BIOLOGY OF REPRODUCTION **61,** 635–642 (1999)

Autoimmune Ovarian Inflammation Triggered by Proinflammatory (Th1) T Cells Is Compatible with Normal Ovarian Function in Mice[1]

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ABSTRACT

The detection of noninfectious ovarian inflammation (oophoritis) and serum ovarian autoantibodies in a patient with premature ovarian failure is indicative of an autoimmune etiology. The mechanisms of autoimmune ovarian injury leading to loss of function are currently unknown. In this study we investigated the impact of oophoritis on ovarian function based on two murine autoimmune ovarian disease (AOD) models. AOD can be induced by thymectomy at Day 3 after birth (d3tx). D3tx mice develop ovarian inflammation and atrophy with loss of oocytes. In these mice, ovarian atrophy and not oophoritis correlated with abnormal estrous cyclicity. The second AOD model is induced by active immunization of adult mice with a murine ZP3 peptide (pZP3) in adjuvant. After active immunization, the zona pellucida antibody titer, not oophoritis, correlated with reduced fertility. To investigate the effect of oophoritis in the absence of antibody response or ovarian atrophy, pZP3-specific T cells were passively transferred into naive syngeneic mice. This recruited cytokine-producing cells into the ovaries so that elevated cytokine production and its effect on ovarian function could be examined. Recipients of pZP3-specific T cells developed severe granulomatous oophoritis, and the diseased ovaries had elevated ovarian mRNA levels of interferon-γ, interleukin-1β, and tumor necrosis factor α. Despite these changes, fertility rates and gonadotropin-induced follicular development remained essentially normal. Therefore, normal ovarian function is compatible with severe ovarian inflammation mediated by autoreactive T cells.

INTRODUCTION

Human autoimmune ovarian disease (AOD) can lead to premature ovarian failure and idiopathic infertility [1], and it is a component of polyendocrine autoimmune syndrome encompassing Addison's disease, autoimmune thyroiditis, insulin-dependent diabetes mellitus, pernicious anemia, and myasthenia gravis [2]. A presumptive diagnosis of AOD in a patient with amenorrhoea, infertility, or other symptoms of premature ovarian failure is based on the detection of autoantibodies to ovarian antigens [3, 4]. Although ovarian inflammation has been documented in AOD patients, our knowledge on the natural history of this disease is limited in scope [5], because early AOD is clinically silent and pathologic studies are rare.

Organ-specific autoimmune inflammatory diseases, including AOD, largely result from the activation of autoreactive, Th1 subset of CD4+ T cells, identified by their capacity to produce interleukin (IL)-2 and interferon-γ (IFN-γ) [6, 7]. Ovaries with AOD are heavily infiltrated with T cells and activated macrophages: cells that produce proinflammatory cytokines and growth factors including IL-1β, tumor necrosis factor α (TNFα), and granulocyte-macrophage colony-stimulating factor (GM-CSF). Because ovarian proinflammatory cytokines have been reported to influence physiological function of normal ovaries, their elevated local concentration, produced by activated T cells and macrophages, may perturb ovarian function [8, 9]. However, in most clinical or experimental autoimmune diseases that affect a single organ, the mechanism whereby immune injury leads to loss of the organ's function is not well understood.

Information on how autoimmune injury of the ovary may lead to ovarian dysfunction is also relevant to fertility control research based on immunization with zona pellucida (ZP) antigens, such as ZP3. Immunization of nonhuman primates with ZP3 leads to infertility, but, in some cases, also ovarian failure by some unknown mechanisms [10, 11]. Therefore, dissecting the causes of ovarian dysfunction versus infertility after ZP3 immunization is critical in the design of a safe and effective ZP3 vaccine.

To gain insight into the mechanism of autoimmune ovarian failure, we have investigated two murine models of AOD. Neonatal thymectomy on Day 3 after birth (d3tx) in susceptible mouse strains results in organ-specific autoimmune diseases affecting multiple organs including the ovary [12, 13]. D3tx leads to oophoritis and ovarian atrophy. Although their causal relation remains unclear, a recent genome-wide search indicated that the occurrences of oophoritis and atrophy in the d3tx mice mapped to distinct chromosomal intervals [14, 15]. AOD also develops in mice immunized with ZP3(330–342) (pZP3), a peptide of the murine primary sperm receptor. Mice immunized with pZP3 develop a T cell response to the peptide, an antibody response to ZP, and inflammation of the ovary. The ovarian inflammation is transferable to normal syngeneic recipients by pZP3-specific T cells and not by ZP antibodies [16, 17]. The present study employed both models of AOD to investigate the effects of ovarian inflammation on ovarian function. The results indicate that inflammation and elevated cytokine expression in the ovaries, mediated by the proinflammatory Th1 T cells, are compatible with normal estrous cyclicity, ovarian follicular development, ovulation, and fertility. Therefore, the loss of ovarian function in AOD depends on mechanisms in addition to, or other than, Th1 cell-mediated oophoritis.

MATERIALS AND METHODS

Materials

Tribromoethanol, Histopaque 1119, eCG, hCG, and hyaluronidase were purchased from Sigma Chemical Co. (St.

Accepted April 13, 1999.
Received February 22, 1999.
[1]The research was supported by NIH U54 HD-29099 and RO1 AI-41236. The study was supported in part by the Cell Science Core and the Molecular Core of the Center for Research in Reproduction (U54 HD96–008). H.B. was supported by a Fogarty Foundation fellowship. Peptide synthesis was supported by a contract of the NICHD Contraceptive Branch.

