TOXICOLOGICAL SCIENCES **85**, 675–682 (2005)
doi:10.1093/toxsci/kfi071
Advance Access publication December 29, 2004

Effects of Sulfasalazine on Sperm Acrosome Reaction and Gene Expression in the Male Reproductive Organs of Rats

Tamio Fukushima,*,†,[1] Masashi Kato,* Tetsuya Adachi,‡ Yoshimasa Hamada,* Masao Horimoto,* Masatoshi Komiyama,†,§ Chisato Mori,† and Ikuo Horii*

*Worldwide Safety Sciences, Nagoya Laboratories, Pfizer Japan Inc., 5-2, Taketoyo, Aichi 470-2393, Japan; †Department of Bioenvironmental Medicine, Graduate School of Medicine, Chiba University, 1-8-1, Inohana, Chiba 260-8670, Japan; ‡Department of Genomic Drug Discovery Science, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto 606-8501, Japan; and §Center for Environment, Health and Field Sciences, Chiba University, Kashiwa 227-0882, Japan

Received November 1, 2004; accepted December 20, 2004

Sulfasalazine (SASP) has been reported to depress the fertility in men and experimental male animals, but the fundamental mechanisms of infertility caused by SASP are still unknown. This study was designed to investigate the mechanisms of infertility in rats treated with SASP at a dose of 600 mg/kg for 28 days, including monitoring of sperm motility using computer associated sperm analysis system and acrosome reaction by FITC-concanavalin A lectin staining. The sperm motility and acrosome reaction, which are important for fertilization, were significantly reduced by SASP. Furthermore, to investigate the molecular mechanisms of infertility induced by SASP, mRNA expression analysis in the testes was performed using cDNA microarray as a first screening. It was revealed that CD59, which is located on the acrosomal membrane and is known to be important for the reproductive function of sperm, was affected in the testes; this was also confirmed by real-time PCR analysis, but the spermatogenesis-related genes examined in this study were not affected. Therefore, we focused on *CD59* and two other acrosome membrane related-genes: *MCP* and *DAF*. *CD59*, *MCP*, and *DAF* in the epididymides of SASP-treated rats were significantly decreased as assessed by real-time RT-PCR analysis and additionally, the expression of CD59 protein was found to be decreased by Western blotting. These results allowed us to hypothesize that the suppression of epididymal acrosomal membrane proteins synthesis with their consequent reduced incorporation to the sperm membrane leads to a depressed sperm motility and acrosome reaction, and thereby leads to infertility in SASP treated male rats.

Key Words: **sulfasalazine; acrosome reaction; *CD59*; mRNA expression.**

Sulfasalazine (SASP, salicylazosulphapyridine) was introduced more than 60 years ago as a drug to treat rheumatoid arthritis (Svartz, 1942). This drug has also been used in the treatment of acute attacks of ulcerative colitis and in Crohn's disease, particularly when the colon is involved (Baron *et al.*, 1962; Summers *et al.*, 1979). However, male infertility was reported by Toth (1979) and Levi *et al.* (1979) in SASP-treated patients and it was known as a side effect of SASP. Several clinical studies have been performed to investigate the mechanisms of SASP-induced infertility. O'Morain *et al.* (1984) evaluated semen obtained from patients who had been treated with SASP for more than three months at a dose of 2000–4000 mg/day, and they found reduced semen quality such as decreased sperm motility or an increase of morphologically abnormal sperm. Assessment of sperm function by *in vitro* fertilization tests using hamster eggs has shown a significantly decreased penetration rate in sperm obtained from patients with inflammatory bowel disease on SASP therapy (O'Morain *et al.*, 1983).

Several studies have been performed in animals to investigate the effects of SASP on fertility and sperm. Treatment-related reduction of fertility or sperm motility was observed in male rats treated with SASP for five weeks, which was equivalent to the period of spermatogenesis in rats, but fertility was restored by three weeks after withdrawal (Pholpramool and Srikhao, 1983). Therefore, it is likely that the target organ of SASP is not only the testis, which is the main organ for spermatogenesis, but also the epididymis, where sperm maturation occurs. Some studies have shown a reduction of sperm concentration and motility in rats treated with SASP (Horimoto *et al.*, 2000; Sharma and Kalla, 1994). But, Hoyt *et al.* (1995) suggested that decreased sperm concentrations and alteration of motility, while contributory, may not be the primary causes of SASP-induced infertility because the impairment of fertility was observed although sperm concentration and motility were unchanged. A decrease of the penetration rate as evaluated by *in vitro* fertilization assay and inability of spermatozoa to undergo the acrosome reaction were observed in SASP-treated hamsters (O'Morain *et al.*, 1985). On the other hand, increased surface abnormalities in rat sperm were reported by Sharma and Kalla (1994). Thus,

[1] To whom correspondence should be addressed at Worldwide Safety Sciences, Nagoya Laboratories, Pfizer Japan Inc., Taketoyo, Aichi 470-2393, Japan. Fax: (81)-569-74-4752. E-mail: tamio.fukushima@japan.pfizer.com.

although several studies regarding the effects of SASP on sperm have been performed, the detailed mechanism of infertility induced by SASP remains unknown.

The present study was performed to investigate the mechanisms of SASP-induced infertility in rats. Sperm functions, such as motility and acrosome reaction, which are known as important factors for fertilization, were investigated in male rats rendered sub-fertile by treatment with SASP at the dose of 600 mg/kg for 28 days. Furthermore, to investigate the cause of the effect of SASP on sperm motility and acrosome reaction at the molecular level, alterations of gene expression in the testes were analyzed using cDNA microarray. The expression of various genes selected by cDNA microarray screening and their related genes was further investigated in the testes or epididymides using real-time reverse transcription and polymerase chain reaction (RT-PCR) analysis.

