—Technology Report—

Successful activation of rat T lymphocytes by sperm specific antigens *in vitro*

Junko NOGUCHI[1)*], **Mitsumi IKEDA**[1)], **Kazuhiro KIKUCHI**[1)], **Thanh Quang DANG-NGUYEN**[1)], **Megumi KASASHIMA**[2)], **Ken-ichiro TATEMATSU**[2)], **Hideki SEZUTSU**[2)] and **Tadashi FURUSAWA**[1)*]

[1)]Division of Animal Sciences, Institute of Agrobiological Sciences, National Agriculture and Food Research Organization (NARO), Ibaraki 305-8634, Japan
[2)]Division of Biotechnology, Institute of Agrobiological Sciences, National Agriculture and Food Research Organization (NARO), Ibaraki 305-8634, Japan

Abstract. Autoimmune orchitis is a condition related to cellular immunity. A disease model involving transfer of T lymphocytes activated by known antigens would be useful for defining pathogenical molecules. Since no method for activating rat T cells using specific antigens is available, we started the study to develop the method. T cells were collected from draining lymph nodes of immunized rats, then co-cultured with syngeneic splenocytes as antigen-presenting cells (APC) in antigen-supplemented medium (= stimulation). The cells were then incubated in medium without antigens and APC (= resting). Repetitive stimulation and resting increased the number of the T cells more than 100-fold. The antigen-specific activation was demonstrated by cell proliferation assay and ELISA assay for interferon gamma. Flow cytometry revealed that > 95% of the cells expressed tumor necrosis factor alpha, a cytokine responsible for autoimmune orchitis. The present method will provide a new procedure to evaluate antigenicity of sperm molecules.
Key words: Antigen activation, Culture, Rat, Sperm, T cell

Since most male germ cells are potentially autoantigenic, spermatogenesis needs to take place in an environment isolated from immune reactions. In that sense, spermatogenesis depends on two factors: a blood-testis barrier, which sequesters autoantigenic germ cells from the immune system, and an immune privileged status whereby regulatory T cells can perform important functions. Several etiological factors can disturb immune tolerance, including systemic and local infections, trauma, noxious chemicals, neoplasms, genetic disorders, systemic diseases and other unknown factors. Once immune tolerance has been disrupted, spermatogenesis may become seriously impaired by the resulting immune response. Testicular biopsies from male partners attending infertility clinics have suggested that such infertility may be due in part to autoimmune orchitis [1].

Autoimmune orchitis can be reproduced in rodents by active immunization with testicular antigen. Such experimental autoimmune orchitis (EAO) has yielded valuable information for understanding how spermatogenesis is damaged under conditions of immune disruption [2]. The affected testis showed marked infiltration of immune cells, including CD4^{+} and CD8^{+} T cells, macrophages and dendric cells, in the interstitium. Inflammatory molecules such as

cytokines and chemokines produced by these cells are considered to impact adversely on spermatogenesis [3]. Currently, the antigenicity of testis-specific molecules is evaluated by active immunization, and several molecules have been implicated in the pathogenesis of autoimmune orchitis [4].

On the other hand, transfer of antigen-specific T cells that respond to testis- or germ cell-derived antigens facilitates the generation of testicular inflammation in normal syngeneic recipient mice [5–7]. Such transfer requires the CD4^{+} subset of T cells [5, 7]. T cell clones established from testicular antigen-specific T cells have been shown to have a pathogenetic role [8], suggesting that passive immunization with antigen molecule-specific T cells could become a useful tool for determining the pathogenicity of the molecule. To our knowledge, however, passive immunity using such antigen molecule-known T cells to elucidate the pathogenesis of orchitis has not yet been reported.

Recently, we have reported that immunization of rats with sperm mixed in lipopolysaccharide (LPS)-supplemented adjuvant induced orchitis associated with impaired spermatogenesis [9]. As in previous EAO models, the pathology is considered due to cell-mediated immunity. To understand the pathogenesis and identify the sperm-derived antigen molecules responsible, it is necessary to establish a procedure for passive immunization. Here we report the *in vitro* proliferation of antigen-specific rat T cells that are sufficient for passive immunization.

Initially, we employed the culture method for rat T lymphocytes reported by Binz *et al.* [10] using ALyS505N-0 (a serum-free medium for human and mouse lymphocytes) instead of the original EHAA medium. However, since the viability of T cells cultured in ALyS505N-0 was very low, we changed the basic medium to

DMEM/F12 supplemented with 10% (vol/vol) fetal calf serum (FCS-DMEM/F12). Co-culture of T cells and APC for stimulation with antigens was undertaken basically according to Yule and Tung [8], except for the medium and added materials. The mouse cells were cultured in RPMI 1640 supplemented with 10% FCS and 1% non-essential amino acids for both for stimulation and resting. For resting culture of rat T cells, supplementation with T cell growth factor (TCGF, spent medium of splenocytes) was necessary. After several attempts, the best results of resting culture were obtained using FCS-DMEM/F12 supplemented with 10% TCGF prepared with ALyS505N-0. ALyS505N-0 does not contain IL-2, but further details of its content are unknown. Addition of either ALyS505N-0 or the TCGF prepared with DMEM/F12 resulted in lower cell viabilities than those obtained using TCGF prepared with ALyS505N-0 (data not shown).

