Defects in the regulatory clearance mechanisms favor the breakdown of self-tolerance during spontaneous autoimmune orchitis

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Pelletier RM, Yoon SR, Akpovi CD, Silvas E, Vitale ML. Defects in the regulatory clearance mechanisms favor the breakdown of self-tolerance during spontaneous autoimmune orchitis. Am J Physiol Regul Integr Comp Physiol 296: R743–R762, 2009. First published December 3, 2008; doi:10.1152/ajpregu.90751.2008.—We identified aberrations leading to spontaneous autoimmune orchitis (AIO) in mink, a seasonal breeder and natural model for autoimmunity. This study provides evidence favoring the view that a malfunction of the clearance mechanisms for apoptotic cell debris arising from imbalances in phagocyte receptors or cytokines acting on Sertoli cells constitutes a major factor leading to breakdown of self-tolerance during spontaneous AIO. Serum anti-sperm antibody titers measured by ELISA reflected spermatogenic activity without causing immune inflammatory responses. Orchitic mink showed excess antibody production accompanied by spermatogenic arrest, testicular leukocyte infiltration, and infertility. AIO serum labeled the postacrosomal region, the mid and end piece of mink sperm, whereas normal mink serum did not. Normal serum labeled plasma membranes, whereas AIO serum reacted with germ cell nuclei. Western blot analyses revealed that AIO serum reacted specifically to a 23- and 50-kDa protein. The number of apoptosis-labeled apoptotic cells was significantly higher in orchitic compared with normal tubules. However, apoptosis levels measured by ELISA in seminiferous tubular fractions (STf) were not significantly different in normal and orchitic tubules. The levels of CD36, TNF-α, TNF-α RI, IL-6, and Fas but not Fas-ligand (L), and ATP-binding cassette transporter ABCA1 were changed in AIO STf. TNF-α and IL-6 serum levels were increased during AIO. Fas localized to germ cells, Sertoli cells, and the lamina propria of the tubules and Fas-L to germ cells. Fas colocalized with Fas-L in residual bodies in normal testis and in giant cells and infiltrating leukocytes in orchitic tubules. Sertoli cells; apoptosis; Fas; IL-6; TNF-α; cytokines

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of the equilibrium of the lymphocyte population (46). Programmed cell death or apoptosis can be induced by signaling pathways inherent (intrinsic) or foreign (extrinsic) to the cell (46). The intrinsic pathway for apoptosis involves the release of cytochrome c from mitochondria into the cytosol, where it binds to apoptotic protease activating factor-1, resulting in the activation of the initiator caspase-9 and the subsequent proteolytic activation of the executioner caspases-3, -6, and -7. The extrinsic pathway for apoptosis involves ligation of the death receptor (such as TNF-α receptor) to its ligand TNF-α. Binding of TNF-α to its receptor induces trimerization of the receptors, which recruit Fas-associated death domain through shared death domains to constitute the death-inducing signaling complex (DISC) (5). DISC assembly induces the cleavage and activation of the initiator procaspase-8 (5), which triggers the execution phase of apoptosis. Binding of Fas-L, a cytokine of the TNF family, to its receptor Fas/Apo1/CD95 has been shown to induce apoptosis in Fas-carrier cells (52). Whether Fas is a primary regulator of germ cell apoptosis (38) or a protagonist in immunotolerance in the testis (6, 38) remains unclear (16, 23, 69). Support for the first view derives from studies on gliding mice, which express a spontaneous point mutation in Fas-L that prevents the ligand from binding to the Fas receptor (84). These mice show reduced toxicant-induced testicular germ cell apoptosis (72). Evidence suggesting Fas is not involved in spontaneous and cryptorchidism-induced apoptosis of germ cells comes from studies using lpr/lpr mice that are deficient in Fas (53, 94). These studies found that experimental cryptorchidism induced further apoptosis of germ cells in both BALB/c and lpr/lpr mice (55). Further complicating this debate, the specificity of many antibodies has been disputed (69), and the exact site of Fas-L localization has varied among reports, even when similar antibodies were used (40, 96).

Fas-L has been widely claimed to be expressed in Sertoli cells and Fas to be confined to germ cells in mice (17) and humans (66). Fas mRNA and protein were found in prepuberal mouse Sertoli cells but not Fas-L mRNA (71). Moreover, Fas-L mRNA was detected in purified populations of meiotic and postmeiotic cells, and the Fas-L was found on the surface of spermatozoa, whereas Fas was demonstrated in mouse and rat Sertoli cells and germ cells (13).

The class I scavenger receptor CD36, which specifically recognizes low-density lipoproteins (67), is required for recognition of apoptotic neutrophils by macrophages that use phosphatidyl receptor (15). CD36 with other phagocyte receptors has been reported to suppress production of inflammatory cytokines and to stimulate the release of anti-inflammatory mediators like TGF-β1 (12). The ATP-binding cassette transporter ABCA1, which is a major regulator of cellular cholesterol and phospholipid homeostasis has been shown to promote engulfment of apoptotic cells and transbilayer redistribution of phosphatidylserine (42). Our study investigates the behavior of CD36 and ABCA1 in spontaneous AIO for the first time.

We hypothesize that a malfunction of the clearance mechanisms for apoptotic cell debris arising from imbalances in phagocyte receptors or cytokines acting on Sertoli cells constitutes a major factor leading to breakdown of self-tolerance during spontaneous AIO.

Our data indicate that the Fas system may contribute to targeted degeneration of residual bodies. In addition, the second peak incidence of apoptosis during natural testicular regression appears unrelated to the Fas system in normal adult mink. Our findings of 1) an overstated increase in serum anti-sperm antibodies; 2) an overload of apoptotic and giant cells, which are accompanied by an imbalance in the levels of TNF-α, IL-6, CD36, and Fas; and 3) leukocyte infiltration may give rise to changes in Sertoli cell-germ cell and Sertoli cell-immune cell interactions resulting in a local immunodepression and the breakdown of self-tolerance causing spontaneous AIO.

MATERIALS AND METHODS

Animals

Male mink (Mustela vison) purchased from Visonnière (St. Damase, PQ, Canada) were individually caged with food, water, and natural lighting. A total of 165 mink were used: 135 normal (10 neonatal, 10 pubertal, and 5 adult for each time interval) and 30 adults with AIO (10 adult for February, March, and July). Anesthesia was carried out by intraperitoneal injection of 0.9 ml/kg body wt of phenobarbital sodium (Somnotol; MCI Pharmaceutical, Mississauga, ON, Canada) and 0.15 ml/kg of a solution of 0.3 g/ml chloral hydrate and blood was obtained by cardiac puncture. The right testis was processed for immunohistochemistry and light and electron microscopy studies and the left testis, for enriched tissue fractionation. The protocol was approved by the Université de Montréal Animal Care Committee.