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Louis, MO). Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, glutamine, nonessential amino acids, and Hanks' Balanced Salt Solution (HBSS) were obtained from Biowhittaker (Walkersville, MD). Fetal calf serum was purchased from Hyclone Laboratories (Logan, UT), RNAzol from Leedo Medical Labs (Houston, TX), and SYBR green dye from FMC Bioproducts (Rockland, ME). Monoclonal antibodies used for immunostaining were from American Type Culture Collection (Rockville, MD).

Animals

Adult and sexually immature (C57BL/6 × A/J) Fl (B6AF1) females were obtained from National Cancer Institute, Bethesda, MD. B6AF1 used for neonatal thymectomy were bred in our mouse colony. Mouse care and treatments were based on approved protocols in accordance with NIH guidelines.

Peptides

The following peptides were used in this study for immunization and generation of T cell lines: pathogenic PZP3(330–342) (NSSSSQFQIHGPR) and its truncated form, pZP3(330–340) (NSSSSQFQIHG), which induce oophoritis, and a nonpathogenic chimeric peptide (CP) 2 (NCAYKTTQANKQAQIHGPR) containing a nonovarian T epitope used as a control. All the peptides were synthesized by Multiple Peptide Systems (San Diego, CA) and were greater than 90% pure by HPLC analysis.

Thymectomy, Estrous Cyclicity, and Immunization

Thymectomy was carried out as previously described [13]. Briefly, 3-day-old mice were anesthetized by hypothermia, and through a midsternal incision the thymic lobes were removed by suction. The sternum and skin were closed with 6–0 silk suture. The operated pups were returned to the nursing mother after several hours. Estrous cyclicity was determined by the vaginal cytology [18]. Vaginal washes in saline were transferred to a glass slide, air dried, fixed in methanol, and stained with methylene blue. The stage of the estrous cycle was graded based on the presence of leukocytes, nucleated epithelial cells, or cornified squamous epithelial cells. For active induction of AOD, mice were anesthetized by i.p. injection of tribromoethanol (0.5 mg/mouse) and immunized subcutaneously, with 50 nM of pZP3 emulsified in CFA (Mycobacterium tuberculosis, H37Ra strain, 0.16 mg/mouse) (Difco Laboratories, Detroit, MI).

Evaluation of Fertility

Fertility was determined in 6- to 8-wk-old, adult female mice either after immunization with pZP3 in CFA, CP2 in CFA, or CFA alone, or as recipients of pZP3-specific T cells. Two female mice (one experimental and one control) were housed with one fertile male and checked for vaginal plugs. Before delivery, the pregnant females were isolated, and the numbers of pups were counted immediately after delivery.

Detection of Antibody to Ovarian ZP by Immunofluorescence

Serum IgG antibody to ovarian ZP was detected by indirect immunofluorescence [17]. Sections (5 μm) of snap-frozen mouse ovaries, fixed in 95% ethanol, were incubated with mouse serum diluted in PBS with 3% BSA. After washing in PBS, antibody bound to ZP was detected by a fluorescein isothiocyanate-labeled goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL). Serial dilutions of serum were tested to determine end-point titer of ZP antibody.

Generation of T Cell Lines and Clones

B6AF1 female mice were immunized with 50 nM of pZP3(330–342) or CP2 in CFA [16]. On Day 14, their lymph node cells were isolated and stimulated in vitro with 30 μM of the respective peptide in DMEM supplemented with 10% heat-inactivated fetal calf serum, 1% 200 mM glutamine, 1% nonessential amino acids, 5×10^{-5} M β-mercaptoethanol, 100 U/ml penicillin, and 100 mg/ml streptomycin. Four days later, viable cells were recovered on a Histopaque 1119 gradient and resuspended in complete medium (10^6/ml) with lymphokine-enriched EL4 supernatant containing 10 U/ml of murine IL-2. After 7–10 days the cells (0.5×10^6/ml) were restimulated with 10 μM peptide in complete medium with irradiated spleen cells (1.5×10^6/ml) from normal B6AF1 and thereafter rested as described above. The T cell lines specific to pZP3 or to CP2 were maintained using this protocol of stimulation and resting. To obtain T cell clones, the cells were plated at one cell per well in 96-well plates, and the same protocol of stimulation and resting was carried out to obtain C4, a pZP3(330–342)-specific T cell clone.

Induction of Oophoritis by Passive Transfer and Effect of Neutralizing Antibody to TNFα

T cells isolated on a Histopaque 1119 gradient at the end of stimulation by their cognate peptide were washed in HBSS and injected i.p. into normal syngeneic adult recipients. Each adult mouse received 10^7 cells, and each prepubertal mouse received 5×10^6 cells. Their ovaries were studied for histology or cytokine at different time points as described.

In some experiments, recipients of pZP3(330–342)-specific pathogenic T cells were also injected with TN3 19.12, a monoclonal hamster antibody that neutralizes murine TNFα (a gift from Dr. Robert Schreiber, Washington University, St. Louis, MO) (1 mg/mouse, i.p.) on the day before cell transfer. Control mice received normal hamster IgG (1 mg/mouse). Ovaries were collected on Day 16 after transfer and examined by histopathology for ovarian disease.

