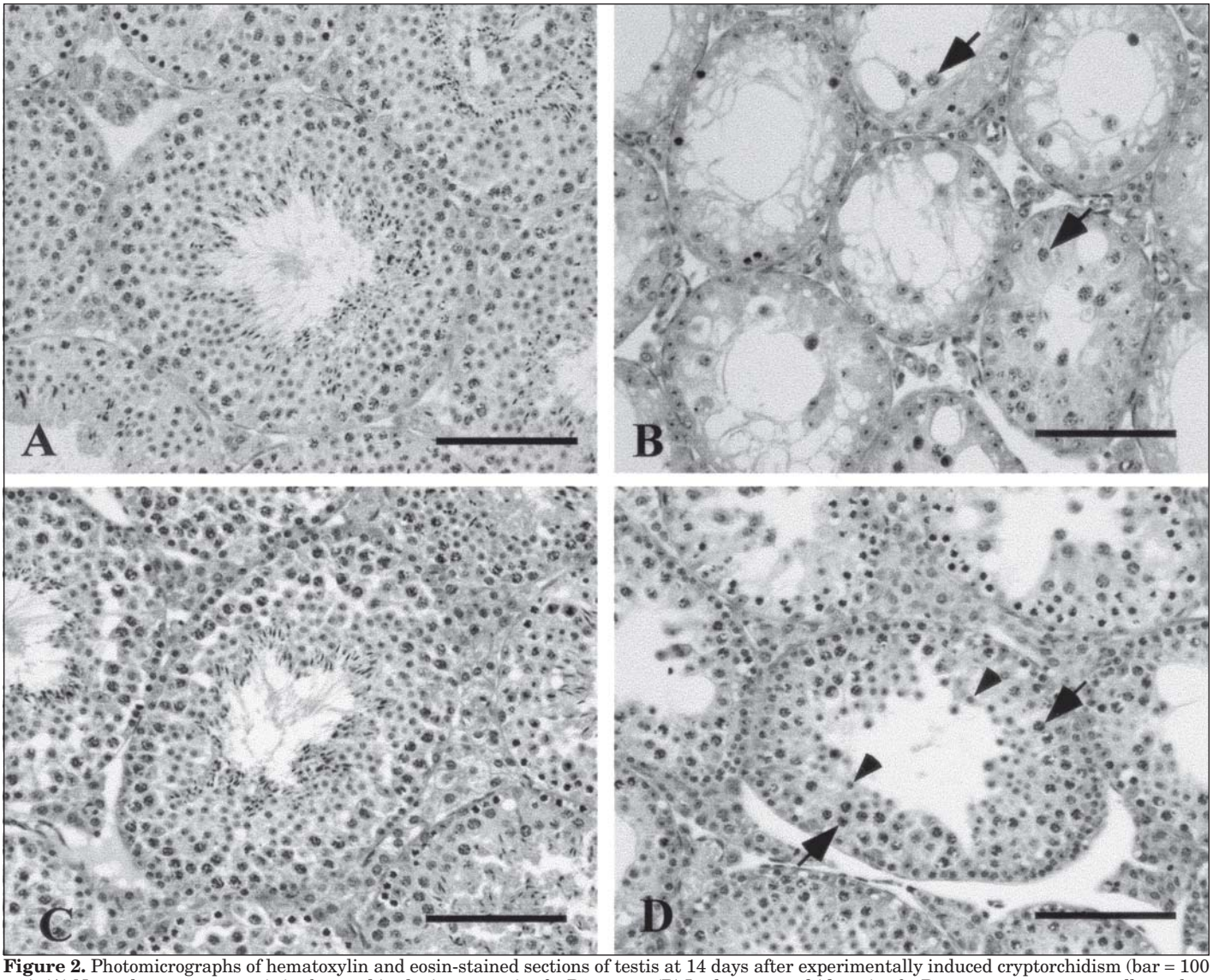

Figure 2. Photomicrographs of hematoxylin and eosin-stained sections of testis at 14 days after experimentally induced cryptorchidism (bar = 100 μm). (A) Normal spermatogenesis is observed in the intact testis of a B6 mouse. (B) In the cryptorchid testis of a B6 mouse, many germ cells are lost and only Sertoli cells, spermatogonia, and a few spermatocytes survive (arrows). (C) Normal spermatogenesis like that of B6 is observed in the intact testis of an M+ mouse. (D) Germ cells, except for elongated spermatids, are observed in the cryptorchid testis of an M+ mouse (arrows = spermatocytes, and arrowheads = round spermatids).

surgery (22). Additionally, strain differences in the response to heat stress in the testis remain unclear. We previously found a strain difference by examining the response to heat stress in five inbred mouse strains, of which MRL/MpJ-+/+ had the most unique phenotype (16). Our results provide additional evidence about the heat stress-resistant trait in many inbred strains, including *lpr* and AKR.