For adoptive transfer in murine models of autoimmune orchitis, cells from the spleen and draining lymph nodes (DLN) of donors have been shown to equally induce the disease in recipients [5]. In the case of rats, more than 2×10^8 cells were collected from the spleen and approximately half of the cells were harvested after nylon wool enrichment. In terms of viable yield after culture, that of DLN-derived cells was better than that of spleen-derived cells (data not shown). Although the reason for the lower yield after the culture of spleen-derived cells was not clear, we obtained donor cells from the DLN in subsequent experiments.

The density of cells in culture is the key to cell viability. Firstly, $6–7.5 \times 10^7$ cells (20–25 ml of medium containing 3×10^6 cells per ml) were incubated in an upright 70-ml flask for resting culture, corresponding to $6–7.5 \times 10^6$ cells per cm^2 attached to the bottom. After 3–4 days of culture (see Fig. 1: experimental schedule), dead cells (determined by trypan blue exclusion) greatly outnumbered viable cells, indicating that the culture conditions did not favor cell viability. Lowering the density by decreasing the cellular density in the medium improved the viability. For resting culture, cells adjusted to $0.2–0.3 \times 10^6$ cells per ml of the medium incubated in flasks at $0.6–0.7 \times 10^6$ cells per cm^2 resulted in higher viability (regularly over 60%). Stimulation culture required higher density than resting culture, assuming that direct contact of T cells and APC would be necessary for antigen presentation. For stimulation culture, T cells combined with APC (1:5) to a final concentration of $2.2–2.5 \times 10^6$ cells per ml were incubated in flasks at $1.4–1.5 \times 10^6$ cells per cm^2.

In the experiment using sperm extract as the antigen, $4–6 \times 10^7$ cells were obtained after nylon column enrichment. The diameter of the cells was less than 10 μm (Fig. 2a, left). As shown in Fig. 2b, the number of cells did not increase (in fact decreased somewhat) in the starting culture, then began to increase after the second series of propagation culture. Cells at this stage were larger than those at the start of culture, which were considered to be lymphoblasts (Fig. 2a, right). The antigen specificity of the cells was investigated by XTT assay. The sperm extract, containing the same antigen as that used in immunization of the donor, activated the cultured cells in a dose-dependent manner, whereas ovarian and testicular extracts did not activate them (Fig. 2c). An abrupt increase was observed upon supplementation with the sperm extract at 1–10 ng/ml, a significant difference from the other two groups being observed at 5 ng/ml and over. Specificity was also confirmed in terms of interferon gamma (INF-γ) production (Fig. 2d). The level of INF-γ in the medium increased sharply at 1–10 ng/ml, in accordance with the result of the XTT assay. Based on production of the cytokine, the cultured cells were considered to be the Th1 subset [11].

Flow cytometry of the expanded cells after antigen stimulation revealed that no less than 97% of cells were TNFα$^+$, corresponding to the sum total of CD4$^+$/CD8$^-$, CD4$^-$/CD8$^+$ and CD4$^+$/CD8$^+$ cells (Fig. 3b). The intensity of TNFα in the cultured cells was relatively high, when compared to that in splenocytes (Fig. 3a). TNFα has been shown to play a role in disrupting spermatogenesis in both active [12] and passive EAO [8]. It is plausible that such expanded cells have the potential to induce orchitis in recipient males when transferred. An experiment to investigate this is currently ongoing in our laboratory.