Semiquantitation of Ovarian Cytokine Levels

RNA was extracted from the ovary by homogenization in RNAzol followed by chloroform extraction and isopropanol precipitation according to manufacturer's instructions. The amount and purity of RNA were determined by absorbance at 260 and 260/280 ratio. Five micrograms RNA was used for reverse transcription using standard protocols (Gibco BRL, Life Technologies, Grand Island, NY). The following primers were used in the polymerase chain reaction: IFN-γ 5′CATTGAAAGCCTAGAAAGTCTG, 3′CTCATGAATGCATCCTTTTTCG; TNFα 5′GTTCTATGGCCCAGACCCTCACA, 3′TACCAGGGTTTGAGCTCAGC; hypoxanthine phosphoribosyl transferase (HPRT) 5′GTTGGATACAGGCCAGACTTTGTTG, 3′GAGGGTAGGCTGGCCTATAGGCT [19]; and IL-1β 5′GCAACTGTTCCTGAACTCA; 3′CTCGGAGCCTGTAGTG-

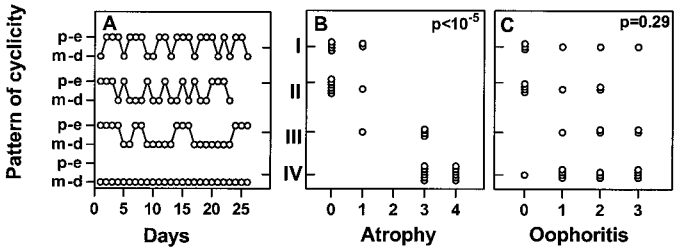

FIG. 1. Abnormal estrous cycles correlated with the extent of ovarian atrophy but not with the severity of oophoritis in d3tx B6AF1 mice. **A**) Mice were studied over 4 wk; the estrous cycles of four representative d3tx mice with increasing degrees of abnormality (I to IV) are shown. Each circle indicates whether the mouse was in proestrus or estrus (p-e) and metestrus or diestrus (m-d). The ovaries of mice with the various estrous patterns were graded for the extent of atrophy (**B**) and oophoritis (**C**), and each circle denotes a mouse.

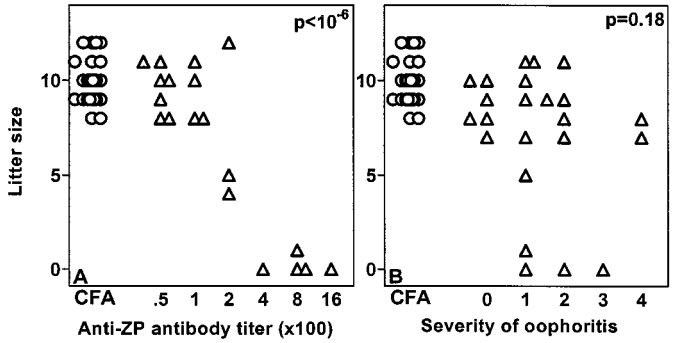

FIG. 2. Reduced fecundity correlated with antibody titer to the ZP (**left**; adapted from Lou et al., The Journal of Immunology 1996; 156:3535–3540, Figure 6 [24]), but not with the extent of oophoritis (**right**), in B6AF1 mice actively immunized with ZP3(330–340) in CFA (triangles). Control mice were immunized with CFA (circles).

CAG [20]. The numbers of cycles for each were optimized for semiquantitative analysis: 23 for HPRT, 40 for IFN-γ, and 30 for both IL-1β and TNFα. The polymerase chain reaction products were resolved on a 2% agarose gel in Tris-acetate-EDTA buffer, visualized with SYBR green dye under UV illumination, and photographed (Polaroid [Cambridge, MA] Type 55 film). Image densitometric analysis was performed using Image Quant 3.3 software (Molecular Dynamics, Sunnyvale, CA), and background optical density (OD) was subtracted. The results are presented as (Cytokine OD/HPRT OD) × 100.

Assessment of Ovarian Pathology

The ovaries were fixed in Bouin's fixative for 24 h and embedded in paraffin. Serial 5-μm sections, stained with hematoxylin and eosin, were examined as coded specimens by an independent observer, blind to experimental details. Oophoritis was graded with increasing severity from 1 to 4 (1, focal inflammation in interstitial space; 2 and 3, increasing multiple inflammatory foci and/or granuloma between or within follicles; and 4, ovarian atrophy). In d3tx mice, ovarian atrophy included a gradual loss of oocytes from the ovaries resulting in empty follicular nests associated with a reduction of ovarian size, and the appearance of hyperplastic and luteinized interstitial glands. The increasing extent of these changes was graded from 1 to 3. Grade 4 atrophic ovaries were completely devoid of oocytes.

Immunocytochemical Staining of Ovarian Infiltrates

Sections (5 μm) of ovarian frozen section, fixed with 95% ethanol, were incubated with antibody to murine CD3 (145–2C11) (1:10 dilution), macrophage (F4/80) (1:100 dilution), or major histocompatibility complex (MHC) class II molecule (M5/114.15.2) (1:10 dilution). After washing twice in PBS with 3% BSA, the sections were treated with biotinylated goat anti-hamster IgG or goat anti-rat IgG, respectively. Bound antibody was detected with streptavidin-horseradish peroxidase (ABC kit; Vector, Burlingame, CA) followed by diaminobenzidine and hydrogen peroxide substrate (BioGenex, San Ramon, CA). The slides were counterstained with methylene blue (0.1% in 9.5% ethanol), dehydrated in an ascending gradient of ethanol followed by xylene, and mounted in Cytoseal 60 (Stephens Scientific, Riverdale, NJ).