Normal fertile male. Tissue samples were collected at 30-day intervals from: 1) neonatal (60 to 90 days old); 2) pubertal (120, 150, 210, 240 days old); and 3) adults older than 270 days old. In addition, tissues were collected from 2- to 3-year-old fertile adults the last week of every month of the annual reproductive cycle. The dynamics of the germ cell population during development and the annual reproductive cycle are depicted in Fig. 1. The germ cells were identified by the method of Pelletier (58).

Infertile mink with AIO. Black and sapphire (genetically related to black) mink were used. Males that did not sire a litter following their first mating season in March due to primary infertility were excluded from the study (89). Only mink of 2–3 yr that mated and sired five or more litters in the previous year but were sterile during the current year and diagnosed with secondary infertility due to spontaneous AIO were employed.

Clinical criteria of fertility. The ejaculated semen recovered from vaginal lavage was evaluated under the light microscope and the morphology, motility, and number of spermatozoa were assessed for each male mink in March. In addition, anti-sperm antibody levels in serum were measured (see below). Only mink with low sperm counts or immobile spermatozoa, high antibody levels, and histopathology of the testis with leukocyte infiltration and destruction of the seminiferous epithelium at autopsy were diagnosed with secondary infertility of immunological etiology.

Reagents

Diaminobenzidine tetrachloride (DAB), orthophenylene diamine, p-nitrophenyl phosphate, PMSF, and 1,4-diazabicyclo-[2.2.2.]octane were from Sigma (Oakville, ON, Canada). MEM was from GIBCO-BRL (Oakville, ON, Canada), FBS from GIBCO-Invitrogen (Burlington, ON, Canada) and Bradford reagent from Bio-Rad (Mississauga, ON, Canada). Collagenase D, chemiluminescence substrate (Luminol Light Plus), and Tris buffered saline solution (TBST) were from Sigma, and horseradish peroxidase was from SIGMA. The secondary antibody for Western blot was HRP-conjugated goat anti-rabbit IgG (Molecular Probes). Diaminobenzidine tetrachloride (DAB) and orthophenylene diamine were from Sigma (Oakville, ON, Canada). MEM was from GIBCO-BRL (Oakville, ON, Canada), FBS from GIBCO-Invitrogen (Burlington, ON, Canada) and Bradford reagent from Bio-Rad (Mississauga, ON, Canada). Collagenase D, chemiluminescence substrate (Luminol Light Plus), and the cell death detection kit ELISA kits were from Roche (Laval, PQ, Canada). The human TNF-α ELISA kit (Quantikine HS human TNF-α) and a human IL-6 ELISA kit (Quantikine HS human IL-6) were from R&D Systems (Minneapolis, MN). p-Nitroblue tetrazolium chloride and 5-bromo-4-chloride-3-indolyl phosphate-toluidine salt were from Roche (Laval, PQ, Canada). The testosterone ELISA kit was from ALPCO (American Laboratory Products, Windham, NH).
Fig. 1. A and B: diagrams of the changes in the permeability status of the blood-testis barrier, the size of the lumen, and the germ cell population. The data were recorded every 30-day (d) interval (represented by a vertical column) during development (A) and the seasonal annual reproductive cycle in adult normal mink (B) [modified after Pelletier et al. (58)]. The shaded areas show the time periods when the blood-testis barrier allows entry of vascularly infused permeability tracers, whereas the nonshaded areas show the periods when the barrier forbids it. The permeability status of the barrier relates to the presence of a lumen (represented by an inner circle atop the diagram) in the tubule, not to that of a particular generation of germ cells in the epithelium (58) (G = gonocytes; Pre-A = pre-type A spermatogonia; A = type A spermatogonia; PL = preleptotene spermatocyte; P = pachytene spermatocyte; 7 = step 7 spermatid; 19 = step 19 spermatid). The serum anti-sperm Ab levels measured by ELISA are shown during development (C), the annual reproductive cycle in adult normal fertile (D), and in normal vs. mink with autoimmune orchitis (AIO) in February, March, and July (E). Results are expressed in the fold dilution of mink serum in which absorbance values were 0.5 mg. Bars represent the means ± SE of 6 independent experiments. C: Ab levels are significantly higher from 90 to 270 days after birth than by 60 days after birth (*P < 0.05 vs. 60 days). D: levels are significantly increased by March and April in normal adults (*P < 0.05, March-April vs. February), and then, they decrease until July. E: however, levels are significantly increased in mink with AIO in February and March but not in July (***P < 0.001, February; ****P < 0.0001, March). F: controls using the secondary Ab alone showed a weak staining in the middle piece of the tail of spermatozoa (Spz). Labeling is stronger over the acrosomal cap (a) of the head, and principal piece (p) of the tail of the epididymal Spz when orchitic sera (H) than when normal sera (G) are used. In addition, only orchitic sera labeled the postacrosomal region of the head (pa) and the middle (m) and end (e) piece of the tail (F–H; magnification, ×1400). I: Western blot analyses representative of 6 independent experiments in which sera from normal adult and mink with AIO were reacted with mink epididymal Spz. Total homogenate (20 mg) of each sample were loaded on polyacrylamide gels. A 23 kDa and a 50 kDa were detected in Spz (arrows) when probed with sera of mink with AIO (right lane) but not with normal adult mink sera (left lane).
Antibodies

Mouse monoclonal anti-human Fas Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and mouse monoclonal anti-human Fas-L Ab was from Transduction Laboratory (Lexington, KY). Horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin (IgG) and anti-rat IgG, biotinylated anti-mouse IgG and FITC-conjugated anti-rat IgG, and alkaline phosphatase-conjugated anti-rabbit IgG were obtained from Jackson Immunotechnology (West Grove, PA). HRP-conjugated streptavidin was from Molecular Probes (Eugene, OR), monoclonal Ab against single-stranded DNA F7-26 (apopstain) was from Alexis (San Diego, CA), and goat anti-guinea pig IgG was from Antibodies (Davis, CA). Polyclonal rabbit anti-mink IgG was a generous gift from Dr. Marshall E. Bloom (National Institutes of Health, Rocky Mountain Labs, Hamilton, MT). Polyclonal rabbit anti-CD36 was kindly provided by Dr. M. Febbraio (Cell Biology, Lerner Research Institute, Cleveland, OH). The rabbit polyclonal ABCA1 antibody raised against a peptide antigen corresponding to residues 1100–1300 was purchased from Novus Biologicals (Littleton, CO). Monoclonal anti-human TNF-α and polyclonal anti-human TNF-α were purchased from R&D Systems (Minneapolis, MN). Monoclonal anti-human TNF-α RI antibody was purchased from Santa Cruz Biotechnology. Polyclonal anti-human IL-6 was a gift from Dr. Choi from The Korean Research Institute of Biosciences and Biotechnology.