MATERIALS AND METHODS

Animals and treatment. Nine-week-old male ($n = 40$) and 11-week-old female ($n = 20$) Crj:CD(SD)IGS rats were purchased from Charles River Japan, Inc. (Hino, Shiga, Japan). The animals were acclimated to our laboratory for seven days prior to treatment and then randomly assigned to a control group and an SASP treatment group according to their body weight. The animals housed in individual cages were maintained under controlled conditions of light (12 h light/dark cycle) and temperature (23 to 25°C) and allowed to eat pelleted feed and drink municipal drinking water *ad libitum*. The males were treated with sulfasalazine (SASP) (Sigma, St. Louis, MO) once daily by oral gavage at a dose of 600 mg/kg for 28 days. The control animals were treated with 0.5% methylcellulose. We selected this dose and treatment period because it was shown previously to affect sperm motion and fertilization (Horimoto *et al.*, 2000). For the mating test, the males were further treated with the drug during cohabitation with untreated females after the dosing for 28 days. Half of the males purchased were used for the mating test and the remaining males were used to determine reproductive organs weight, for the sperm motility analysis, to detect acrosome reaction and for gene expression and Western blot analysis. Experiments were designed to minimize the number of animals and the study was in agreement with the Institute of Animal Care & Use Committee of Pfizer Inc.

Mating test. All males ($n = 10$) in each group were placed in cages to cohabit with untreated females (14 weeks old at the time of copulation) in the proestrus in the late afternoon of the last dosing day. Next morning, mating was confirmed by the presence of a copulatory plug and/or sperm in the vaginal saline lavage. The day on which mating was confirmed was designated as Day 0 of gestation. Mated females were subjected to cesarean section on Day 14 of gestation and the number of corpora lutea and implants was counted. The conceptuses were classified into resorption, dead fetuses, and live fetuses.

Sample collection. The ten SASP-treated and ten control males were euthanized on the next day of the last dosing. The testes, epididymides, prostate, and seminal vesicles were removed and weighed. The left testis was prefixed in 10% neutral buffered formalin and then fixed in bouin for pathological examination. Sperm was collected from the left cauda epididymis for sperm motility analysis and acrosome reaction analysis. The right testis and epididymis were stored in liquid nitrogen until use for gene expression analysis.

Pathological examination. The fixed testis was trimmed, dehydrated, embedded in paraffin, sectioned, mounted on glass slides, stained with hematoxylin and eosin, and examined by light microscopy.

Sperm motility analysis. The left cauda epididymis was washed with 2 ml phosphate buffered saline (PBS) (Invitrogen Corp., Carlsbad, CA) warmed at 37°C and cut in 3 ml Krebs-Ringer modified medium containing 0.5% bovine serum albumin to allow the sperm to disperse in the medium (Toyoda and Chang, 1974). After sperm concentration was determined using a haemocytometer, aliquots of the sperm suspension were transferred to 1 ml fresh medium to obtain a final concentration of 1.0×10^6 cells/ml, and incubated in an atmosphere of 5% CO_2 and 95% air at 37°C for 10 min.

Just after incubation, the sperm suspensions were loaded into flat 100 μM deep cannulae (HTR 1078; VitroCom Inc., Mountain Lake, NJ) for sperm motility analysis using an HTM-IVOS motility analyzer (Hamilton Thorne Research, Beverly, MA) with software version 10.8. For each rat, ~450 sperm tracks (range of 392–549) were measured. The setting conditions were as follows: frames acquired = 30, frame rate = 60 Hz, minimum contrast = 85, minimum cell size = 2 pixels, minimum static contrast = 25, straightness (STR) threshold = 50.0%, path velocity (VAP) cutoff = 25.0 μm/s, prog. min VAP = 150.0 μm/s, straight line velocity (VSL) cutoff = 7.0 μm/head size, nonmotile = 8 pixels, cell intensity = 70, static head size = 0.21 to 4.98, static head intensity = 0.10 to 2.28, static elongation = 1 to 25, slow cells motile = no, magnification = 0.82, video frequency = 60, bright field = no, LED illumination intensity = 3100, temperature set = 37.0°C, chamber depth = 100 μm, % motile sperm = the ratio of number of motile sperm to total number of sperm (%) and % progressively motile sperm = the ratio of number of motile sperm with both path VAP > 150 μm/s and STR > 50.0%.

Detection of acrosome reaction. Sperm samples were prepared in the same manner as described above and incubated for 0, 1, 3, and 5 h. Then the sperm suspensions were centrifuged at 10,000 × *g* for a few seconds to collect sperms. After washing the sperms twice with 1 ml PBS, they were stained with 0.2 mg FITC-concanavalin A lectin (Vector Laboratories, Inc., Burlingame, CA) at room temperature for 30 min. The sperms were washed once with PBS and collected by centrifugation. A drop of the stained sperms was placed on a slide glass and covered with a cover slip. Then the spermatozoa were examined under a fluorescence microscope (BX51; Olympus Optical Co. Ltd., Tokyo, Japan) equipped with a B excitation unit for wavelengths between 460 and 490 nm and an O530 emission filter passing wavelength over 530 nm. Figure 1 shows sperm cells with or without acrosome reaction. Acrosome-reacted sperm show especially bright acrosome and obvious darkness in the head region (Fig. 1A), whereas acrosome-intact sperm displays bright fluorescence over the entire sperm head (Fig. 1B). Three-hundred spermatozoa were examined and the ratio of sperms showing acrosome reaction was calculated in each group. In a preliminary study, we confirmed the validity of this staining method as follows. The epididymal sperm prepared from a mature rat were incubated for 1 h in the same medium as sperm motility analysis. Calcium ionophore A23187 (Sigma, St. Louis, MO) was added to the sperm suspension at a final concentration of 0.01 μM and the suspension was incubated for 30 additional min. The sperms were stained with FITC-concanavalin A lectin in the same manner and the number of acrosome-reacted and acrosome-intact sperm was determined. Figures 1A and 1B show representative photomicrographs of sperm incubated with or without calcium ionophore. The ratio of acrosome-reacted sperm was 49.8% in the calcium ionophore added group while it was only 16.1% in the control group.