Gonadotropin-Induced Follicular Development and Ovulation

Pathogenic pZP3-specific T cells or nonpathogenic CP2-specific T cells were transferred, i.p., into 17- to 20-day-old female B6AF1 mice, 5×10^6 cells per recipient. Ten days later, the mice received 2 IU eCG s.c., followed 48 h later by 1 IU hCG s.c. Some mice were studied before the eCG treatment; others were examined at 24 or 48 h after eCG, and at 24 h after hCG treatment. Serial sections were cut through the entire ovary, and at least 12 nonadjacent sections were evaluated for the numbers of follicles at different stages of development. Healthy and atretic follicles were counted based on classification previously described by Pederson and Peters [21] and Byskov [22]. Healthy follicles were classified as primordial (no granulosa cell layers), 1–5 granulosa cell layers, antral (small antrum not forming single coalesced cavity), and preovulatory (single large antrum with cumulus oophorus adjacent to mural granulosa or near center of antrum). Atretic follicles were graded as type 1 (few granulosa cells with pyknotic nuclei close to follicular cavity, evidence of granulosa cell division, no leukocytes), type 2 (many pyknotic nuclei with few dividing granulosa cells, numerous nuclear fragments, discontinuous basement membrane, leukocytes, follicular cavity larger than in type 1), and type 3 (follicles contracted, no follicular activity, hypertrophy of theca and remaining granulosa cells, numerous leukocytes, discontinuous basement membrane).

Oocytes were recovered from fallopian tubes of gonadotropin-treated mice 14 h after hCG treatment. The cumulus was dispersed by treatment with hyaluronidase (300 μg/ml) in PBS with 3% BSA, and the number of oocytes was counted.

Statistical Methods

Student's *t*-test, chi-square test, and ANOVA were used for data analysis.

RESULTS

Abnormal Estrous Cyclicity Correlated with Ovarian Atrophy but Not Oophoritis in D3tx Mice

B6AF1 female mice are genetically susceptible to autoimmune oophoritis. In B6AF1 mice thymectomized on Day 3 after birth, oophoritis is apparent by 3–4 wk; as the inflammation progresses, oocytes and ovarian follicles