We applied sperm adhesion molecule 1 (SPAM1, a sperm-specific molecule), as the antigen for the immunization of donors and the *in vitro* activation system. SPAM1 (formerly named PH-20) is the first sperm-specific protein found to be pathogenic for autoimmune orchitis [13]. Using the silkworm expression system, recombinant rat SPAM1 with truncation of the signal peptide and the putative
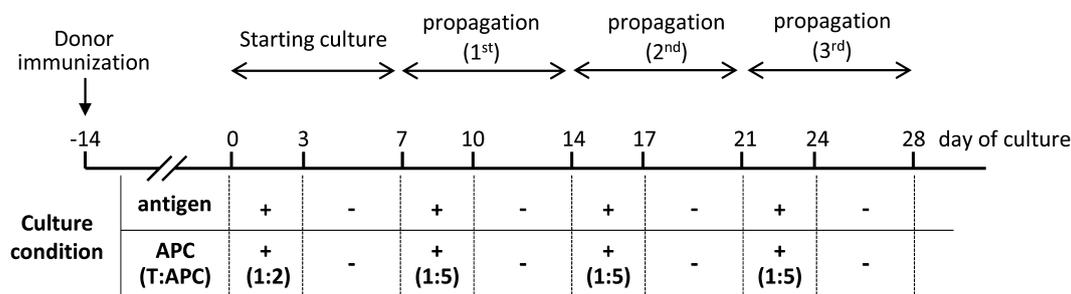
Fig. 1. Experimental schedule. Donor animals were injected with antigens emulsified in adjuvant two weeks before the onset of culture (day 0). T cells collected from the donors were subjected to antigen stimulation in starting culture for 3 days, and then to 4-day resting culture. One cycle of the propagation consisted of 3-day stimulation and then a 4-day rest. The conditions for each culture are shown below the schedule. APC, antigen-presenting cells. Normal spleen cells were treated with mitomycin c to diminish their mitotic activity, then used as APC. The ratio of T cells and APC is shown in parenthesis.
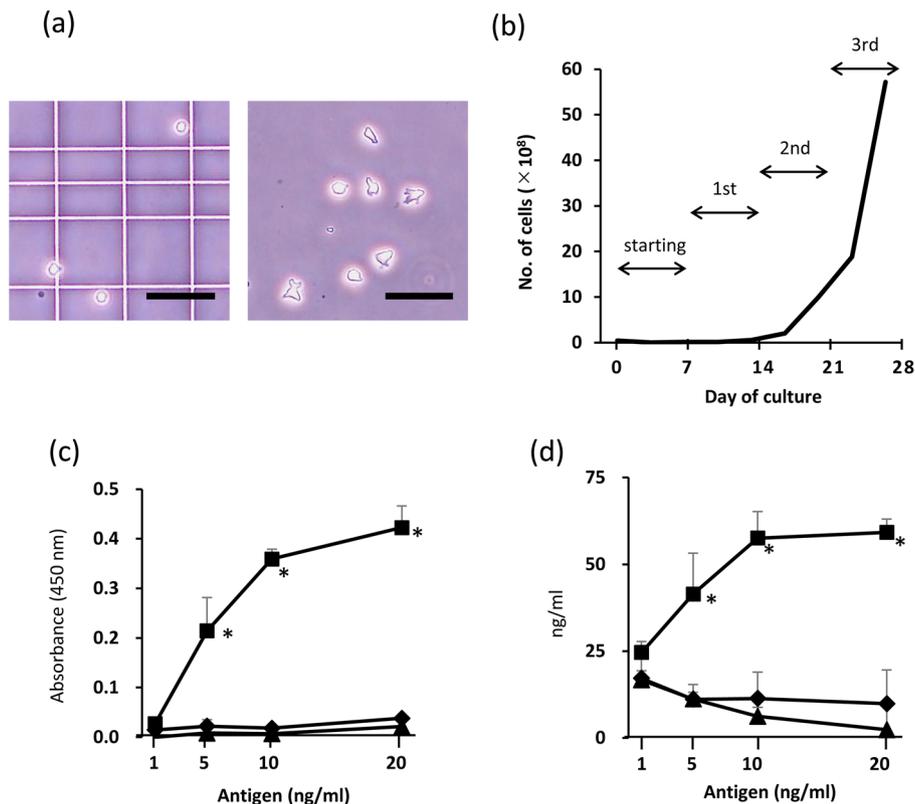
Fig. 2. Proliferation and antigen specificity of sperm extract-specific T cells. T cells from donor's draining lymph nodes were enriched using a nylon wool column. The T cells in PBS supplemented with 0.1% trypan blue were loaded in a hemocytometer (a, left). The cells were then stimulated by antigen presentation via antigen-presenting cells. Viable cells were separated by density gradient centrifugation after stimulation (a, right). The cells were irregularly shaped and larger than cells in the left-hand photo. Within hours, the cells had changed to an oval shape. Bar = 50 μm. Repeated stimulations with the antigen induced proliferation of the cells. A representative result is shown in b. The culture was started with 0.4 $\times 10^8$ donor T cells. Double-ended arrows indicate a cycle of cultures (starting and propagation cultures). In the second cycle of propagation, the number of cells started to increase and finally reached 57×10^8. In the XTT assay (c), the cells showed dose-dependent activation with the sperm extract (■), but not with testicular (♦) and ovarian extracts (▲). ELISA of INF-γ using the spent medium after cell culture confirmed the result of the XTT assay (d). An antigen-specific increase of INF-γ was seen. * Significant differences (P < 0.01) were detected between the groups supplemented with sperm extract and those supplemented with other antigens. The assays were repeated more than 3 times, and the representative data was shown in c and d.

transmembrane domain [14] has been produced (Fig. 4a). The protein was extracted from about 40 cocoons, yielding 12 mg of purified protein that was required for a series of experiments. In a previous study of mouse autoimmune orchitis, $12–15 \times 10^6$ clonal T cells specific for testis antigen were needed for efficient induction of the disease [8]. As shown in Fig. 4c, a series of experiments yielded more than 20×10^8 specific cells. This number would be sufficient for adoptive transfer in rats, even taking into account the difference in body size.