Epididymal Spermatozoa

Epididymal spermatozoa from normal adult mink killed in February were obtained and processed as described earlier (2).

Isolation of Seminiferous Tubule-Enriched Fractions

Freshly decapsulated testes were processed for isolation of seminiferous tubule-enriched fractions (STF) and interstitial tissue-enriched fractions (ITF) as described earlier (65). Each fraction was characterized under the microscope (1). The protein content in each sample was measured with the Bradford reagent (Bio-Rad) and BSA as standard.

Electrophoresis and Immunoblotting

STF were washed and homogenized in PBS (137 mM NaCl, 3 mM KCl, 8 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.4) containing 2 mM PMSF, 1 mM EDTA, 2 μg/ml leupeptin, and 2 μg/ml aprotinin, on ice. The samples were processed as described elsewhere (2, 34). Each fraction was characterized under the microscope (1). The protein content in each sample was measured with the Bradford reagent (Bio-Rad) and BSA as standard.

Western blot analyses were performed on epididymal mink spermatozoa lysates by using serum from normal and from mink with AIO killed in February or March to ascertain spermatozoan antigens that reacted with antibodies in the orchitic mink serum. Lysates of mink spermatozoa killed in February were loaded into 8% SDS polyacrylamide gel and subjected to electrophoresis before being transferred onto a nitrocellulose membrane. Blocking was done with 5% skim milk in TBS for 1 h at 37°C. The membrane was incubated with mink serum (1:200 dilution in TBS containing 1% BSA) at 4°C overnight, washed in 0.05% TBST before being incubated with rabbit anti-mink IgG (from Dr. Marshall E. Bloom from the Laboratory of Persistent Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT) (1:2,000 in TBS containing 5% skim milk) at RT for 1 h, and then washed with alkaline phosphatase conjugated anti-rabbit IgG (1:2,000 in TBS containing 5% skim milk) for 1 h at RT. After extensive washing with TBST, the reaction was developed with a substrate solution containing p-nitroblue tetrazolium chloride and 5-bromo-4-chloride-3-indolyl phosphate-toluidine salt.

Quantification of Apoptosis by ELISA

Apoptosis was measured with a cell death detection ELISA kit in accordance with the manufacturer’s instruction and as described elsewhere (2). The kit allows the quantitative evaluation of mononucleosome and oligonucleosomes-bound DNA in the cytoplasmic fraction of a cell homogenate caused by DNA fragmentation during apoptosis. The results are expressed in absorbance unit per milligram of STF. Samples were assayed in duplicate.

TNF-α and IL-6 Measurements in Mink Sera

The concentration of TNF-α and of IL-6 in sera was measured by a human TNF-α ELISA kit (Quantikine HS human TNF-α) and a human IL-6 ELISA kit (Quantikine HS human IL-6) from R&D Systems according to the manufacturer’s instructions. Samples were run in triplicate, and means were calculated.

Detection and Localization of Anti-Sperm Antibodies by Immunofluorescence

Spermatozoa diluted in PBS were plated on poly-l-lysine-coated glass cover slips at a density of 1×106 cells/ml, air-dried, and fixed with −20°C methanol. Nonspecific binding was blocked with 3% skim milk in PBS. Smeared spermatozoa were sequentially incubated with serial dilutions of mink sera (from 1:100 to 1:2,000) in 3% skim milk in PBS for 1 h at 37°C, with rabbit anti-mink IgG (1:3,000 dilution) for 1 h at 37°C, and with FITC conjugated anti-rabbit IgG (1:400 dilution). Controls included the omission of the mink sera and
of each secondary antibody. Coverslips were mounted in PBS-glycerol (1:1) containing 5% DAB. Micrographs were taken with 400 ASA T-MAX Eastman Kodak films (Rochester, NY) on an Axiophot 2 Carl Zeiss fluorescence microscope.

Preparation of Tissues for Immunolabeling

Perfusion-fixed testes and epididymides with Bouin’s fixative were immunolabeled using the protocol described elsewhere (52). Sections were incubated with the same anti-human Fas-L (1:4 dilution) or anti-human Fas (1:60 dilution) used in the Western blot studies and with biotinylated anti-mouse IgG (1:2,000) and (1:200 dilution) HRP-conjugated streptavidin. In addition to the controls described above in the Western blot studies, further controls were done that included the use of the primary antibody alone and the use of the second Ab alone. Photographs were taken on Eastman Kodak Technical Pan films with an Axiophot 2 Carl Zeiss microscope. For confocal microscopy, a Zeiss LSM510 was used.

Immunohistochemistry with Sera from Normal Mink and Mink with AIO Incubated with Tests and Epididymis Sections

The sera of six normal and mink with high anti-sperm antibody levels obtained in February and March were incubated with normal mink testis sections as described elsewhere (65). The sections were incubated with mink serum (1:200–1:1,000 dilution in TBS) overnight, washed, incubated first with rabbit polyclonal anti-mink IgG (1:3,000 dilution in TBS) for 1 h at RT, washed next with biotinylated anti-rabbit IgG, and then exposed to HRP-conjugated streptavidin. The reaction was detected with DAB.

Detection of Apoptosis by Immunolabeling

Apoptotic germ cells were labeled with an antibody against single-stranded DNA (45). Bouin-fixed and 4% PBS-buffered, formaldehyde-fixed, paraffin-embedded testis sections were incubated 20 min with 0.1 mg/ml saponin in PBS, next, for 20 min with 20 μg/ml protease K at room temperature (RT), washed in distilled water, and processed according to the manufacturer’s protocol. Sections were incubated 20 min in a 50% aqueous solution of formamide at 56°C, washed 5 min in cold PBS, and then exposed 5 min to 3% H2O2 in PBS and rinsed in distilled water. Nonspecific binding was blocked with 3% skim milk at RT. Sections were incubated with monoclonal Ab F7-26 (apostain; 1:50 dilution) at RT for 2 h, and next, with an anti-mouse IgM conjugate (1:100 dilution) at RT for 1 h. The quantification of apostain-positive cells was carried out on 20 tubules selected at random obtained from five different mink per experimental group. The apostain-positive cells were counted without knowing from which experimental condition the slides were taken.

Electron Microscopy of Thin Sections

The testes were perfusion-fixed with Karnovsky’s fixative (35) in 0.1 M Na cacodylate buffer pH 7.3 and processed for thin sectioning as previously described (59). Thin sections collected on formvar-coated, carbon-stabilized grids were examined at 80 kv with a Philips 420 electron microscope.

Determination of Serum Testosterone Levels

Testosterone concentration in mink sera was measured by ELISA using ALPCO kits as described (33). The sensitivity of the method was 0.07 ng/ml. All samples were evaluated in a single assay.