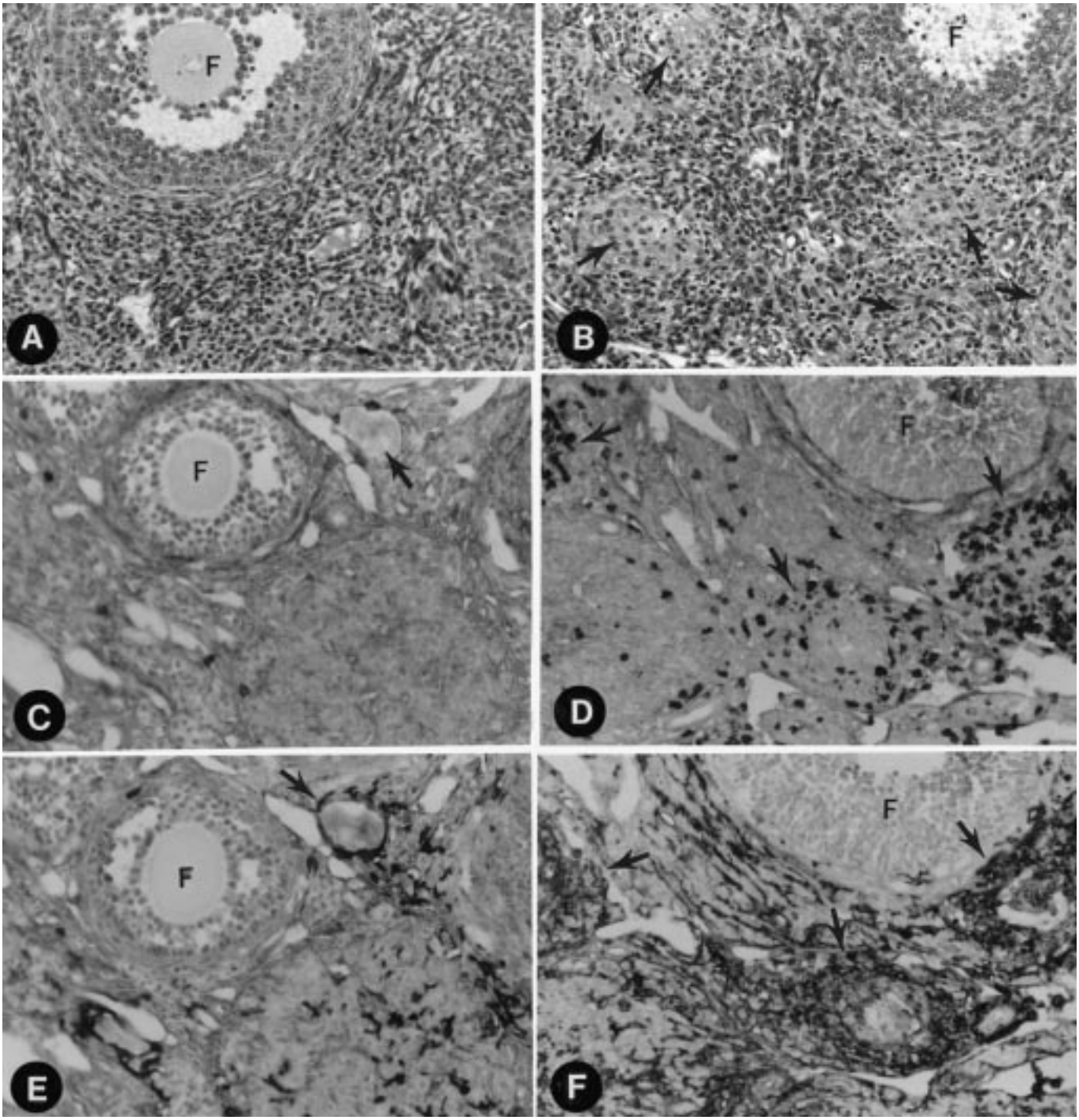

FIG. 3. Immunopathology of autoimmune oophoritis that resulted from adoptive transfer of pZP3-specific, Th1 T cell line. **Left panels**) Normal ovary; **right panels**) ovary with granulomatous oophoritis. In **B,** note the numerous granulomata (arrows) in the ovarian interstitium, each consisting of a central pale-staining collection of activated macrophages surrounded by lymphocytes. Ovarian inflammation spared the growing and mature follicles (**F**). **C** and **D** show immunoperoxidase staining for T cells (CD3+). Normal ovary (**C**) showed few T cells, while inflamed ovary (**D**) showed clusters of T cells in atretic follicles. **E** and **F** show immunoperoxidase staining for MHC II expression. Normal ovary (**E**) showed few positive cells, while inflamed ovary (**F**) showed an up-regulation of MHC II expression, particularly in areas adjacent to T cell infiltration. **C** and **E** are adjacent sections of normal ovary, and **D** and **F** are adjacent sections of an inflamed ovary. Arrows in **C–F** point to atretic follicles. **A, B**) Hematoxylin and eosin; **C–F**) immunoperoxidase stain; **A–F,** ×100 (published at 90%).

gradually disappear [13]. After 14 wk, the inflammatory cells regress, leaving behind severely atrophic ovaries. To assess the ovarian function of d3tx mice, 30 B6AF1 female mice were thymectomized on Day 3 after birth. Between the ages of 9 and 13 wk, their vaginal smears were staged for estrous cyclicity for 1 mo, and the ovarian pathology was studied. Age-matched normal B6AF1 female mice in our colony had regular cycles each lasting 4–6 days, with proestrus (1 day), estrus (1–2 days), metestrus (1 day), and diestrus (1–2 days) (data not shown). The patterns of estrous cycles with increasing degree of abnormality (I–IV) and their incidence in d3tx mice were as follows: I) normal:

20%; II) regular cycles with shortened estrus: 23%; III) irregular cycles with prolonged diestrus and normal or prolonged estrus: 17%; and IV) no cyclicity: 40% (Fig. 1A). As shown in Figure 1, B and C, abnormal cyclicity correlated significantly with the presence and extent of ovarian atrophy characterized by loss of developing and primordial follicles ($p = 10^{-5}$) but not with the extent of ovarian inflammation ($p = 0.29$).

Infertility in Mice Immunized with pZP3 Correlated with Anti-ZP Antibodies and Not with Ovarian Inflammation

We next examined female B6AF1 mice immunized with ZP3(330–340), a pZP3 peptide that contains a pathogenic T cell epitope. Despite the lack of a native ZP3 B cell epitope in ZP3(330–340), the immunized mice produced antibody to ZP. Previously, we have shown that these unusual ZP autoantibodies reacted with distant B cell epitopes of ZP3, and the response was triggered by endogenous ovarian antigen [23]. Notwithstanding the mechanism of antibody induction, as had been previously shown, the reduced fertility in these mice was found to correlate with ZP antibody titers ($p < 10^{-6}$) [24]. However, the reduction in infertility did not correlate with the extent of oophoritis ($p = 0.18$) (Fig. 2). While mice given CFA alone had 8–12 pups per litter, the experimental mice with high ZP antibody titers had significantly fewer pregnancies and smaller litter sizes.

The results of the two foregoing studies suggest that oophoritis per se is not responsible for ovarian failure. However, the evidence is circumstantial, confounded by the fact that ovarian atrophy and ZP antibody often coexisted with oophoritis. Thus, in order to study the effect of oophoritis on ovarian function, we turned to the recipients of pZP3-specific T cells. The pathology of T cell-mediated oophoritis and cytokine responses in ovaries of T cell recipients are described in the next two sections.

ZP3 Peptide-Specific T Cell Lines and Clones Adoptively Transferred AOD Without Inducing Anti-ZP Antibodies in Normal Syngeneic Recipients

PZP3-specific T cell lines and T cell clones were derived from mice immunized with pZP3. When these T cells were activated by pZP3 in vitro, they responded by secreting IL-2 and IFN-γ but not IL-4 or IL-5 (data not shown). They were therefore of the Th1 functional phenotype [25]. The pathogenic T cells also expressed the integrin α4β1 (VLA-4), required for homing of the activated T cells into the ovaries to initiate oophoritis ([26]; unpublished results). As control nonpathogenic cells, we studied: 1) C4, a pZP3-specific T cell line that secreted IL-2 and IFN-γ but expressed a low level of VLA-4; and 2) CP2, a Th1 T cell line that recognizes a foreign, nonovarian T cell peptide [17].