Flow cytometry analysis revealed that nylon-wool-nonadherent cells were enriched in T cells (CD3+ cells, Fig. 5a). The cells comprised CD4+/CD8− (= helper T cells) and CD4−/CD8+ cells (= cytotoxic T cells) at the onset of culture (about 70 and 20%, respectively, in Fig. 5a). After propagation, a decrease in proportion of CD4−/CD8+ cells was observed, while the proportion of CD4+/CD8+ cells tended to increase (from about 4% to about 30%, in Fig. 5b). We confirmed the increase of the double positive cells after propagation in a repeated experiment. Such double-positive cells have been found in

mouse EAO [15] and could be CD4+ cytotoxic T cells (CD4 CTLs). Common to helper T, but not cytotoxic T cells, CD4 CTLs receive antigen presentation via MHC class II [16]. It is thought that antigen presentation by MHC II-expressing APC occurs during propagation, resulting in expansion of the CD4+/CD8− and CD4+/CD8+ cells. CD4 CTLs have been shown to exacerbate autoimmune disease through perforin-mediated cytolytic activity [16]. Further study is necessary to characterize the CD4+/CD8+ cells and clarify their contribution to the pathogenesis of autoimmune orchitis.

We have succeeded in establishing a procedure for *in vitro* activation of rat T cells using specific antigens. Now that antigen-specific T cells are available, passive immunization will help to clarify the etiology of autoimmune orchitis and identify the antigens responsible.

Methods

Experimental animals
All the animals used were produced at the Institute of Agrobiological
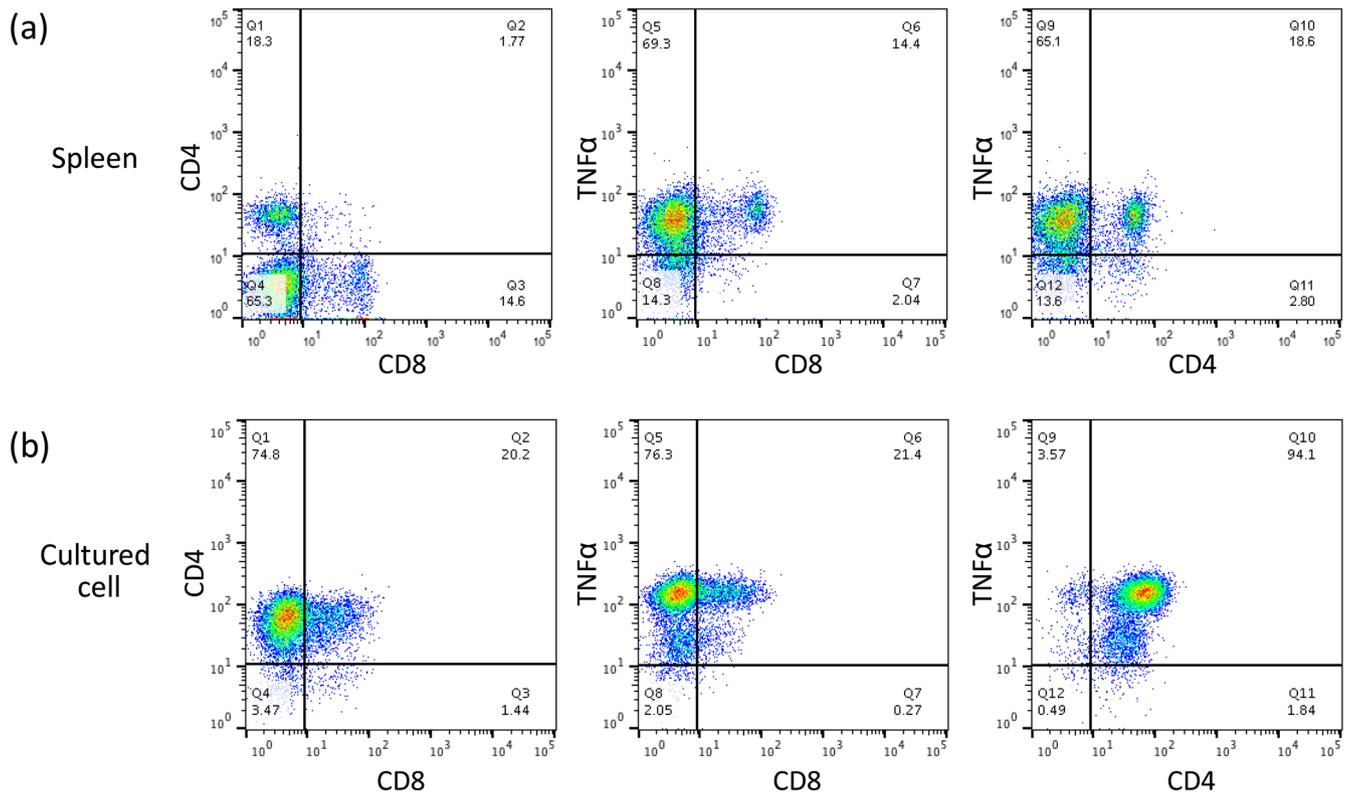
Fig. 3. Expression of tumor necrosis factor alpha (TNFα) by the expanded cells upon stimulation with the sperm extract. Flow cytometry of normal splenocytes (control, a) and expanded cells (b). Note that most of the expanded cells were CD4$^+$/CD8$^-$ or CD4$^+$/CD8$^+$. CD4$^+$-dependent TNFα expression was evident. The experiment was performed twice, and the representative data was shown.
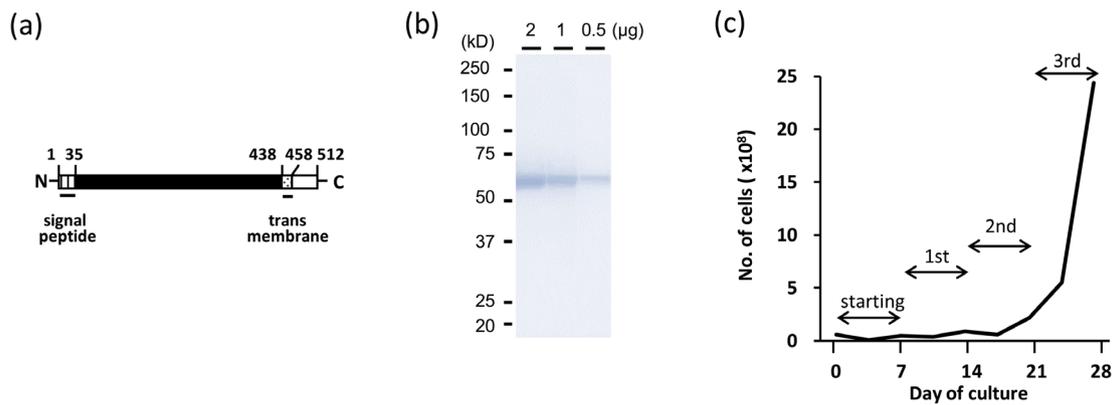
Fig. 4. Sperm adhesion molecule 1 (SPAM1)-specific T cell activation. Shaded bar represents the recombinant rat SPAM1 produced using the silkworm expression system (a). 6 × His was added at the C terminus of the protein. Purified recombinant rat SPAM1 (b). Separated with SDS-PAGE and Coomassie blue stained. Loaded amount per lane was shown at the top. Repeated stimulation by SPAM1 induced proliferation of the specific T cells. A representative result is shown in c. The culture was started with $0.6 × 10^8$ cells. Double-ended arrows indicate a cycle of cultures (starting and propagation cultures). In the second cycle of propagation, the number of cells started to increase and finally reached $24 × 10^8$. The culture was repeated more than 3 times, and the representative data was shown.