Data and Statistical Analysis

Relative protein content of scanned immunoreactive bands in Western blots was estimated by densitometry (Scion Image Software, Scion, MD). Means ± SE of more than six independent experiments for Fas-L and Fas and of more than three independent experiments for SR-BL, SR-BII, CD36, and ABCA1 are presented. For the statistical analyses of data, three groups were considered: 1) development: 60- to 270 days old, 2) normal adults obtained from August-July, and 3) normal adults and infertile adult mink with AIO obtained in February, March, and July. For each variable, one-way ANOVA was used with time as the factor and contrasts were performed with the Bonferroni (Dunn) adjustment for groups 1 and 2. Comparisons between fertile/infertile mink were analyzed by two-way ANOVA followed by t-tests for each month. Pearson’s correlation coefficients were calculated for some pairs of variables without taking into account the factor time for subsets 1 and 2, whereas the time was considered for subset 3. Analyses were done with Statview software (Stata, College Station, TX). For SR-BL, SR-BII, CD36, and ABCA1 the data are presented as the means ± SE and evaluated with the Student’s t-test.

RESULTS

Calendar of Changes in Germ Cell Population and of Permeability of Blood-Testis Barrier in Normal Mink

Figure 1 depicts the monthly changes in the germ cell population, the permeability status of the blood-testis barrier to vascularly infused permeability tracers, the size of the tubular lumen during postnatal development (Fig. 1A), and the annual reproductive cycle in normal adult mink (Fig. 1B). Gonocytes and pretype A spermatogonia are present during the neonatal period from birth (May) to 90 days. As defined by criteria developed in other animal models (37, 57), in the mink the appearance of type A spermatogonia in tubules marked the onset of puberty, whereas completion of spermatogenesis by day 240 after birth marked the end (58). Spermatocytes appear in the epididymis 12–14 days later.

Measurements of Serum Anti-Sperm Antibody Levels

In normal males, serum anti-sperm antibody levels increased significantly by the onset of puberty and reached a plateau from 150 days after birth to adulthood (Fig. 1C). Similarly, the pattern of serum anti-sperm antibody levels paralleled that of the seasonal spermatogenic activity; levels increased significantly from February to March and then declined from April to May while remaining low during testicular regression in normal adults (Fig. 1D). Nonetheless, mink with AIO showed significantly elevated anti-sperm antibody levels in February and March but not in July (Fig. 1E).

Localization of Anti-Sperm Antibodies by Immunofluorescence

Serum anti-sperm antibodies from normal mink labeled the acrosome of the head of epididymal spermatozoa, the annulus and principal piece of the tail (Fig. 1G), whereas antibodies from mink with spontaneous AIO labeled the same regions of spermatozoa but with greater intensity (Fig. 1H), plus the postacrosomal region over the nucleus in the head and the middle and end piece in the tail (Fig. 1H). These results reveal a difference in the amounts as well as in the quality of the anti-sperm antibodies measured by Western blot analysis in the orchitic with respect to the normal serum.

Western Blot Analyses of Sera from Normal Adult and From Mink with AIO

To identify the new spermatozoan antigens that reacted with antibodies in the orchitic mink serum, Western blot analysis was
performed on spermatozoa lysates by using serum from normal and from mink with AIO killed in February or March. A 23- and a 50-kDa band were detected with the serum of mink with AIO (Fig. 1, I, right) but not with the normal serum (Fig. 1, I, left). The 50-kDa band was detected also in the heart, liver, ovary, salivary gland, spleen, STf, adrenal gland, and the epididymis where the band was particularly intense (data not shown). However, the 23-kDa band was only detected in the spermatozoa (Fig. 1, I, right).

**Immunoperoxidase Staining Studies with Normal vs. Orchitic Mink Sera Incubated with Normal Adult Mink Testis and Epididymis and Other Tissue Sections**

The sera from normal adult mink killed in February and March labeled germ cell surfaces (Fig. 2, A–C), the limiting membrane of tubules (Fig. 2, A–C and 2G), blood vessels, interstitial cells, and macrophages (Fig. 2, B, C, and G) in the testis. However, normal sera labeled spermatozoa (Fig. 2I), intratubular lymphocytes (Fig. 2H), and macrophages of the interstitium (Fig. 2J) but labeled very weakly the limiting membrane of tubules (Fig. 2, H–J) in the epididymis.

The serum of mink with AIO labeled the nucleus of pachytene spermatocytes (Fig. 2, D–F), round and elongated spermatids (Fig. 2, D and E), residual bodies (Fig. 2E), as well as the limiting membrane of the seminiferous tubules. The interstitium (Fig. 2M) and macrophages in the epididymis were labeled (Fig. 2, K and M) but not epididymal cells (Fig. 2L). In addition, nuclei in the kidney, liver, and spleen were stained (data not shown).

**Immunoperoxidase Labeling of Testicular Germ Cell Apoptosis with Monoclonal Ab F7-26 (apostain)**

There were very few free apoptotic bodies in February (the zenith of spermatogenic activity) in normal adult testis (data not shown), but by March (Fig. 3A) and May (Fig. 3B) isolated apostain-labeled germ cells were detected. In contrast, numerous apostain-positive round spermatids, spermatocytes, and multinucleated giant cells appeared in the lumen of the tubules in February and March. (Fig. 3C). The number of apostain-labeled apoptotic cells per seminiferous tubule was significantly higher in the orchitic than in the normal testis sections in March and June (Fig. 3D). Moreover, apostain-positive cells were significantly more numerous in June than in March in the normal seminiferous tubules (Fig. 3D).

**Measurements of Nucleosome Release in STf by ELISA**

The nucleosome release in STf increased significantly from 240 to 270 days after birth with the completion of spermatogenesis (Fig. 3E). Two successive significant increases in the nucleosome release incidences were recorded during the annual reproductive cycle, first in March and then in July (Fig. 3F), followed by a decrease from August to November (Fig. 3F). The nucleosome release in STf measured by ELISA in normal and orchitic tubule-enriched fractions showed no significant differences in February and in March (Fig. 3G).

**Measurements of Phagocyte Receptors (ABCA1 and CD36) in STf**

By comparison with the ABCA1 levels in normal STf, there was a tendency toward an increase in ABCA1 levels in orchitic STf in March (Fig. 3H). The CD36 levels were significantly lower in orchitic than in normal STf in March (Fig. 3I).