Normal mice that received the pathogenic T cells developed oophoritis 2 days later. Lymphocytic infiltrate appeared initially in the ovarian hilum and then spread throughout the ovarian interstitium. Inflammation targeted the atretic follicles but spared the developing and mature follicles. By Day 6, the inflammatory cells were organized into numerous granulomata (Fig. 3B), each consisting of clusters of T cells (Fig. 3D) and macrophages (data not shown). The macrophages had the activated phenotype, as they expressed the class II MHC molecules (Fig. 3F). Ovaries of pathogenic T cell recipients were not atrophic, and antibodies to ZP were not detected in the serum or bound to the ovarian ZP. Inflammation (Fig. 3A), T cell infiltration (Fig. 3C), or up-regulation of MHC class II (Fig. 3E) was absent in recipients of control cell lines.

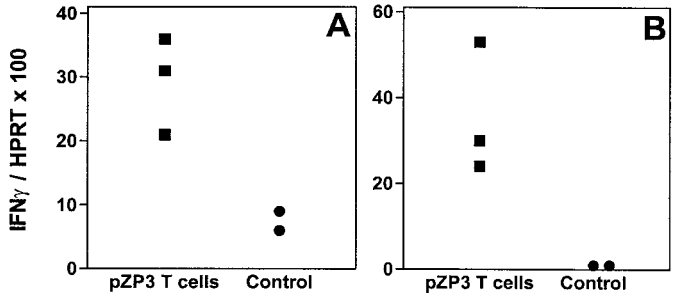

FIG. 4. Semiquantitation of ovarian IFN-γ mRNA by RT-PCR. The ovaries of pZP3-specific (squares) or control (circles) T cell recipients were studied 14 days after cell transfer. Total RNA was isolated from the ovaries and assayed for level of IFN-γ, and each symbol represents one animal, studied at diestrus (**A**) or at estrus (**B**). One of two different experiments is shown.

Increased Ovarian Expression of IFN-γ, IL-1β, and TNFα and a Pathogenic Role for TNFα in Autoimmune Oophoritis

The ovaries from mice with oophoritis were semiquantified for the mRNA of IFN-γ, IL-1β, and TNFα by reverse transcription-polymerase chain reaction (RT-PCR). Recipients of pZP3-specific T cells continued to have normal cyclicity and were monitored by daily vaginal smears; on Day 14, their ovaries were studied for histological evidence of oophoritis and for ovarian cytokine mRNA. The vaginal cytology assigned mice to the active (proestrus, estrus) or the quiescent (diestrus, metestrus) phase of the estrous cycle at the time they were killed. Ovaries from untreated mice, or from mice given nonpathogenic T cells (CP2), were studied in parallel.

The ovaries with inflammation were found to have elevated ovarian IFN-γ mRNA levels, and this was noted in ovaries collected at both quiescent (Fig. 4A) and active (Fig. 4B) phases of the estrous cycle. In addition, ovaries with oophoritis from T cell recipients also had elevated IL-1β and TNFα mRNA levels compared with controls; and similarly, the elevated cytokine levels were independent of estrous cyclicity (Fig. 5).

We next determined whether the proinflammatory cytokines expressed in the diseased ovaries were required for the development of oophoritis. Mice (n = 10) were injected with TN3 19.12, a neutralizing antibody to TNFα. Control mice received hamster IgG (1 mg/mouse). All the mice received a pathogenic T cell line on the following day. The ovaries were harvested 16 days later and studied for disease severity. Oophoritis in mice treated with TNFα antibody was significantly reduced (5 of 10; mean disease severity: 1.2) as compared to that in control mice (9 of 10; mean disease severity: 2.5) ($p < 10^{-6}$). Thus ovarian expression of a number of proinflammatory cytokines was enhanced in autoimmune oophoritis, and TNFα was involved in the oophoritic process.

Severe Oophoritis and Proinflammatory Cytokine Expression Did Not Affect Gonadotropin-Induced Follicular Development or Gonadotropin-Induced Ovulation in Immature Mice

Having evaluated the pathological change and cytokine expression in pZP3 T cell recipients, we next evaluated the

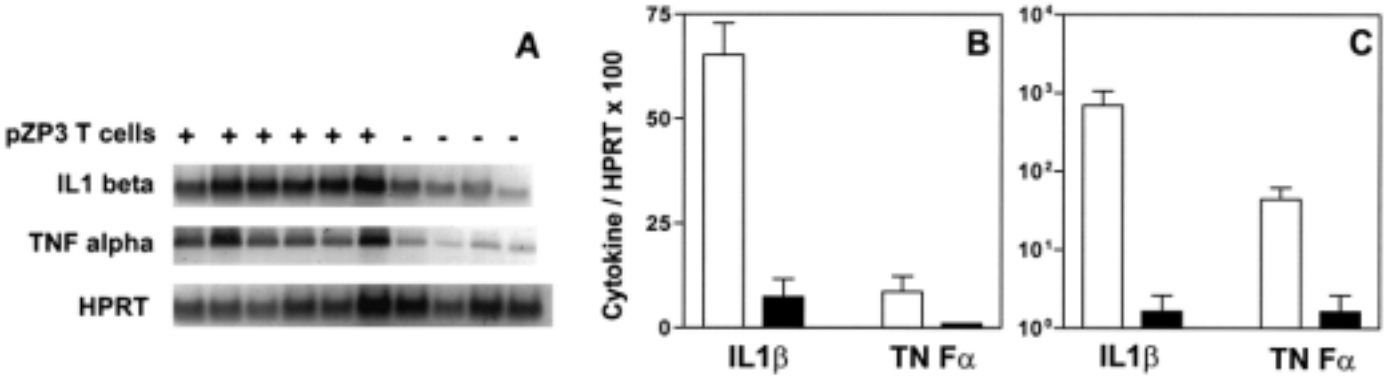

FIG. 5. Semiquantitation of ovarian IL-1β and TNFα by RT-PCR. **A**) Comparison of ovarian cytokine mRNA expression in the presence or absence of oophoritis (pZP3-specific T cell recipients). RT-PCR products of IL-1β, TNFα, and HPRT from ovaries of mice that received pathogenic (lanes marked +) are compared with those of nonpathogenic T cell recipients (lanes marked −). **B** and **C**) Densitometric analysis of ovarian cytokine IL-1β and TNFα expression in diestrus (**B**) and estrus (**C**) after transfer of pathogenic T cells (open bars) or control T cells (filled bars). The results, expressed as (cytokine/HPRT) × 100, represent the mean ± SEM of 4–8 mice per group.