Sciences, NARO. They were obtained by mating normal rats in an inbred Wistar strain harboring a partial deletion in the gene encoding FK506 binding protein 6. The mutant locus was named '*as*'.

Males homozygous for *as* show testicular atrophy due to arrested spermatogenesis at the pachytene spermatocyte stage, while heterozygous and wild-type males and all the females show no abnormal
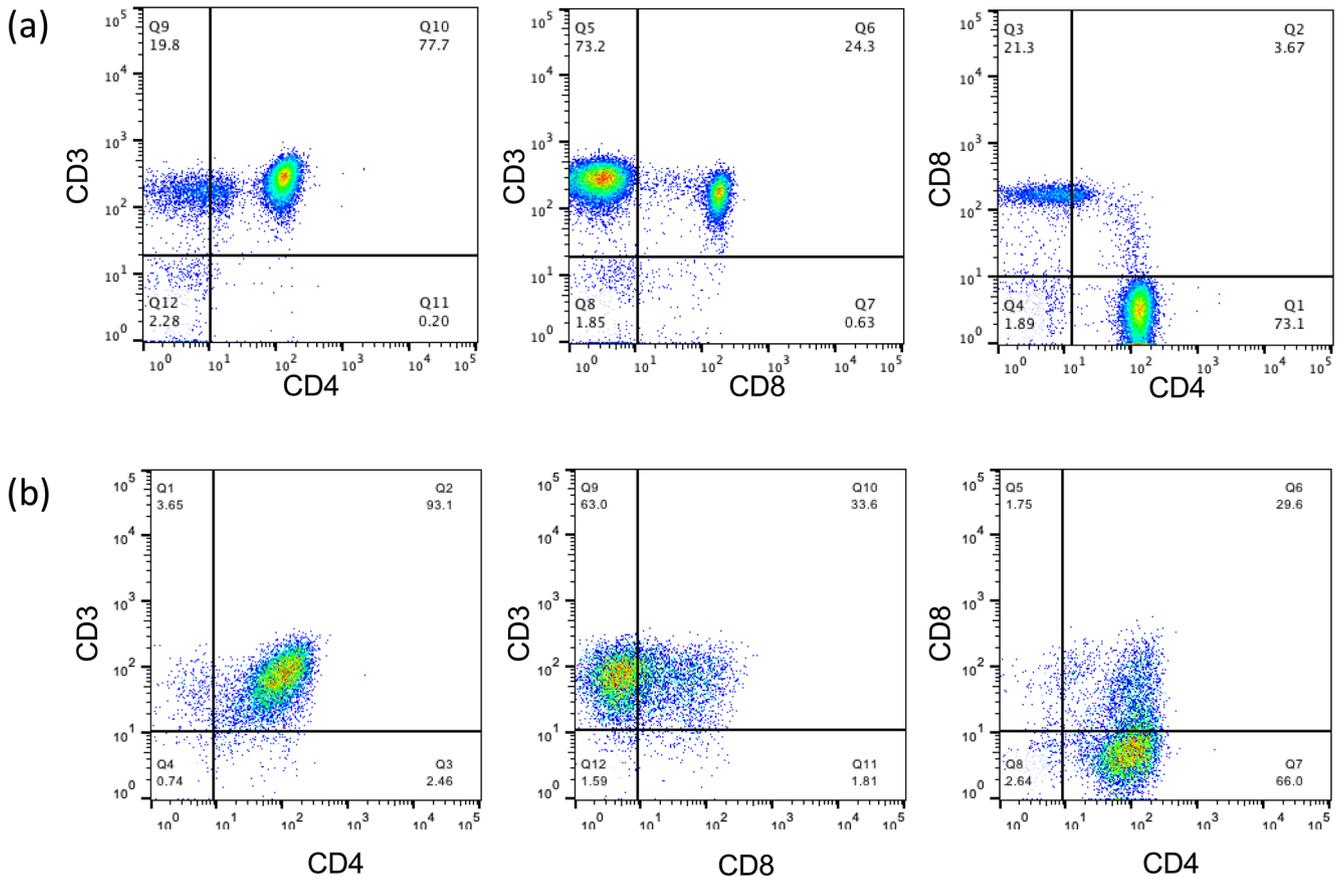
Fig. 5. T cell subset distribution in sperm adhesion molecule 1 (SPAM1)-specific T cells. Flow cytometry of the SPAM1-specific T cells enriched using the nylon wool column (a). More than 95% of the cells were CD3+. Flow cytometry of the expanded cells after antigen stimulation (b). Note the marked increase of CD4+/CD8+ cells, compared to those in (a). The experiment was performed twice, and the representative data was shown.