RNA extraction and purification of poly(A)$^+$ RNA. Total RNA was extracted from testes and epdidymides of each animal using TRIZOL Reagent (Invitrogen Corp.). Prior to the extraction, the epididymides were cut at the center of the corpus and divided into the cranial half (caput region) and caudal half (cauda region). Poly(A)$^+$ RNA was purified from the total RNA using Oligotex-dT30 <Super>mRNA Purification kit (TaKaRa, Tokyo, Japan). The quality of the poly(A)$^+$ RNA was checked using Agilent 2100 Bioanalyzer (Agilent Technologies, Tokyo, Japan).

Microarray analysis of mRNA expression in the testes. Ten arrays (one array per animal) each were used for each of the control and SASP-treated groups. Approximately 3 μg of poly(A)$^+$ RNA from each animal was converted to cyanine 5-dUTP (Perkin Elmer Lifescience, Kanagawa, Japan) labeled

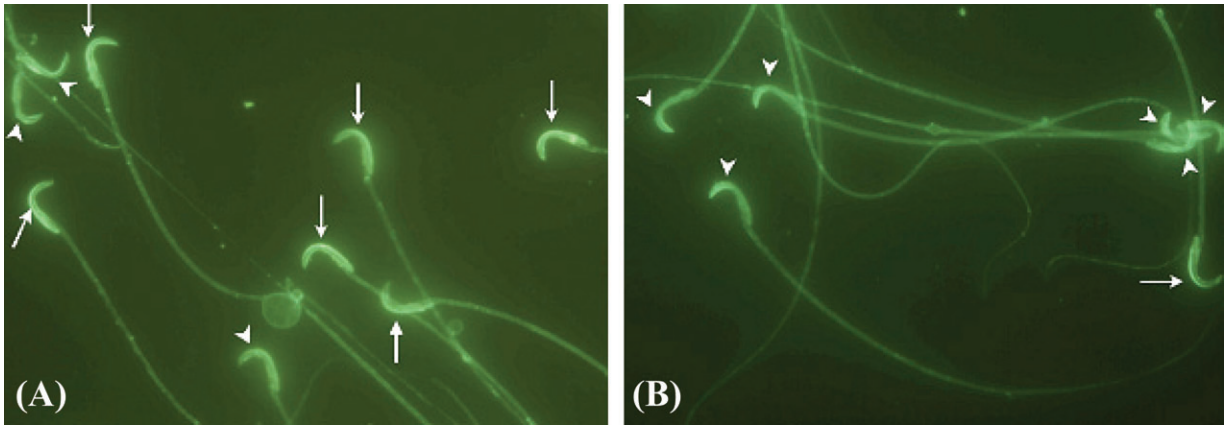

FIG. 1. Fluorescence photomicrographs of sperms stained with FITC-concanavalin A lectin. The arrows point to acrosome-reacted sperm with the head exhibiting the especially bright acrosomal region. The arrowheads point to acrosome-intact sperm without the specific staining pattern in the head, indicating no acrosome reaction. (A) A photomicrograph of sperm incubated with calcium ionophore for 30 min. (B) A photomicrograph of sperm incubated without calcium ionophore.

cDNA using SuperScript II RNase H$^-$ reverse transcriptase (Invitrogen Corp.) and oligo $(dT)_{12–18}$ primer (Invitrogen Corp.). The labeled cDNA was purified using a MiniElute PCR Cleanup kit (Qiagen, Tokyo, Japan).

The labeled cDNA was hybridized to an ED array version 1 (Asahi Techno Glass, Co. Ltd., Chiba, Japan) overnight at 60°C. The ED array version 1 carries total 1833 probes: 864 rat and 889 mouse genes. The glass arrays were washed once in 2× SSC/1% SDS for 10 min, twice in 0.2× SSC/0.1% SDS for 10 min each time, once in ethanol, and finally they were dried up. The microarrays were scanned using a fluorescence laser-scanning device, ScanArray (GSI Lumonics, Tokyo, Japan). The fluorescence intensity of the genes was quantified using a microarray analysis software, QuantArray version 2.1 (GSI Lumonics, Tokyo, Japan), and the intensity data file was imported into Microsoft Excel for data analysis. The data was normalized across arrays using the total of the intensity values of all features on each array. The normalized intensity values were averaged among the 10 arrays to obtain the mean intensity of each gene expression in each group. The change in each gene expression was calculated by determining the fold change (ratio) of the mean intensity of each group. A 1.5-fold or more increase or decrease of the change in the SASP-treated group compared with the control was regarded as significant change in gene expression.