ovarian function of these animals. Ovarian function was determined in sexually immature female mice with oophoritis. Pathogenic pZP3-specific T cells were transferred into 2.5-wk-old female mice, and the cell recipients were studied 10 days later. Ovarian follicular development was induced by eCG, and ovulation was stimulated by hCG. The ovaries, obtained at 0, 24, and 48 h after eCG injection and at 24 h after hCG treatment, were examined for oophoritis and quantitation of ovarian follicles as previously described [27]. As shown in Table 1, despite severe oophoritis, the numbers of growing, antral, and atretic follicles between oophoritic mice (n = 7) and control mice (n = 3) did not differ significantly at the time points studied. In addition, the ovulation rate, indicated by the number of oocytes recovered from the fallopian tubes, was not affected by granulomatous oophoritis (Table 1).

TABLE 1. Effect of oophoritis on gonadotropin-induced follicular development and ovulation in sexually-immature mice.

Findings	Recipients of pathogenic T cell line	Recipients of nonpathogenic T cell line
Oophoritis		
Incidence	7/7	0/3
Severity (mean ± SEM)	3.6 ± 0.3	—
Quantitation of ovarian follicles 48 hours after PMSG treatment[a]		
Growing and mature follicles[b]		
Primordial	17.17 ± 3.72	14.76 ± 2.91
1 granulosa cell layer	2.25 ± 0.56	2.15 ± 0.20
2 granulosa cell layers	0.83 ± 0.0	0.54 ± 0.15
3–5 granulosa cell layers	1.44 ± 0.19	0.68 ± 0.12
Antral	0.36 ± 0.06	0.46 ± 0.08
Preovulatory	0.36 ± 0.03	0.21 ± 0.05
Atretic follicles		
Type 1	0.22 ± 0.10	0.30 ± 0.06
Type 2	0.83 ± 0.30	0.59 ± 0.09
Type 3	2.69 ± 0.33	1.22 ± 0.26
Ovulation rate after PMSG and hCG treatment		
Incidence	14/14	13/13
No. of ovulated oocytes (mean ± SEM)	11.5 ± 1.5	16.5 ± 1.9

[a] Stages of follicular development and atresia are based on Pederson and Peters [21] and Byskov [22] classification as described in *Materials and Methods*.
[b] No. of follicles/section (mean ± SEM).

Transfer of Pathogenic T Cells Induced Severe Oophoritis but Not Infertility

To evaluate the effect of severe oophoritis on fertility, adult female B6AF1 mice that received 10^7 pathogenic T cells were mated with fertile males 6 days later, at the peak of oophoritis severity. As shown in Figure 6A, their cumulative litter sizes did not differ from those of mice that received nonpathogenic T cells. All experimental and control mice became pregnant, and the mean litter sizes were 9.7 and 9.3, respectively. Thus, severe oophoritis did not appear to affect the ability of the mice to ovulate, to conceive, and to sustain pregnancy. To positively control this study, we evaluated the fertility of mice immunized with a chimeric ZP3 peptide that induces antibodies to ZP without concomitant ovarian inflammation [17]. As shown in Figure 6B, these mice had reduced fertility rates, and their litter sizes were reduced as compared to those of control mice given CFA alone.

DISCUSSION

The immunopathology of AOD consists of ovarian atrophy, oophoritis, and serum autoantibodies to ovarian an-

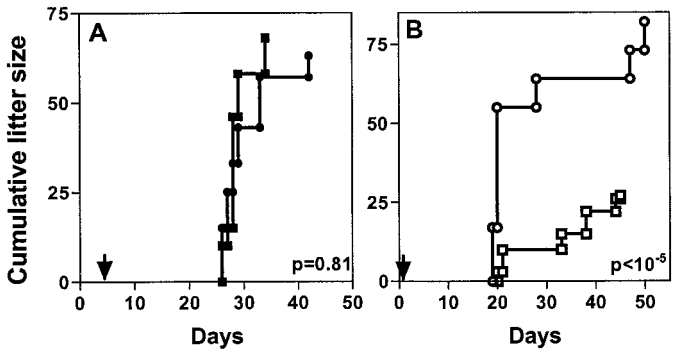

FIG. 6. Effect of T cell-mediated ovarian inflammation on fertility. **A**) Cumulative litter sizes of adult female B6AF1 recipients of pathogenic (solid squares) or nonpathogenic (solid circles) T cells are shown (n = 7 per group). Note that mating with fertile males began 6 days after cell transfer (arrow). **B**) Positive control: cumulative fecundity rate (n = 10 per group) was reduced in females immunized with CP2 (open squares) in CFA that had high titers of anti-ZP antibody. Control mice (open circles) received CFA alone.

tigens [28]. The combined effects of these changes are likely responsible for the complex clinical presentation in patients with premature ovarian failure. Herein, we have used two murine models of AOD to investigate the immunological mechanisms responsible for loss of ovarian function in AOD. The results indicate that ovarian function is compromised when ovaries become atrophic with loss of ovarian follicles, and that infertility is associated with a high serum titer of ZP antibody. In contrast, neither ovarian function nor fertility is compromised in the presence of T cell-induced ovarian inflammation.