phenotype [17, 18]. Phenotypically normal rats in this strain were used. Experiments involving animals were undertaken in strict accordance with the recommendations in 'Standards Relating to the Care and Keeping and Reducing Pain of Laboratory Animals' (Ministry of Environment, Japan). Animal experiments were approved by the Institute of Agrobiological Sciences, NARO Animal Care and Experiment Committee (H28-007).

Preparation of antigens

Extracts of sperm, testes and ovaries were prepared as follows. The 1×10^8 epidydimal sperm per ml of DMEM/F12 (12400-024; Thermo Fisher Scientific, Waltham, MA, USA) were homogenized in a blade-type homogenizer then frozen and thawed three times. After centrifugation ($17,000 \times g$ for 30 min at 4°C), the supernatant was collected and stored at –80°C until use. Approximately 0.1 g of the testis and ovary in 1 ml of DMEM/F12 was treated in the same way as the sperm to prepare the extracts. The protein concentrations of the extracts were measured using a Coomassie® protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Recombinant rat SPAM1 protein was produced using the transgenic silkworm technique [19]. The protein in the cocoon was extracted in 1% Triton X-100 PBS

(Dulbecco's PBS. 05913; Nissui, Tokyo, Japan) then purified using a His-tagged protein purification kit (TALON®; Clontech Laboratories, Mountain View, CA, USA) in accordance with the manufacturer's instructions. The harvested protein solution was dialyzed in PBS then stored until use (–80°C).

Immunization of donors and preparation of antigen-specific T cells

Antigen solution (sperm extract or recombinant rat SPAM1 protein) was emulsified with an equal volume of Montanide™ ISA71VG (a gift from Seppic, Paris, France) supplemented with LPS (from *E. coli* O127:B8. L4516; Sigma-Aldrich, St. Louis, MO, USA). The emulsion, 100 µl per head, included 100 µg of the sperm extract or the SPAM1 protein and 20 µg of LPS. Donor animals (adult female or male rats; BW about 220–250 g) were anesthetized with isoflurane and then the emulsion was injected subcutaneously in both hind footpads. About 2 weeks after the injection, the animals were sacrificed in CO_2 gas and their DLN (popliteal and inguinal lymph nodes; [20]) were collected. They were aseptically ground using glass slides in 0.1% glucose-supplemented PBS (gPBS) and cells were pelleted by centrifugation ($900 \times g$ for 5 min at 4°C). Erythrocytes present were

lysed in 155 mM NH_4Cl, 10 mM $KHCO_3$, and 1 mM EDTA/4Na (pH 7.4). After washing with gPBS, the cells were suspended in 5 ml of DMEM/F12 containing 10% FCS (FCS-DMEM/F12) at 37°C. A nylon-wool column for T cell enrichment was prepared following Mahi-Brown *et al.* [5] with some modifications. Briefly, 1.5 g of nylon wool fiber (Polysciences, Warrington, PA, USA) was set in a 20-ml disposable syringe with a three-way stop cock and a 22 G needle. Following sequential washing with 50 ml of gPBS and DMEM/F12, the column was equilibrated with FCS-DMEM/F12 in 7% CO_2 at 37°C. The cell suspension was applied to the column then incubated for 1 h in 7% CO_2 at 37°C. Pre-warmed FCS-DMEM/F12 (about 30 ml) was added to the column, and eluted cells (T cells) in the pass-through fraction were harvested by centrifugation.