Confirmation of mRNA expression in the testes and the epididymides by real-time RT-PCR. Expression of genes selected by microarray analysis and of their related genes was quantified in the testes and the epididymides by real-time reverse transcription and polymerase chain reaction (RT-PCR) using an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Briefly, total RNA in each group was reverse-transcribed individually with SupperScript II reverse transcriptase (Invitrogen Corp.) and oligo-dT. Expression of the following five genes was measured: *CD59* (accession no. NM_012925, Rushmere *et al.*, 1994), *membrane cofactor protein* (*MCP*; accession no. NM_019190, Miwa *et al.*, 1998), *decay accelerating factor* (*DAF*; accession no. AB026905, Miwa *et al.*, 2000), *heat shock protein 70-2* (*HSP70*; accession no. X77208, Walter *et al.*, 1994) and *spermatogenic cell-specific glyceraldehyde 3-phosphate dehydrogenase* (*Gapd-s*; accession no. AJ297631). *CD59* and *HSP70* were on the ED array but not the other three genes. HSP70 and Gapd-s are indispensable for spermatogenesis in mice (Eddy, 1999; Mori *et al.*, 1992). In the present study, rat homologues of these two genes were used as spermatogenesis-related genes. The rat specific primers for the above genes (Table 1) were designed using Primer Express software (Applied Biosystems). A standard curve was created using appropriate dilutions of a sample of cDNA synthesized from testicular total RNA of a control animal. The samples were quantified using the values calculated from standard curves for each gene. The SYBR Green PCR Master mix (Applied Biosystems) was used for real-time RT-PCR analysis. Expression of 18s ribosomal RNA gene was quantified using ribosomal RNA control (18sRNA) and TaqMan Universal PCR Master mix for internal controls. Real-time RT-PCR analysis was performed in triplicate for each group. Individual values were normalized using the value of 18s ribosomal RNA, and then the relative values in the SASP-treated group were calculated by setting the control at 1.

CD59 protein expression analysis by Western blot. The cauda epididymides from four rats of the control or SASP treated group were homogenized with 1 ml of M-PER Mammalian Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL) containing 1% protein inhibitor mix (Amersham Life Sciences, Piscataway, NJ) and incubated for 1 h on ice. After incubation, debris was removed by centrifugation at 15,000 × *g* for 10 min and the lysates were stored at −80°C until used. The total protein concentration in the lysates was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). The proteins (1 μg per lane) were mixed with an equal volume of NuPAGE LDS sample buffer (Invitrogen Corp.) and separated by SDS-PAGE under no-reducing conditions using NuPAGE 4–12% Bis-Tris Gels (Invitrogen Corp.) and then, electrotransferred to Hybond-P PVDF membrane (Amersham Bioscience, Arlington Heights, IL). The membrane was blocked with blocking buffer containing bovine milk protein (Nakalai tesque, Kyoto, Japan) for 1 h and incubated overnight at 4°C with mouse anti-Rat CD59 clone (TH9) (Research Diagnostics, Flanders, NJ) and then, the membrane was washed with Tris buffered saline containing 0.05% Tween-20 (TBS-T). CD59

TABLE 1
Primer Pairs

Gene (Accession no.)	Primer pair (Upper, sense primer; lower, antisense primer)	PCR product (bp)
CD59	5′-GTCCTCTGTTCCACAGGTGTT-3′	98
(NM_012925)	5′-GCATCCAGGTTAGGAGAGCA-3′	
MCP	5′-TTCAGTGTTTGGAGGGATTTTACA-3′	100
(NM_019190)	5′-GGACCTTTAAGACATTGTGGGATAG-3′	
DAF	5′-TCCCTCAAAAAGGAGTATCGTAACA-3′	82
(AB026905)	5′-GATTTTCCTGTGCGTGAAGTTACTT-3′	
HSP 70-2	5′-GTACTGGGAGTGTGTCTTTGCAAT-3′	91
(X77208)	5′-CGTGCCTGTCAAAAGAAATAACTTC-3′	
Gapd-s	5′-GTGCCAACCCCAAACGTATC-3′	91
(AJ297631)	5′-CTGCTTTCACAGCCTCCTTGA-3′	

was visualized using an ECL detection system (Amersham Bioscience) after the membrane was incubated at room temperature for 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA). The signals were quantified densitometrically using Las1000 detection system (Fuji film, Tokyo, Japan).

Statistical analysis. Data were statistically analyzed using Aspin-Welth's *t*-test. Probability less than 5% was considered statistically significant.

RESULTS

Effect of SASP on Reproductive Status

Decreases in the number of implantation sites and live fetuses were noted in the SASP-treated group (Table 2). However, there was no difference in the number of females copulated nor in the number of corpora lutea (Table 2).

Effect of SASP on the Weight of Reproductive Organs and Histological Changes in the Testes

There were no differences in the weight of testes, epididymides, prostate, and seminal vesicles between the control and the SASP-treatment group (Table 3). In the pathological examination of the testes, there were also no significant differences between the SASP-treated group and the control (data not shown).

Effects of SASP on Sperm Motility Parameters

Table 4 shows the results of sperm motility analysis. Percentage of motile sperm in the SASP-treated group was comparable to that in the control. On the other hand, the percentage of progressively motile sperm and velocity parameters such as path velocity (VAP), straight line velocity (VSL), and curvilinear velocity (VCL) were significantly decreased by SASP. Parameters of linearity of sperm motion such as straightness (STR) and linearity (LIN) were not changed in the two groups, but beat cross frequency (BCF) was increased in the SASP-treated group.

TABLE 2
Fertility Index in Female Rats Mated with Control or SASP-Treated Male Rats

	Control	SASP
Number of females mated	10	10
Number of females copulated	10	10
Number of females pregnant	8	9
Number of corpora lutea	15.9 ± 2.6	15.3 ± 1.6
Number of implantation sites	14.3 ± 1.3	11.4 ± 2.2**
Number of live fetuses	13.3 ± 1.0	11.3 ± 2.2*

Note. Each value represents the mean ± SD. The asterisk (*) indicates significant difference from the control group ($p < 0.05$).