The ratios of testis weight and the Sertoli cell index at 14 days after surgery were compared among ten strains. Results indicated that the decrease of cryptorchid testis weight in the heat stress-sensitive group was due to the loss of germ cells, especially late spermatocytes and round and elongated spermatids. On the other hand, in the resistant group, the number of round spermatids in the cryptorchid testis was about half that of the intact testis, and almost all of the late spermatocytes survived. These results indicated that, in the resistant group, germ cell loss was inhibited at the phase of transition from early to late spermatocytes. However, even in the resistant group, the initial decrease of round and elongated spermatids suggests that there are some pathways in which the heat stress induces the loss of germ cells in spermatogenesis, and that the main pathway acting after 10 days is blocked by unknown factors. This hypothesis is in agreement with previous reviews indicating that spermatogenesis is controlled by many factors, including hormones, testis-specific genes, and some apoptosis-associated factors (12, 24, 26).

In the case of heat stress associated with cryptorchidism, it is well-known that pathologic germ cell death occurs via an apoptotic mechanism (9, 11, 28). Nothing else about the effect of heat stress on cell proliferation has been reported to our knowledge, although PCNA-positive nuclei are observed during the G1, S, and G2/M phases of the cell cycle (29). For this reason, the mechanism of germ cell loss in cryptorchidism should be reexamined, including previous reports in accordance with cell death/cell proliferation balance. In any case, the low rate of apoptosis and