**Western blot analyses of Fas and Fas-L**

Western blot analyses performed with the Fas antibody on Jurkat cells used as positive control showed the 40-kDa immunoreactive band corresponding to Fas, demonstrating that the mouse monoclonal anti-human Fas antibody used recognized the Fas receptor in normal (Fig. 4A) and orchitic mink tissues (Fig. 4C). In addition, the 40-kDa Fas immunoreactive band was detected in normal epididymal spermatozoa (Fig. 4A) as well as in ITf (data not shown). The mouse monoclonal Fas-L antibody detected a 35-kDa immunoreactive band corresponding to Fas-L in the positive control human endothelial cells (Fig. 4D), and analyses performed with anti-Fas-L detected an identical 35-kDa band in mink STf (Fig. 4, D and F) as well as in ITf (data not shown), epididymal spermatozoa (Fig. 4D), and fractions of normal testicular macrophages (Fig. 4D).

**Quantification of Fas and Fas-L Protein Levels in STf**

Fas protein levels were elevated with the completion of puberty (Fig. 4, A and B). During testicular recrudescence, Fas levels increased from November to April when they peaked (Fig. 4, A and B). In orchitic STf, Fas was significantly decreased in February and March (Fig. 4, A and C).

Fas-L levels increased significantly in STf from 120 to 150 days after birth but declined significantly by 270 days after birth (Fig. 4, D and E). Fas-L levels in normal mature mink STF positively related to the spermatogenic activity and the number of germ cells in the tubules: the levels were significantly elevated from November to May and lowered from June to August (Fig. 4, D and E). However, Fas-L levels in normal and orchitic STf were not significantly different in February and March (Fig. 4F).

**Measurements of TNF-α, TNF-α RI, and IL-6 Levels**

Measurements of TNF-α levels in orchitic mink serum by ELISA showed a significant increase in February (Fig. 4G). In Western blot analyses the TNF-α antibody detected a 17-kDa immunoreactive band in mink STf (Fig. 4H). TNF-α levels were significantly increased in orchitic STf in March (Fig. 4, H and H'). The TNF-α RI (p55) antibody detected a 50-kDa band.
immunoreactive band in mink STF (Fig. 4I'). Compared with normal STF, orchitic STF showed significantly increased levels of this receptor in February and March (Fig. 4I and I'). Measurements of IL-6 levels in mink serum by ELISA showed an increase in February and March (Fig. 4G). Western blot analyses detected two forms of IL-6 in normal mink STF: a 26-kDa band and 17-kDa immunoreactive band (Fig. 4K'). The 17-kDa IL-6 immunoreactive band was absent in orchitic STF.
and this absence accounted for most of IL-6 decrease (Fig. 4, K and K').

Immunolocalization of Fas in Testis Sections

**Normal testis.** Fas immunolocalized to Sertoli cells throughout development and the annual reproductive cycle (Fig. 5, A–I). In addition, Fas localized to spermatids, residual bodies (Fig. 5D), and degenerating germ cells (Fig. 5, A–I). During regression, numerous apoptotic cells with Fas-positive intracytoplasmic clumps (Fig. 5, E–G) were exfoliated toward the lumen of the tubules (Fig. 5, E–F) leaving as many vacuoles surrounded by Fas-positive Sertoli cell processes (Fig. 5, G–H).

**Orchitic Testis.** During the onset of spontaneous AIO, germ cells typically lost contact with neighboring Sertoli cells, leaving spacious gaps at times filled with amorphous material (Fig. 6, A and B). Fas labeling decreased in Sertoli cells (compare Figs. 5A with 6A). Many individual Fas-positive germ cells were exfoliated in the lumen and collected to form giant cells (Fig. 6, A and B). Infiltrating lymphocytes were detected lumenal to Sertoli cell junctional complexes and near spermatids (Fig. 6, C and D). Some infiltrating cells observed in the interstitium and in the most damaged seminiferous tubules were Fas-positive (Fig. 6C).

Immunolocalization of Fas-L

**Normal testis.** Fas-L localized to clumps in the vicinity of the nucleus in gonocytes (Fig. 7A), spermatogonial stem cells (Fig. 7B), spermatocytes (Fig. 7, C–F), and spermatids (Fig. 7, C–F). Minute Fas-L-positive dots occurred at the periphery of
the spermatids (Fig. 7, D, E, and G). In addition, Fas-L was localized to residual bodies by stage VII of the cycle of the seminiferous epithelium (Fig. 7F). During testicular regression, the signal was present in dying spermatocytes and spermatids (Fig. 7, G–J) and near the nucleus of the stem spermatogonia in August (Fig. 7K).

Orchitic Testis. Apoptotic spermatids and spermatocytes exfoliated in the lumen, collected into giant cells or lodged deep within the epithelium contained sizeable Fas-L positive granules (Fig. 8, A–C). At times, the Fas-L positive material obscured the intracytoplasmic content, whereas at others, a thick coat of reaction product covered the cell surface (Fig. 8C). The cisternae of endoplasmic reticulum of plasmocytes near the tubules lamina propria were usually distended (Fig. 8, D and inset).

Confocal Microscopy Analyses of the Colocalization of Fas and Fas-L in Normal Testis

Confocal microscopy was specifically used to assessing colocalization of Fas and Fas-L either in the normal during specific stages of the spermatogenesis or in the orchitic testis.

Fas during stages VII and VIII. Fas was localized near the round and elongated spermatids surface (Fig. 9, A–C). The residual bodies near the apex or the base of the Sertoli cells by the end of stage VII were Fas-positive (Fig. 9, A and B). Labeling in this location was extinguished with the disappearance of the bodies during stage VIII (Fig. 9C). In Sertoli cells, the distribution of Fas was stage specific (Fig. 9, A–C), weakly positive (Fig. 9A) in stage I–early VII, and strongly labeled by stage VIII (Fig. 9C). The limiting membrane of the tubules was Fas-positive during stage I to early VII (Fig. 9, A–C).

Fas-L during stages VII and VIII. Fas-L antibody labeled elongated spermatids, residual bodies, and meiotic and postmeiotic cells (Fig, 9, A’, B’, and C’). The cytoplasm of spermatogonial was labeled (Fig. 9A’), whereas the plasma membrane of the meiotic cells was strongly positive (Fig. 9, B’ and C’), whereas the plasma membrane of the postmeiotic cells was weakly Fas-L-positive (Fig. 9C’).

Colocalization of Fas and Fas-L during stages VII. The colocalization of Fas and Fas-L proteins was observed in two locations: the elongated spermatids (Fig. 9A”) and the residual bodies engulfed by the Sertoli cells and collected near the base of these cells by the end of stage VII (Fig. 9B”). No colocalization of Fas and Fas-L was observed by stage VIII (Fig. 9A”).

Confocal Microscopy Analyses of the Colocalization of Fas and Fas-L in Orchitic Testis

Fas. Fas labeling significantly decreased in Sertoli cells (Fig. 10, A–C), whereas apoptotic germ cells and giant cells remained intensely positive (Fig. 10B). The epithelium of damaged tubules was filled with large-sized vacuoles, few labeled apoptotic germ cells, and infiltrating cells (Fig. 10C). Fas positive material surrounded the thickened basal lamina during the acute phase (Fig. 10B).