In our experimental system, the recipients of the pZP3-specific Th1 T cells did not have either ovarian atrophy or ZP antibodies, allowing us to study, in isolation, the effect of the Th1 T cell-mediated inflammation on ovarian function. The pZP3 pathogenic T cell lines have been characterized as CD4 T cells of the Th1 subset. They express cell surface VLA4 integrin molecules, which interact with endothelial cell vascular cellular adhesion molecule 1 to permit the entrance of the T cells into the ovaries where they encounter endogenous pZP3. That T cell clusters within the inflamed ovaries are invariably surrounded by activated (MHC II positive) macrophages strongly implies that T cell-derived cytokines like IFN-γ have recruited and activated macrophages, which in turn produce proinflammatory cytokines including TNFα and IL-1β. This possibility is supported by the detection of elevated mRNA for these cytokines in the ovaries of the pZP3 Th1 cell recipients and by the reduction in disease incidence and severity by neutralizing antibody to TNFα. This finding is consistent with the TNFα antibody effect on other autoimmune diseases, including experimental autoimmune orchitis [29].

There is compelling evidence for the participation of cytokines in ovarian physiology [30]. Granulosa cells and ovarian macrophages in normal ovaries secrete IL-1, GM-CSF, IFN-γ, TNFα, and epidermal growth factor [8]. IL-1 is important in the inflammatory process of ovulation; and in the presence of gonadotropins, IL-1 significantly enhances ovulation in immature rats [31, 32]. The up-regulation of the pro-apoptotic Fas molecule on granulosa cells, induced by IFN-γ, appears to play a role in follicular atresia [33]. Atresia is enhanced by TNFα, presumably by affecting the blood supply to ovarian follicles [34]. Elevation of ovarian TNFα following lipopolysaccharide administration results in the inhibition of gonadotropin-stimulated ovarian function [35]. Given these and other observations, it has been postulated that abnormal cytokine production may lead to premature ovarian failure [9, 36]. The increased expression of MHC II antigens (HLA DR3) on the granulosa cells in ovaries of patients with premature ovarian failure was interpreted as a response to increased ovarian IFN-γ [37]. In our adoptive transfer experiments, we placed cells actively producing cytokine within the ovarian microenvironment. This allowed us to examine directly the in vivo effect of elevated ovarian proinflammatory cytokine levels on ovarian functions. Although there was a slight reduction in the gonadotropin-induced oocyte numbers in diseased mice, it did not differ significantly from that in controls. Moreover, the fertility rates and litter sizes of oophoritic mice were unaffected. Therefore, despite elevated ovarian cytokine expression, the mice ovulated normally; and they mated, conceived, and maintained normal pregnancy.

There are several explanations for the somewhat unexpected finding of normal ovarian functions in the presence of enhanced ovarian proinflammatory cytokines and severe ovarian inflammation. First, an intact hypothalamo-pituitary-gonadal axis may override the influence of local cytokine imbalance. Second, the pathophysiological elevation of cytokine levels in oophoritis may be different from the levels achieved by the injected doses of the cytokines used in earlier studies. Third, we measured cytokine mRNA and not their proteins. Because transcription and translation of cytokines are independently regulated, mRNA detection might not mirror cytokine production [38]. However, this is not a likely explanation, for two reasons: 1) there was recruitment and activation of inflammatory cells to the ovaries by the Th1 cells, a process dependent on the production of functional cytokines and chemokines; and 2) TNFα antibody significantly reduced disease severity. Fourth, the ovarian cytokine influence may be compartmentalized and confined to the microenvironment of the inflammatory cells. Because the Th1 T cells target only atretic follicles, the developing and mature follicles may be protected by tissue barriers or through differential cytokine receptor expression.

The present study, which has focused on Th1 response and inflammation, indicates that this effector mechanism alone is insufficient to cause functional failure in organ-specific autoimmune disease. This finding has support from studies on other models of autoimmunity including the non-obese diabetic mouse [39], and in murine experimental autoimmune encephalomyelitis [39, 40]. In patients with autoimmune oophoritis, the situation is likely to be more complex, where other types of effector T cells and different classes of antibodies also participate. Their concerted action may be required to bring about ablation of ovarian function. For example, pZP3-specific Th2 cells, which produce IL-4 and IL-5, also induced a severe form of oophoritis associated with heavy eosinophilic infiltrates ([41]; personal communication with Alard). In addition, autoantibody to ZP does not induce oophoritis or affect follicular development, while T cells specifically target atretic follicles. However, cotransfer of pathogenic T cells with ZP autoantibody retargets the T cell-mediated oophoritis from the atretic follicles to the growing and mature follicles resulting in oocyte loss ([42]; unpublished results). Thus, the loss of ovarian function in AOD may be manifest only when there is coexpression of the multiple pathways of the autoimmune response. Finally, the distinctive genetic mapping of chromosomal intervals for oophoritis and ovarian atrophy also suggests that nonimmune mechanism may participate in the pathogenesis of ovarian atrophy as well [15].

ACKNOWLEDGMENTS

The authors thank Melissa Bevard and Angela Miller for excellent histology support.

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