Starting culture

The eluted T cells were cultured with twice the number of APC in stimulation medium [FCS-DMEM/F12 supplemented with antigen (final concentration 10–15 µg/ml), 1% fresh rat serum and 5×10^{-5} M 2-mercaptoethanol] in 7% CO_2 at 37°C for 3 days. For preparation of APC, the spleen from normal rats was treated according to the same procedure as that for the DLN, and any erythrocytes present were lysed. The cells were then incubated in DMEM/F12 containing 0.1 mg/ml mitomycin c (Kyowa Kirin, Tokyo, Japan) for 25–30 min at 37°C to diminish their mitotic activity. After 3-day culture, viable cells were separated using Lympholyte®-Rat (Cedarlane, Burlington, Canada), in accordance with the manufacturer's instructions. The viable cells were cultured in resting medium [FCS-DMEM/F12 supplemented with 10% TCGF and 5×10^{-5} M 2-mercaptoethanol] in 7% CO_2 at 37°C for 4 days (= resting culture). The TCGF was prepared following Binz *et al.* [10], except for the use of ALyS505N-0 (Funakoshi, Tokyo, Japan) as the medium.

Propagation culture

The cells after the starting culture were co-cultured with 5 times the number of APC in the stimulation medium for 3 days (stimulation culture). The viable cells were separated as mentioned above then cultured in the resting medium for 4 days. The stimulation and resting cultures were repeated alternatively to propagate the T cells. The experimental schedule is shown in Fig. 1.

Cell activation assay

Cellular metabolic activity was measured using an XTT kit (Biological Industries, Cromwell, CT, USA) in accordance with the manufacturer's instructions. Briefly, T cells were mixed with APC (1:5) and then a total of 1×10^5 cells in 250 µl of stimulation medium were added to each well of a 96-well plate (92196; Techno Plastic Products; Trasadingen, Switzerland) and incubated in 7% CO_2 at 37°C for 2 days. After the incubation, 150 µl of the medium was removed and stored at –25°C for assay of INF-γ. The reaction solution (50 µl per well) was added, and then the cells were incubated further until the medium became colored. The absorbance of the medium was measured at 450 nm.

ELISA of INF-γ

To measure INF-γ in the medium, 96-well ELISA plates (467466; Thermo Fisher Scientific) were coated with 2 µg/ml anti-INF-γ monoclonal antibody (MAB5851; R&D Systems, Minneapolis, MN, USA) in PBS overnight at 4°C then blocked with 1% BSA-PBS. Sample or serial dilution of recombinant INF-γ (585-IF; R&D Systems) was added to the wells then incubated for 90 min at room temperature. Following serial incubations with the biotin-labeled second antibody (BAF585; R&D Systems) and avidin-HRP (A-2014; Vector Laboratory, Burlingame, CA, USA), an ELISA substrate solution (5120-0053; Seracare Life Sciences, Milford, MA, USA) was added and allowed to react for 15 min. The reaction was terminated with 1 M H_3PO_4 and then the absorbance at 415 nm was determined.

Flow cytometric analysis

Cells (1×10^6 cells) in 500 µl of staining buffer [Hank's balanced salt solution (14025092; Thermo Fisher Scientific), supplemented with 10 mM HEPES and 2% FCS] were reacted with monoclonal antibodies using a rat compensation set (58517; BD Biosciences, Franklin Lakes, NJ, USA) for 1.5–2 h on ice. The compensation set comprises of APC-labeled anti-rat CD3, PE-labeled anti-rat CD4 and FITC-labeled anti-rat CD8. After reaction with cell surface markers, the cells were washed and suspended in staining buffer supplemented with propidium iodide then analyzed by flow cytometry (MoFlo Astrios, Beckman Coulter, Brea, CA, USA). For staining of tumor necrosis factor alpha (TNFα), cells were cultured in medium supplemented with protein transport inhibitor (554724; BD Biosciences) for the final 4 h. As a control, spleen from an intact rat was prepared as mentioned above and the collected spleen cells were incubated in resting medium in 7% CO_2 at 37°C overnight. Cells treated with the inhibitor were stained with surface markers (anti-CD4 and -CD8), then fixed and permeabilized using a Fixation/Permeabilization kit (554714; BD Biosciences) in accordance with the manufacturer's instructions. After reaction with a monoclonal antibody against TNFα-eFluor® 660 (50-7423-82; Thermo Fisher Scientific), the cells were analyzed by flow cytometry.

Statistics

Data from the XTT assay and ELISA were expressed as means ± SEM and analyzed statistically using SAS add-in 7.1 for Microsoft® Office. When significance was detected by one-way ANOVA, the significance of differences in means among the groups was determined by Tukey's test (P < 0.05).