Fas-L. Fas-L was detected in spermatids, giant cells, spermatagonia, and infiltrating cells (Fig. 10, A’–C’). Colocalization of Fas and Fas-L. Fas colocalized with Fas-L in the round and elongated spermatids that persisted following the disruption of the seminiferous epithelium. (Fig. 10A”). Colocalization was striking in the giant cells (Fig. 10B”) and observed in some apoptotic cells and infiltrating cells (Fig. 10, A” and C”). However, the Fas-L positive material in the limiting membrane of the tubule did not colocalize with Fas (Fig. 10B”).

Serum Testosterone Levels

Testosterone serum levels peaked in February in normal mink (9.58 ± 1.74 (n = 7, February); 1.65 ± 0.62 (n = 8, March); 0.64 ± 0.08 (n = 4, July)). However, the testosterone peak was significantly lower in mink with AIO [1.93 ± 0.52 (n = 6, Feb; P < 0.01 vs. normal mink); 0.33 ± 0.09 (n = 9, March; P < 0.05 vs. normal mink); 0.44 ± 0.09 (n = 4, July)].

Discussion

Anti-sperm antibody levels in normal mink. This study provided the first quantification of the levels of antibodies to sperm antigens during the normal development and annual reproductive cycle. The prevalence of high serum anti-sperm antibody titers recorded during periods when spermatozoa were plentiful and low titers during periods when autoantigens-bearing germ cells were seasonally depleted is reflective of the spermatogenic activity. This natural formation of antibodies to spermatozoa is evidence that B lymphocytes were allowed access to autoantigens-bearing germ cells without triggering an immune inflammatory response or a destruction of the seminiferous epithelium. The fact that the majority of matings were successful is proof that a natural rise in serum anti-sperm antibody titers documented here coincide with periods when the blood-testis barrier has been shown to be incompetent in blocking blood-born substances (58, 59). Significantly, lymphocytes were reported not to infiltrate the seminiferous tubules even during these time periods of the annual reproductive cycle when the blood-testis barrier was permeable (58, 59). Thus, contrary to a previous suggestion (31), the blood-testis barrier does not act as an immunological barrier (63), since local immunosuppressor factors were more efficient at restricting entry of leukocytes than inter-Sertoli cell tight junctions. In fact, not only does the barrier not segregate all the autoantigens, but in most instances their availability to the immune cells in the interstitium of the testis is not accompanied by an autoimmune aggression (Ref. 98, and present study).

Anti-sperm antibody levels in mink with spontaneous AIO. Our studies confirmed and extended an earlier report (89) by showing a significant increase in anti-sperm antibodies in mink with spontaneous AIO in February, thus, while the blood-testis (58, 59) and blood-epididymis (60, 61) barrier were competent in sequestering autoantigens-bearing germ cells. Experimental AIO studies consistently reported unaltered permeability of the blood-tubular barrier (9, 64, 95), suggesting that the disease did not result from a leak in the barrier.

The sustained production of antibodies by B cells requires a continuous exposure to antigens. The anti-sperm antibody increase we recorded in normal mink signifies that B cells were naturally exposed to germ cell autoantigens. The same stimulus caused an excessive activation of autoreactive lymphocytes and antibody production in orchitic mink, suggesting that a
greater number of germ cells would be targeted and opsonised in these, rather than in normal mink. Significantly, our observation that spermatozoa lysates probed with sera from mink with AIO detected a 23- and a 50-kDa band not detected with normal mink sera suggests changes in the composition of auto-antibodies. The AIO serum labeled the postacrosomal region, the mid and end piece of mink sperm, whereas normal mink serum did not. In addition, AIO serum reacted with the germ cell nuclei, whereas normal serum labeled the plasma membranes. On the one hand, these results suggest that B cells in orchitic mink were exposed to these regions of the spermatozoa. Autoantigens against mature spermatozoa could have
been absorbed by epididymal cells and then released into the interstitium and exposed to immunocompetent cells, causing an autoimmune inflammation of the epididymis, since many of these mink showed epidemicmitis with autoreactive lymphocyte infiltration. On the other hand, our observations indicate that intracellular autoantigens were exposed to lymphocytes.

The impaired clearance of apoptotic cells allowed large amounts of self-antigens, in particular, nuclear antigens to stimulate the immune system. The appearance on the plasma membrane of apoptotic cells of blebs enriched in nuclear antigens, some of which as potential targets of the immune system, is another documented step of apoptosis (8). Our observation that tubule fractions and spermatozoa probed with mink sera with AIO detected a 23-kDa protein, which, based on molecular mass comparison, was similar to the UI small nuclear ribonucleoprotein particle C of somatic cells (49) and whose mRNA has been reported in meiotic and postmeiotic germ cells (50) is further indication that these mink may have produced auto-antibodies for antigens of nuclear origin. This could have resulted from damages to the germ cell plasma membranes and secondary necrosis following prolonged exposure of cellular corpses bearing surface protrusions with intracellular components. The rapid engulfment of cellular corpses has been shown to prevent an inflammatory response to self-antigens due to apoptotic overload and secondary necrosis (46).

**Apoptosis.** Nearly 75% of the potential number of spermatozoa were reported lost in normal testis (29). The low number of apoptotic-positive germ cells we recorded in normal mink reflects the efficiency of Sertoli cells to engulf dying cells, while the significantly increased numbers of these germ cells in orchitic tubules is evidence this phagocytic activity was altered. This result echoes the report of an increased numbers of in situ end-labeling positive apoptotic cells following experimental induction of AIO in rat testis (87). However, it contrasts with our observation of no significant differences between the apoptosis levels measured by ELISA in normal and orchitic mink tubules. The two technical approaches used measured different substrates accounting in part for this discrepancy. Here, apoptotic germ cells were labeled with apostain, an antibody against single-stranded DNA, which provided specific results for the detection of apoptosis in the testis (45). Our ELISA measurements allowed quantification of mononucleosome- and oligonucleosome-bound DNA in the cytoplasmic fraction of a cell homogenate caused by DNA fragmentation during apoptosis. Apostain labeling and in situ end labeling or even the less reliable (25) terminal deoxynucleotidyld transferase biotin-dUTP nick-end labeling, which was shown to be not specific for apoptosis (11, 22) all identify dying apoptotic cells in situ, that is to say, of damaged cells nonengulfed by Sertoli cells. In contrast, our ELISA measurements reflect the nucleosome release in the tubule fractions not the phagocytic activity of Sertoli cells. Apoptosis may involve chromatin condensation without DNA fragmentation in some germ cells. In fact, apoptotic spermatocytes and elongated spermatids do not show nuclear features typical of apoptosis (27). Some apoptotic cells may escape terminal deoxynucleotidyld transferase assay detection or turn out to be false positives, or still, a number of cells with apoptotic features could switch to necrotic features by the end of their death process. Time-related differences in DNA cleavage could occur particularly when the phagocytic capacity is exceeded. The fragmentation of apoptotic cells into apoptotic bodies was altered during spontaneous AIO as evidenced by our finding of numerous difficult-to-engulf giant cells whose sizes favored their remaining for longer periods in the tubules and thereby clearance impairment. Fragmentation is one of many steps of apoptosis (4), and the size of apoptotic corpses could affect their ability to be phagocytosed (14, 56). That local regulatory clearance mechanisms did not rid the epithelium of apoptotic cell debris is evidence that the phagocytic capacity of Sertoli cells were exceeded.