TABLE 3
Reproductive Organs Weight (g) in Control and SASP-Treated Male Rats

	Control	SASP
Testes	3.29 ± 0.19	3.24 ± 0.30
Epididymides	1.16 ± 0.06	1.11 ± 0.07
Prostate	1.43 ± 0.27	1.54 ± 0.28
Seminal vesicles	1.40 ± 0.15	1.43 ± 0.31

Note. Each value represents the mean ± SD of 10 rats.

Effect of SASP on the Acrosome Reaction

In the control, the ratios of sperms showing acrosome reaction were 10.9, 14.3, 25.8, and 31.2 at 0-, 1-, 3-, and 5-h incubation, respectively (Table 5). Thus the ratio of acrosome-reacted sperm was increased thorough the incubation time.

In the SASP-treated group, the ratios of sperms showing acrosome reaction were 8.0, 9.9, 14.2, and 18.1 at 0-, 1-, 3-, and 5-h incubation, respectively, with a tendency increase depending on the time. The ratio at 0-h incubation was comparable with that of the control group, while the ratios at 3- and 5-h incubation were significantly lower than those of the control group, with the ratio after 5-h incubation being approximately 50% of the control level.

cDNA Microarray Analysis of mRNA Expression in the Testes

As a result of cDNA microarray analysis, it was observed that in the SASP-treated testes 69 genes were up-regulated and 4 genes were down-regulated, compared to the control (Table 6). The affected genes were classified into 10 functional categories referred to as ''Gene Ontology.'' Expression of one acrosome membrane-related gene, namely *CD59*, was up-regulated, but those of spermatogenesis- and apoptosis-related genes were unchanged.

TABLE 4
Effect of SASP on Sperm Motility

Parameters	Control	SASP
% Motile sperm	83.9 ± 5.2	80.1 ± 4.6
% Progressively motile sperm	23.0 ± 8.3	8.3 ± 2.5**
VAP (μm/s)	130.9 ± 7.7	107.6 ± 7.0**
VSL (μm/s)	81.6 ± 8.0	67.6 ± 7.6**
VCL (μm/s)	299.3 ± 22.1	259.1 ± 18**
ALH (μm)	23.2 ± 1.6	23.9 ± 1.7
BCF (Hz)	31.3 ± 1.4	35.8 ± 2.4**
STR (%)	59.9 ± 3.1	59.1 ± 3.8
LIN (%)	26.2 ± 1.9	24.9 ± 2.3

Note. Each value represents the mean ± SD. The asterisk (**) indicates significant difference from the control group ($p < 0.01$).

TABLE 5
Ratio (%) of Acrosome-Reacted Sperm in Control and SASP-Treated Male Rats

Incubation time	Control	SASP
0 h	10.9 ± 4.2	8.0 ± 2.1
1 h	14.3 ± 4.9	9.9 ± 3.2
3 h	25.8 ± 5.8	14.2 ± 3.9**
5 h	31.2 ± 6.0	18.1 ± 3.7**

Note. Each value represents the mean ± SD. The asterisk (**) indicates significant difference from the control group ($p < 0.01$). Ten males were used in each group.

Real-Time RT-PCR Analysis of mRNA Expression in the Testes

Among the genes that exhibited altered expression levels in the SASP-treated group by the microarray analysis, we focused on *CD59* since the CD59 protein is known to localize in the acrosomal membrane region of the sperm head (Simpson and Holmes, 1994). In addition to *CD59*, we also investigated the expression of two other acrosome membrane-related genes—*MCP* and *DAF*—which have functions similar to that of *CD59*, as well as spermatogenesis-related genes, including *HSP70* and *Gapd-s*. In the SASP-treated testes, it was observed that the expression of *CD59* was slightly increased while *MCP* and *DAF* were not changed (Fig. 2). On the other hand, expression of spermatogenesis-related genes was not affected (Fig. 2).

Effect of SASP on Gene Expression in the Epididymides

We investigated the expression of the acrosomal membrane-related genes *CD59*, *MCP,* and *DAF* in the epididymides using real-time RT-PCR. In the caput region of the epididymides from SASP-treated males, expression of *CD59*, *MCP,* and *DAF* was significantly down-regulated to approximately half that in the control group (Fig. 3). In the cauda region of the epididymides from SASP-treated males, the expression of *CD59* and *MCP* was also significantly down-regulated when compared to the control. *DAF* expression in the cauda region tended to decrease in the SASP-treated group, although the decrease was not statistically significant (Fig. 3).

TABLE 6
Number of Genes with Altered Expression Levels in SASP-Treated Rat Testes as Revealed by Microarray Analysis

	Number of	
Categories	Up-regulated genes	Down-regulated genes
Acrosome membrane related	1	0
Spermatogenesis	0	0
Apoptosis	0	0
Drug metabolism	5	0
Growth factor	3	0
Signal transduction	9	0
Cell structure	1	0
Transporter and channels	13	0
Others	22	2
Unknown (EST)	15	2
Total	69	4

Note. The affected genes were classified into 10 functional categories referred to as "Gene Ontology."

Changes of the Expression of CD59 Protein in the SASP-Treated Cauda Epididymis

The expression of CD59 protein in the SASP-treated cauda epididymis was statistically decreased when compared to the control (Fig. 4).

DISCUSSION

At the beginning of the study, male rats with reduced fertility were obtained by treatment with sulfasalazine (SASP) at 600 mg/kg for 28 days. When these SASP-treated male rats were mated with untreated females, the number of implantation sites and live fetuses was significantly decreased, indicating reduced fertility was observed. These results are the same as those of some previous studies (Horimoto *et al.*, 2000; Pholpramool and Srikhao, 1983).