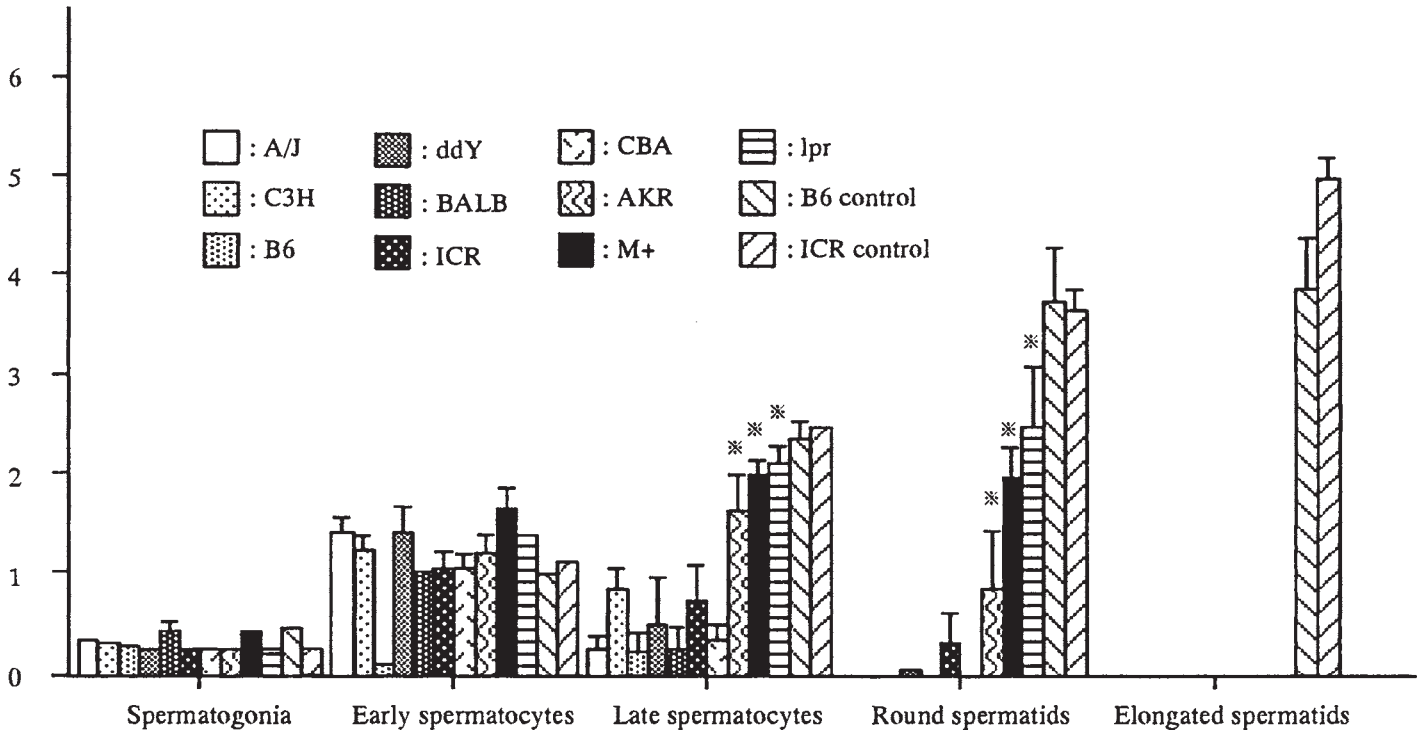

Figure 3. Sertoli cell index in the cryptorchid testes at 14 days after experimentally induced cryptorchidism of 10 mouse strains and in the control testes of B6 and ICR mice. Sertoli cell index was estimated by counting the numbers of spermatogonia, early spermatocytes, late spermatocytes, round spermatids, or elongated spermatids per Sertoli cell in 20 cross sections of seminiferous tubules from each cryptorchid strain. Values are mean ± SEM. *Significant difference compared with values for testes of the heat stress-sensitive group ($P < 0.05$).

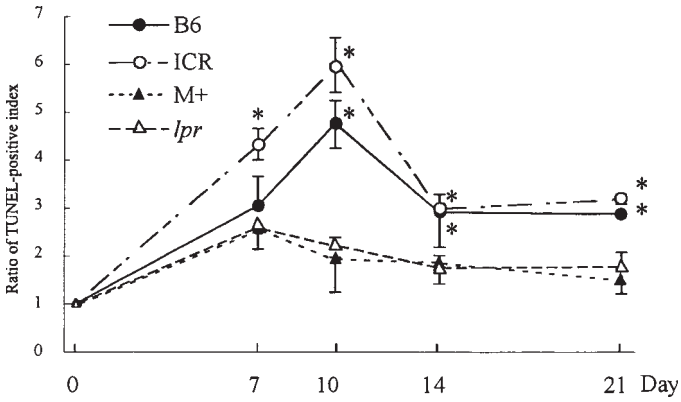

Figure 4. Changes in numbers of TUNEL-positive cells at 0 to 21 days after experimentally induced cryptorchidism in B6, ICR, M+ and *lpr* mouse strains. The vertical value shows the ratio of the TUNEL-positive index described in Materials and Methods. Values are mean ± SEM. *Significant difference compared with testes of the heat stress-resistant group ($P < 0.05$).

high rate of proliferation in the testis of heat stress-resistant strains identified in this study could indicate additional specialized mechanisms for spermatogenesis.

The homozygous mutation, *lpr* (lymphoproliferation) is the insertion of a transposon into chromosome 12, causing a deficiency of the Fas receptor, and has been used as a model for lupus erythematosus (1, 20). In the study reported here, significant difference in the resistance against heat stress was not observed between mouse strains M+ and *lpr*, both of which had the same genetic background. These results indicated that the main cause of heat stress resistance did not involve the Fas-Fas ligand pathway, but the genetic background of MRL. It is not surprising that AKR mice also manifested heat-stress resistance, because the MRL mouse originated from LG (75.0%), AKR (12.6%), C3H (12.1%), and B6 (0.3%) backgrounds (Mouse Genome Informatics, http://www.informatics.jax.org).

In the study reported here, despite the sensitivity in C3H and B6 mice, the AKR mouse manifested heat stress resistance like M+ and *lpr* mice, suggesting the possibility that a factor(s) causing heat stress resistance in the testis might be involved in the genetic background of the MRL/MpJ mouse, especially in the 12.6% of the genome that originated from the AKR mouse.

Somatic cells are protected from thermal insult by inducing a set of heat shock proteins (*hsps*), which function as molecular chaperones to maintain proteins in their native folded structure (7). Of all *hsp* genes, *hsp70i* (inducible), which belongs to the *hsp70* multigene family, usually has the highest expression level in cells exposed to heat shock. In spermatocytes and spermatids, the induction level of expression of the *hsp70i* genes seems to be lower than that in somatic cells, and it has not yet been determined whether the increased level of HSP70i expression could have any protective effect on these cells (5). Our results raised the possibility that a heat stress-resistant factor(s) existing in the heat stress-resistant mouse strains was not involved the *hsp* multiple gene family. In any case, the goal of this study was to find the real heat stress resistance gene by using analysis of quantitative trait loci, and we are about to do it with F2 and backcross progeny.

In conclusion, we confirmed the strain difference in the degree of cell loss in the seminiferous tubules associated with experimentally induced cryptorchidism and observed heat stress resistance in the testes of the AKR, M+, and *lpr* mice. Identification of

the heat stress resistance factor(s) is important for more detailed understanding of the mechanism of spermatogenesis and development of a therapy for male infertility.

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