**Phagocyte receptors.** We propose that defect in the clearance machinery evidenced here by an overload of apoptotic cells potentially triggered by an imbalance in phagocyte receptors [CD36 (present study) or SR-BI (2)] or cytokines (TNF-α and IL-6, present study) implicated in the local immunoregulation by Sertoli cells constitutes a mechanism for the breakdown of self-tolerance during spontaneous AIO. This study has investigated the possibility that the spontaneous AIO-induced inflammation triggers Sertoli cells to modify their cytokine-dependent cell-to-cell interactions implicated in blocking entry of foreign cells and in cell recognition and elimination. This study reports findings regarding the expression in the serum and seminiferous tubules of factors (IL-6, TNF-α, TNF-α RI, Fas, Fas-L, and CD36) known to be involved in immunoregulation, apoptosis, and self-tolerance in normal conditions and assesses the deregulation of these mechanisms during spontaneous AIO.
Fig. 5. Fas immunolocalization in a 210-day-old mink testis (A) and in an adult, in a stage VII (B and D) and stage VIII (C) in normal testis in February. Labeled residual bodies are identified (white feathered arrow near the lumen in D). E–I: Fas labeling in normal testis in April (E and F), May (G), June (H), and August (I). Fas localizes to Sertoli cells (thin arrows, A–D; F and G) and to the surface of spermatocytes, round spermatids (wide arrows, E) and elongated spermatids (zigzagged arrows, B and E), as well as intracellularly (wide arrows, E and F) in the degenerating germ cells. Degenerating and exfoliating spermatids and spermatocytes contain variously sized intracytoplasmic Fas-positive clumps. v = vacuoles. Magnification, ×1,110.
Fig. 6. Typical pathological features by the onset (A), middle (B), and end (C) of AIO in February. Diffuse Fas-positive material is identified near the base of Sertoli cells (thin arrows, A and B). Apoptotic germ cells gathered in clusters form giant cells (G) also contain a diffuse Fas-positive material (A and B). Wide arrows point to Fas-positive degenerating spermatocytes (A) and round spermatids (B). Large white feathered arrows identify Fas-positive infiltrating cells in tubules in which destruction of the epithelium is advanced (C). Arrowheads in A–C show regions of the epithelium where roomy spacing separates normally contiguous germ cells and Sertoli cells (magnification ×1,110). D: electron micrograph showing an infiltrating cell (I) from the immune system, which is located below Step 1 round spermatids and Step 13 elongated spermatids and lumenally to an apparently intact Sertoli cell junctional complex joining adjacent Sertoli cells labeled Ser1 and Ser2. Magnification, ×11,960.
Fas-L immunolocalization in a 60 (A)-, 90 (B)-, 180 (C)-, 210 (D)-days-old mink testis in an early stage VII (E), middle stage VII (F), and stage X (G), in February (H, I), April (J), June, and August (K) in normal adult testis. In germ cells, Fas-L-positive clumps are identified near the nucleus in gonocytes (A), some spermatogonia (B, D, K), and spermatocytes (C, D, F, J, arrowheads). In addition, Fas-L-positive crusts are scattered on the plasma membrane (D, H, I, wide arrows). Plentiful, but minute, cytoplasmic Fas-L-positive granules are sprinkled in the vicinity of the plasma membrane in round and elongated spermatids (E, G, H, I, zigzagged arrows). Residual bodies are labeled (F arrows). Many spermatocytes (wide arrows) and spermatids (zigzagged arrows) contain Fas-L positive intracytoplasmic granules, which increase in size with the advance of cell death (H, I, J). Magnification, ×1,110.

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Fig. 8. Distribution of Fas-L in adult mink testes with AIO in February. A–C: sequence of the micrographs is representative of progress of damage to epithelium. Numerous spermatids (arrow), spermatocytes, and spermatogonia (arrowheads) contain sizeable Fas-L-positive intracytoplasmic granules that sometime completely filled the cell. C: Fas-L-positive material is detected at the cell surface. Giant cells (G) containing large Fas-L-positive granules are identified (magnification, ×1,110). D: a plasmocyte (P) is identified next to a very infolded (arrowheads) limiting membrane of a tubule with AIO in the electron micrograph (magnification, ×11,960). Inset: shows a high magnification of the dilated cisternae of endoplasmic reticulum coated with ribosomes contained in this plasmocyte (magnification, ×49,450).
Fig. 9. Confocal microscopy showing immunolocalization of Fas (A–C, red), Fas-L (A’–C’, green), and the exact colocalization of Fas with Fas-L (A”–C”, yellow) by the beginning (A) and the end (B) of stages VII and by VIII (C) in normal adult testis in February. A: Fas localizes to the mature spermatids and neighboring residual bodies (dots) facing the lumen. In addition, the round spermatids are faintly labeled together with the adjacent Sertoli cell cytoplasmic processes. B: reaction is slightly intensified in the Sertoli cell processes but it is particularly strong in the residual bodies collected near the base of the cell by the end of stage VII. C: by stage VIII, residual bodies disappear and Fas labeling intensifies in the Sertoli cell cytoplasmic processes. Fas-L Ab labels (A’–C’) mature spermatids and residual bodies lying either (A’, B’) close to the lumen or en route toward the base of the tubule (B’). A’–C’: labeling is apparent chiefly in meiotic cells in the lower third of the tubules, although postmeiotic cells are also faintly labeled. A’: Fas-L labeling is seen in the cytoplasm of the young spermatogonia next to the limiting membrane of the tubule, whereas in the meiotic cells, labeling is associated with the plasma membrane in (B’ and C’). Fas and Fas-L colocalize in (A” and B”) the mature spermatids and in (B”), the residual bodies that have been engulfed in the basal third of the Sertoli cells (B”) although in this localization, colocalization disappears (C”) with the disappearance of the bodies by stage VIII. Magnification, ×1,110.
Cytokines. Cell survival or death is in part governed by cytokines that influence cells of the immune and of other systems. IL-6, which is implicated in immunoglobulin secretion by B lymphocytes, was shown to activate T and B lymphocytes (28). Using IL-6 antibodies, we were the first to identify a 26- and a 17-kDa immunoreactive bands in normal tubule fractions and to report the absence of the 17-kDa immunoreactive band in orchitic tubules. The existence of such an alternatively spliced form of IL-6 mRNA encoding for a polypeptide missing the membrane glycoprotein (gp)130 interactive (signal-transducing) domain, while retaining the IL-6 receptor (p80) domain, has been reported in another cell systems (36). Since the gp130 interactive domain facilitates signal transduction and generation of high-affinity receptor sites for a particular cytokine (74, 83), its absence in the 17-kDa IL-6 form suggests that this alternative form could theoretically bind to IL-6 receptor (p80) but would act as a natural antagonist of IL-6, because it is unable to interact with gp130 (36). The production of the 17-kDa IL-6 form could represent a control mechanism that would prevent IL-6 producer cells like B lymphocytes and Sertoli cells (82) to be continually activated by IL-6 in normal but not orchitic tubules in which this alternative form was lacking. The IL-6 serum levels increase, the decrease in total IL-6 protein levels, the