Many factors which reduce male fertility have been reported as follows: underdevelopment of reproductive organs, reduced number of normal sperms, suppressed sperm motility, abnormal sperm motion, and reduced acrosome reaction (Perreault and Cancel, 2000). In the present study, however, there were neither significant changes in the weight of reproductive organs nor remarkable pathological findings in the testes of the rats with suppressed fertility induced by the SASP treatment. From these results, it is suggested that SASP does not affect the morphology of male reproductive organs. It has also been reported that SASP does not affect the sperm count at the same dose as used in the present study (Horimoto *et al.*, 2000). Sperm motility analysis showed this was altered, as indicated by a decrease of the percentage of progressively motile sperm, sperm velocity and an increase in the beat frequency of the sperm head. In alpha chlorohydrin treated rats, Kato and coworkers (2002) have observed remarkable decreases in the percentage of progressively motile sperm and sperm velocity, both of which are considered as causes of infertility. The depression of the percentage of progressively motile sperm was also remarkable in this study, but the decrease of sperm velocity was less than that induced by alpha chlorohydrin. Therefore it is suggested that the reduction of the percentage of progressively motile sperm might be related to the infertility induced by SASP, with less contribution of the reduction of sperm velocity.

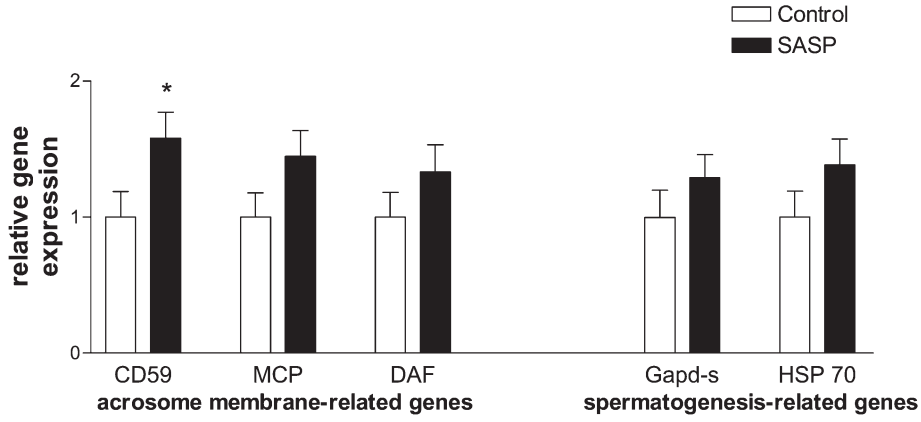

FIG. 2. Effect of SASP on mRNA expression in the testes. The expression of the five genes investigated was confirmed using real-time RT-PCR. The asterisk (*) indicates significant difference from the control group ($p < 0.05$). *CD59*, *membrane cofactor protein* (*MCP*) and *decay accelerating factor* (*DAF*) are acrosome-related genes, while spermatogenic *cell-specific glyceraldehyde 3-phosphate dehydrogenase* (*Gapd-s*) and *heat shock protein 70-2* (*HSP70*) are spermatogenesis-related genes.

On the other hand, the remarkable reduction of the acrosome reaction ratio was observed in the SASP-treated group. The spontaneous acrosome reaction ratio in the control group increased with time and reached the highest value at 5 h of incubation; this time point coincided with that of the beginning of sperm penetration into eggs in the *in vitro* fertilization study (Toyoda and Chang, 1974). In the SASP-treated group, the acrosome reaction ratio at the 3- and 5-h time points was about half the ratio observed in the control group. Suppression of fertility in rats treated with genistein or nimodipine has been reported to correlate with a marked decrease of the acrosome reaction ratio (Kumi-Diaka and Townsend, 2003; Saha *et al.*, 2000). In addition, the analysis of acrosome reaction in human sperm is recommended to estimate the fertilization ability of human sperm in the clinical field (Ohashi *et al.*, 1995). Therefore, the data obtained by those authors and our present results suggest that a reduction of the acrosome reaction ratio is one of the mechanisms of induction of decreased fertility by SASP as well as depressed percentage of progressively motile sperm.

Furthermore, to investigate molecular mechanisms underlying the reduction of male fertility caused by SASP, we performed a genomic analysis of the reproductive organs. mRNA expression changes in the testes were analyzed by cDNA microarray as the first screening, expecting to detect the effect of SASP on genes related to spermatogenesis. However, SASP did not remarkably affect the expression of spermatogenesis-related genes; this was also confirmed by real-time RT-PCR analysis. On the other hand, up-regulation of some genes related to the acrosomal membrane composition, such as *CD59,* was detected by microarray analysis. *CD59* is one of the complement regulatory proteins that are important for the reproductive function of sperm, and it

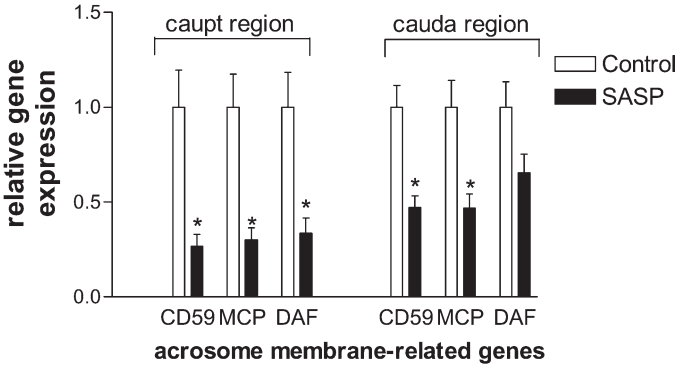

FIG. 3. Effect of SASP on mRNA expression in the epididymides. The expression of the three acrosome-related genes was confirmed using real-time RT-PCR. The asterisk (*) indicates significant difference from the control group ($p < 0.05$).