References

1. **Schuppe HC, Meinhardt A, Allam JP, Bergmann M, Weidner W, Haidl G.** Chronic orchitis: a neglected cause of male infertility? *Andrologia* 2008; **40**: 84–91. [Medline] [CrossRef]

2. **Fijak M, Pilatz A, Hedger MP, Nicolas N, Bhushan S, Michel V, Tung KSK, Schuppe**

HC, Meinhardt A. Infectious, inflammatory and 'autoimmune' male factor infertility: how do rodent models inform clinical practice? *Hum Reprod Update* 2018; **24**: 416–441. [Medline] [CrossRef]

3. Pérez CV, Theas MS, Jacobo PV, Jarazo-Dietrich S, Guazzone VA, Lustig L. Dual role of immune cells in the testis: Protective or pathogenic for germ cells? *Spermatogenesis* 2013; **3**: e23870. [Medline] [CrossRef]

4. Fijak M, Iosub R, Schneider E, Linder M, Respondek K, Klug J, Meinhardt A. Identification of immunodominant autoantigens in rat autoimmune orchitis. *J Pathol* 2005; **207**: 127–138. [Medline] [CrossRef]

5. Mahi-Brown CA, Yule TD, Tung KSK. Adoptive transfer of murine autoimmune orchitis to naive recipients with immune lymphocytes. *Cell Immunol* 1987; **106**: 408–419. [Medline] [CrossRef]

6. Feng ZY, Ming W, Li LD, Louis J, Fei WY. Adoptive transfer of murine autoimmune orchitis with sperm-specific T lymphoblasts. *Arch Androl* 1990; **24**: 51–59. [Medline] [CrossRef]

7. Itoh M, Mukasa A, Tokunaga Y, Hiramine C, Hojo K. New experimental model for adoptive transfer of murine autoimmune orchitis. *Andrologia* 1991; **23**: 415–420. [Medline] [CrossRef]

8. Yule TD, Tung KSK. Experimental autoimmune orchitis induced by testis and sperm antigen-specific T cell clones: an important pathogenic cytokine is tumor necrosis factor. *Endocrinology* 1993; **133**: 1098–1107. [Medline] [CrossRef]

9. Noguchi J, Watanabe S, Nguyen TQ, Kikuchi K, Kaneko H. Development of a lipopolysaccharide (LPS)-supplemented adjuvant and its effects on cell-mediated and humoral immune responses in male rats immunized against sperm. *J Reprod Dev* 2017; **63**: 111–115. [Medline] [CrossRef]

10. Binz H, Fenner M, Engel R, Wigzell H. Studies on chemically induced rat tumors. II. Partial protection against syngeneic lethal tumors by cloned syngeneic cytotoxic T lymphocytes. *Int J Cancer* 1983; **32**: 491–500. [Medline] [CrossRef]

11. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 1986; **136**: 2348–2357. [Medline]

12. Theas MS, Rival C, Jarazo-Dietrich S, Jacobo P, Guazzone VA, Lustig L. Tumour necrosis factor-alpha released by testicular macrophages induces apoptosis of germ cells in autoimmune orchitis. *Hum Reprod* 2008; **23**: 1865–1872. [Medline] [CrossRef]

13. Tung KS, Primakoff P, Woolman-Gamer L, Myles DG. Mechanism of infertility in male guinea pigs immunized with sperm PH-20. *Biol Reprod* 1997; **56**: 1133–1141. [Medline] [CrossRef]

14. Hou ST, Ma A, Jones R, Hall L. Molecular cloning and characterization of rat sperm surface antigen 2B1, a glycoprotein implicated in sperm-zona binding. *Mol Reprod Dev* 1996; **45**: 193–203. [Medline] [CrossRef]

15. Nicolas N, Michel V, Bhushan S, Wahle E, Hayward S, Ludlow H, de Kretser DM, Loveland KL, Schuppe HC, Meinhardt A, Hedger MP, Fijak M. Testicular activin and follistatin levels are elevated during the course of experimental autoimmune epididymoorchitis in mice. *Sci Rep* 2017; **7**: 42391. [Medline] [CrossRef]

16. Takeuchi A, Saito T. CD4 CTL, a cytotoxic subset of CD4+ T cells, their differentiation and function. *Front Immunol* 2017; **8**: 194. [Medline] [CrossRef]

17. Noguchi J, Toyama Y, Yuasa S, Kikuchi K, Kaneko H. Hereditary defects in both germ cells and the blood-testis barrier system in as-mutant rats: evidence from spermatogonial transplantation and tracer-permeability analysis. *Biol Reprod* 2002; **67**: 880–888. [Medline] [CrossRef]

18. Crackower MA, Kolas NK, Noguchi J, Sarao R, Kikuchi K, Kaneko H, Kobayashi E, Kawai Y, Kozieradzki I, Landers R, Mo R, Hui CC, Nieves E, Cohen PE, Osborne LR, Wada T, Kunieda T, Moens PB, Penninger JM. Essential role of Fkbp6 in male fertility and homologous chromosome pairing in meiosis. *Science* 2003; **300**: 1291–1295. [Medline] [CrossRef]

19. Tatematsu K, Kobayashi I, Uchino K, Sezutsu H, Iizuka T, Yonemura N, Tamura T. Construction of a binary transgenic gene expression system for recombinant protein production in the middle silk gland of the silkworm Bombyx mori. *Transgenic Res* 2010; **19**: 473–487. [Medline] [CrossRef]

20. Tilney NL. Patterns of lymphatic drainage in the adult laboratory rat. *J Anat* 1971; **109**: 369–383. [Medline]