Fig. 10. Confocal microscopy micrographs (A–C) are displayed in a sequence representative of the progressive damage to the adult mink testes during spontaneous AIO. Immunolocalization of Fas (A–C, red), Fas-L (A’–C’, green), and Fas- Fas-L colocalization (A”–C”, yellow). The Fas labeling is diminished in the Sertoli cells (A–C). A: scattered elongated spermatids are Fas-positive. B: apoptotic cells clustered in giant cells are intensely Fas-positive. Fas is also detected in round spermatids (A) and infiltrating cells (C). Fas-L is detected in elongated spermatid (A’) and in the younger germ cells near the limiting membrane of the tubule (C’) and in giant cells (B’). Precise colocalization of Fas with Fas-L is viewed in yellow in round and elongated spermatids (A”), giant cells (B”), and few infiltrating cells (C”). Magnification, ×1,110.
overload of apoptotic germ cells, and the leukocyte infiltration in the 17-kDa IL-6-deficient orchitic tubules exemplify changes in Sertoli cell-germ cells and Sertoli cell-immune cells interactions underlying the local immunoderegulation.

TNF-α levels were high in serum and tubules, whereas IL-6 levels were high in serum while being low in tubules, suggesting that these orchitic mink responded to TNF-α stimulation but that the Sertoli cell response was altered. Cultured mouse Sertoli cells were reported to respond to TNF-α by enhancing their surface expression of the binding molecules for lymphocytes intercellular adhesion molecule 1 and vascular cell adhesion molecule 1, which binds β-2- and β-1-integrins on leukocytes while increasing their secretion of biologically active IL-6 (70). TNF-α likely contributes significantly to the pathogenesis of spontaneous AIO since induction of AIO by transfer of testis and sperm antigen-specific T cell clones was reduced following injection of TNF-α neutralizing antibodies (100).

*Fas and Fas-L.* Our observation of Fas and Fas-L in spermatocytes and spermatids agrees with the reports of a death receptor and its corresponding ligand in germ cells (10, 13, 54) but contrasts with studies that viewed Sertoli cells as the sole source of Fas-L (97). Our novel observation of Fas and Fas-L colocalized in residual bodies engulfed in Sertoli cells suggests that Fas-mediated apoptosis contributes to the targeted disposal of these cytoplasmic fragments and implies a nuclear-independent apoptosis. Significantly, experimental impairment of the elimination of residual bodies by Sertoli cells in line TGR(mRen2)26 transgenic rats was associated with infertility (80).

The apoptosis peak incidences we recorded in March were accompanied by an increase in Fas and Fas L tubular levels, whereas the second apoptosis peak incidences in July were accompanied by a decrease in the abundance of both, suggesting that the Fas system was not related to the second peak. This and the apopain labeling of spermatids and aged spermatocytes during the first peak incidence and of young spermatocytes and spermatogonia during the second are suggestive of either a differential sensitivity to Fas-mediated apoptosis or that Fas-L activity differs among germ cells.

Fas-L is known to exist as a cell surface ligand as well as to be released in soluble form (76, 85). Here, we localized Fas in the normal tubule basal lamina and Fas-L in the redundant extracellular matrix during the acute phase of spontaneous AIO. Sertoli cells and myoid cells contribute to the formation of the tubular lamina interna (47, 91); thus, either or both cells could have contributed local Fas and Fas-L secretion in response to stimuli. The role of the basement membrane in the differentiation of epithelia (81) and the integrity of the seminiferous epithelium (43) has been confirmed. Soluble Fas-L was reported in the extracellular matrix of the ciliary body of the anterior chamber of the eye (3), and its contribution to the mechanism of immune privilege has been demonstrated (24). The capacity to induce apoptosis of soluble Fas-L was shown less than that of membrane-bound Fas L in vitro (76) but not in vivo where hepatocyte apoptosis (68) leaded to liver failure (86). Our observation of local amounts of Fas-L in the orchitic tubules’ basal lamina is significant since soluble Fas-L binds directly to fibronectin (3), a natural constituent of the tubular basement membrane and germ cells except spermatogonia (75). In addition, interaction of the extracellular matrix with soluble Fas-L together with the capacity of the matrix to enhance its efficacy by increasing its local concentration and retention have been shown (3). Our finding of Fas-L in orchitic mink suggests a contribution of the tubular extracellular matrix to Fas-mediated cell death.

**Perspectives and Significance**

The production of antibodies to spermatozoa we reported in normal mink is evidence that B cells are naturally exposed to germ cell autoantigens without causing immune inflammatory responses, destruction of the seminiferous epithelium, or male infertility. The overactivation of autoreactive B lymphocytes and overproduction of antibodies in orchitic mink exemplifies an immunoderegulation. Our novel observation that Fas and Fas-L colocalized in residual bodies engulfed in Sertoli cells suggests a contribution of Fas-mediated apoptosis to the targeted disposal of these cytoplasmic fragments. The observation is significant considering that experimental impairment of the elimination of these bodies has been associated with infertility. The study provided evidence that a malfunction of the local regulatory mechanisms needed to clear the orchitic seminiferous epithelium of apoptotic cell debris following imbalances in phagocyte receptors or cytokines acting on Sertoli cells constitutes a major factor leading to breakdown of self-tolerance during spontaneous AIO. Our finding of infiltrating cells from the immune system that possess the capacity of inducing apoptosis in the targeted cells via perforine-granzyme B-mediated mechanisms are indicative of a diminished ability of the Sertoli cells to forbid lymphocytes entry, not the immunological surveillance by lymphocytes at these sites.

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