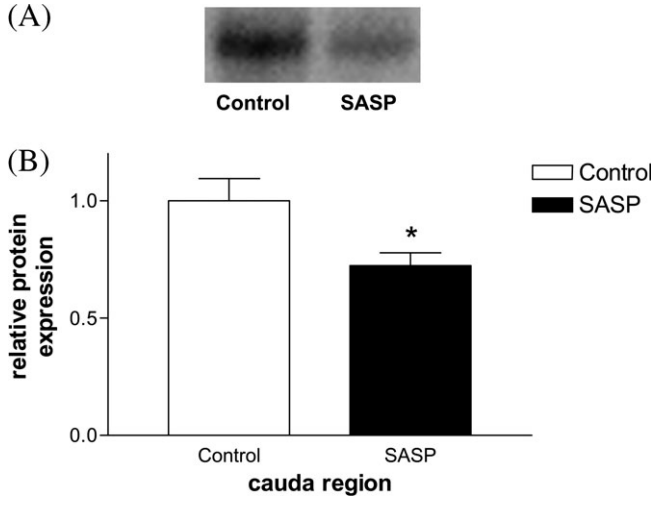

FIG. 4. Effect of SASP on CD59 protein expression in the cauda epididymides. (A) Anti-CD59 Western blot analysis. Left band is representative band from the control group and right is SASP-treated animal. One μg of total protein per lane was used. (B) Densitometric analysis of CD59 immunoreactive bands in the control rats (open bar) and in the SASP-treated group (solid bar). The asterisk (*) indicates significant difference from the control group ($p < 0.05$).

appears to be required in the testes during normal spermatogenesis (Simpson and Holmes, 1994). This protein is also known to be located in the acrosomal region of the sperm head and seems to protect the sperm from complement mediated damage in the female reproductive tract (Simpson and Holmes, 1994). The proteins that have functional similarity to CD59, membrane cofactor protein (MCP) and decay accelerating factor (DAF) are also known to be located in the inner acrosomal membrane (Simpson and Holmes, 1994). In addition to the protective function, these proteins seem to be relevant to sperm motion (Jiang and Pillai, 1998). Contribution of MCP to the sperm-oocyte interaction and to the acrosome reaction has been reported as well (Anderson *et al.*, 1993; Inoue *et al.*, 2003). Therefore real-time RT-PCR analysis was carried out to confirm the expression of *CD59* that was on the microarray, *MCP* and *DAF* that were not on it, in the testes, and it was revealed that only *CD59* was slightly increased among the acrosomal membrane-related genes, confirming the result of cDNA microarray analysis. The fact that *CD59* was slightly increased and that spermatogenesis-related genes such as *HSP70-2* and *Gapd-s* were not affected in the testes suggested that the testes were not the main organ affected by SASP.

On the other hand, real-time RT-PCR analysis revealed that mRNA expression of *CD59*, *MCP,* and *DAF* was decreased to half the level observed in the control, both in the caput and caudal regions of the epididymis of the SASP-treated rats. Furthermore, the expression of CD59 protein in the cauda epididymis was also decreased. These three complement regulatory proteins are reported to be synthesized in the male genital tract epithelium, transported to the fluid secretions, and then incorporated into the sperm membrane (Kirchhoff and Hale, 1996). Based on the above-mentioned evidence, SASP treatment in male rats may affect the complement regulatory protein synthesis and fluid secretion required for sperm maturation in the epididymis. The present results suggest that SASP suppresses the synthesis of complement regulatory proteins (at least *CD59*, *MCP,* and *DAF*) in the male reproductive tract. The suppressed synthesis of these proteins, with their consequent reduced secretion into the fluid, may affect the distribution of sperm membrane proteins. The disorder of the distribution of sperm membrane proteins could possibly lead to changes of cytoplasmic calcium levels and to a decreased sperm motility and acrosome reaction by SASP treatment.

In summary, decreases in the percentage of progressively motile sperm and in the acrosome reaction ratio were observed in rats treated with SASP. The mRNA expression of *CD59*, *MCP,* and *DAF* was decreased in the epididymides of the affected rats, while there was no apparent change in the expression of genes related to spermatogenesis in the testes. In addition, a decrease in CD59 protein expression was observed in the epididymides from SASP-treated rats. The expression of MCP and DAF proteins remains to be elucidated.

ACKNOWLEDGMENTS

We gratefully thank Dr. Yasushi Sato, Pfizer Global Research & Development, for pathological examination of tissues and Dr. Hitoshi Tainaka, Asahi Techno Glass, Inc., for his help with the microarray analysis.

REFERENCES

Anderson, D. J., Abbott, A. F., and Jack, R. M. (1993). The role of complement component C3b and its receptors in sperm-oocyte interaction. *Proc. Natl. Acad. Sci.* **90,** 10051–10055.

Baron, J. M., Connell, A. M., Lennard-Jones, J. E., and Jones, F. A. (1962). Sulphasalazine and salicylazosulphadimidine in ulcerative colitis. *Lancet* **1,** 1094–1096.

Eddy, E. M. (1999). Role of heat shock protein HSP70-2 in spermatogenesis. *Rev. Reprod.* **4,** 23–30.

Horimoto, M., Isobe, Y., Isogai, Y., and Tachibana, M. (2000). Rat epididymal sperm motion changes induced by ethylene glycolmonoethyl ether, sulfasalazine, and 2,5-hexandione. *Reprod. Toxicol.* **14,** 55–63.

Hoyt, J. A., Fisher, L. F., and Swisher, D. K. (1995). Short-term male reproductive toxicity study with sulfasalazine in the rat. *Reprod. Toxicol.* **9,** 315–326.

Inoue, N., Ikawa, M., Nakanishi, M., Matsumoto, M., Nomura, M., Seya, T., and Okabe, M. (2003). Disruption of mouse CD46 causes an accelerated spontaneous acrosome reaction in sperm. *Mol. Cell. Biol.* **23,** 2614–2622.

Jiang, H., and Pillai, S. (1998). Complement regulatory proteins on the sperm surface: Relevance to sperm motility. *Am. J. Reprod. Immunol.* **39,** 243–248.

Kato, M., Makino, S., Kimura, H., Ota, T., Furuhashi, T., Nagamura, Y., and Hirano, K. (2002). In vitro evaluation of acrosomal status and motility in rat epididymal spermatozoa treated with a-chlorohydrin for predicting their fertilizing capacity. *J. Reprod. Develop.* **48,** 461–468.

Kirchhoff, C., and Hale, G. (1996). Cell-to-cell transfer of glycosylphatidylinositol-anchored membrne proteins during sperm maturation. *Mol. Hum. Reprod.* **2,** 177–184.

Kumi-Diaka, J., and Townsend, J. (2003). Toxic potential of dietary genistein isoflavone and beta-lapachone on capacitation and acrosome reaction of epidiymal spermatozoa. *J. Med. Food.* **6,** 201–208.

Levi, A. J., Fisher, A. M., Hughes, L., and Hendry, W. F. (1979). Male infertility due to sulphasalazine. *Lancet* **2,** 276–278.

Miwa, T., Nonaka, M., Okada, N., Wakana, S., Shiroishi, T., and Okada, H. (1998). Molecular cloning of rat and mouse membrane cofactor protein (MCP, CD46): Preferential expression in testis and close linkage between the mouse Mcp and Cr2 genes on distal chromosome 1. *Immunogenetics* **48,** 363–371.

Miwa, T., Okada, N., and Okada, H. (2000). Alternative exon usage in the 3′ region of a single gene generates glycosylphosphatidylinositol-anchored and transmembrane forms of rat decay-accelerating factor. *Immunogenetics* **51,** 129–137.

Mori, C., Welch, J. E., Sakai, Y., and Eddy, E. M. (1992). In situ localization of spermatogenic cell-specific glyceraldehyde 3-phosphate dehydrogenase (Gapd-s) messenger ribonucleic acid in mice. *Biol. Reprod.* **46,** 859–868.

Ohashi, K., Saji, F., Kato, M., Tsutsui, T., Tomiyama, T., Tanizawa, O. (1995). Acrobeads test: A new diagnostic test for assessment of the fertilizing capacity of human spermatozoa. *Fertil. Steril.* **63,** 625–630.

O'Morain, C. O., Smethurst, P., Doré, C. J., and Levi, A. J. (1984). Reversible male infertility due to sulphasalazine: Studies in man and rat. *Gut* **25,** 1078–1084.

O'Morain, C. O., Smethurst, P., and Levi, A. J. (1985). Reversible male fertility induced by sulphasalazine. In *Male Fertility and its Regulation* (Lobet, T. J. and E. S. E. Hafez, Eds.), pp. 49–54. Kluwer Academic Publishers, The Netherlands..

O'Morain, C. O., Smethurst, P., Moore, H., Fraser, L., and Levi, A. J. (1983). Mechanism of action of sulphasalazine induced infertility. *Gastroenterology* **84,** 1410.

Perreault, S. D., and Cancel, A. M. (2000). Significance of incorporating measures of sperm production and function into rat toxicology studies. *Reproduction* **121,** 207–216.

Pholpramool, C., and Srikhao, A. (1983). Antifertility effect of sulfasalazine in the male rat. *Contracepetion* **28,** 273–279.

Rushmere, N. K., Harrison, R. A., van den Berg, C. W., and Morgan, B. P. (1994). Molecular cloning of the rat analogue of human CD59: Structural comparison with human CD59 and identification of a putative active site. *Biochem. J.* **304,** 595–601.

Saha, L., Bhrgava, V. K., Garg, S. K., and Majumdar, S. (2000). Effect of nimodipine on male reproductive functions in rats. *Indian J. Physiol. Pharmacol.* **44,** 449–455.

Sharma, R. K., and Kalla, N. R. (1994). Spermatozoal abnormalities and male infertility in the rat following sulfasalzine treatment. *Int. J. Fertil. Menopausal. Stud.* **39,** 347–354.

Simpson, K. L., and Holmes, C. H. (1994). Differential expression of complement regulatory proteins decay-accelerating factor (CD55), membrane cofactor protein (CD46) and CD59 during human spermatogenesis. *Immunolgoy* **81,** 452–461.

Summers, R. W., Switz, D. M., and Sessions, J. T. (1979). National co-operative Crohn's disease study group. Result of drug treatment. *Gastroenterology* **77,** 847–869.

Svartz, N. (1942). Salazopyrin a new salfanilamide preparation. *Acta. Med. Scan.* **110,** 577–598.

Toth, A. (1979). Reversible toxic effect of salicylazosulfapyridine on semen quality. *Fertil Steril.* **31,** 538–540.

Toyoda, Y., and Chang, M. C. (1974). Fertilization of rat eggs in vitro by epididymal sperm spermatozoa and the development of eggs following transfer. *J. Reprod. Fert.* **36,** 9–22.

Walter, L., Rauh, F., and Gunther, E. (1994). Comparative analysis of the three major histocompatibility complex-linked heat shock protein 70 (Hsp70) genes of the rat. *Immunogenetics* **40,** 325–330.