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Involvement of Tumor Necrosis Factor-α in the Pathogenesis of Autoimmune Orchitis in Rats[1]

María O. Suescun,[3,6] Claudia Rival,[4] María S. Theas,[4] Ricardo S. Calandra,[5,6] and Livia Lustig[2,4]

Instituto Multidisciplinario de Biología Celular,[3] CONICET, La Plata, Argentina
Centro de Investigaciones en Reproducción,[4] Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina
Instituto de Biología y Medicina Experimental,[5] CONICET, Buenos Aires, Argentina
Facultad de Ciencias Exactas,[6] Universidad Nacional de La Plata, La Plata, Argentina

ABSTRACT

We studied the testicular macrophages of rats with experimental autoimmune orchitis (EAO) and analyzed whether the tumor necrosis factor-α (TNFα) is involved in germ cell apoptosis and in Leydig cell steroidogenesis. The EAO was induced in adult male Sprague-Dawley rats by active immunization with testicular homogenate and adjuvants. In the experimental group, a severe orchitis was observed 80 days after the first immunization. ED1- and ED2-positive macrophages were quantified by immunohistochemistry. The TNFα concentration of conditioned media from testicular macrophages (TMCM) was determined by ELISA. The number of apoptotic TNF receptor 1 (TNFR1)-positive germ cells was identified by combining in situ end labeling of apoptotic DNA and immunohistochemical techniques. The effect of TNFα on Leydig cell testosterone production was determined by RIA. In rats with EAO, we observed a significant increase in the number of TNFα-positive testicular macrophages, the TNFα concentration in TMCM, and the number of TNFR1-positive germ cells. Sixty percent of TNFR1-positive germ cells were apoptotic. These results suggest that TNFα could be involved in the pathogenesis of EAO. Acting together with other local factors such as Fas-FasL, TNFα could trigger germ cell apoptosis. We also demonstrated that TNFα inhibited in vitro testosterone production in basal and hCG-stimulated Leydig cells from rats with orchitis.

apoptosis, cytokines, immunology, testis, testosterone

INTRODUCTION

Macrophages constitute almost 20% of the testicular interstitial tissue of several species, including human, monkey, boar, and rat [1]. Distinct subsets of testicular macrophages have been recognized in rats by monoclonal antibodies ED1 (CD68 antigen) and ED2 [2]. ED1 reacts with a transmembrane protein mainly located in macrophage lysosomes that presumably characterize circulating monocytes or recently arrived macrophages [2], whereas ED2 reacts with a membrane antigen of resident macrophages.

Testicular macrophages interact with a variety of cell types, including lymphocytes and Leydig cells. The latter have been shown to regulate the number of testicular macrophages [3]. On the other hand, macrophages and their products have the ability to modulate Leydig cell steroidogenesis [4].

Although the role of testicular macrophages in the immunologically privileged status of the normal testis is not clear, it has been demonstrated that when macrophages are immunoactivated, they acquire an inflammatory pattern expressed by their ability to secrete several proinflammatory cytokines, such as tumor necrosis factor-α (TNFα), interleukin (IL)-1, and IL-6 [5].

The TNFα is a pleiotropic cytokine that modulates inflammatory and immunoendocrine responses and is involved in apoptotic cell death. It exists in two biologically active forms: as a propeptide, associated to the cellular membrane; and as a soluble peptide, generated by the action of specific metalloproteases. It exerts its biological activity by binding to specific receptors localized on the cell surface. Two different receptors are known: TNFR1 (55 kDa), which is mainly involved in the death pathway; and TNFR2 (75 kDa). In mouse testis, pachytene spermatocytes and round spermatids were found to express TNFα mRNA, and the bioactive cytokine was mainly produced by the round spermatids [6]. In the normal testis, TNFα has been found to modulate spermatogenesis [4, 6–8] and Leydig cell steroidogenesis in vitro [9–12] and to stimulate the expression of cell adhesion molecules [13].

Controversial results have been reported in vivo concerning the role of this cytokine in the development of experimental autoimmune orchitis (EAO). Teuscher et al. [14], injecting neutralizing antibodies to TNFα in mice with autoimmune orchitis, suggested that TNFα is not a major factor in the pathogenesis of the disease. In contrast, Yule and Tung [15] were able to reduce testis damage by using the same TNFα antibody therapy in another EAO model, thereby highlighting the importance of this cytokine in the pathogenesis of EAO.

We previously studied different aspects of EAO induced in rats by active immunization with spermatic antigens and adjuvants. In this experimental model, we described an increase in the number of major histocompatibility complex (MHC) II-positive interstitial cells, mainly macrophages [16], and we observed numerous interdigitations and specialized junctions between these cells and Leydig cells [17]. We also reported that testicular macrophage-conditioned

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[2]Correspondence: Livia Lustig, Centro de Investigaciones en Reproducción, Facultad de Medicina, Paraguay 2155, Piso 10, C1121 ABG Buenos Aires, Argentina. FAX: 54 11 59509612; e-mail: llustig@fmed.uba.ar

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media from rats with EAO modulated Leydig cell steroidogenesis [18].

The main target of the immunological attack in this model is the germ cells that undergo sloughing and death. Recently, we demonstrated that germ cell death occurs through apoptosis and that the Fas-FasL system is involved [19]. Because not all apoptotic germ cells expressed Fas, another pathway could be involved in this process. The aim of the present study was to characterize testicular macrophage subpopulations, to study the involvement of TNFα in germ cell apoptosis, and to examine the effect of this cytokine on Leydig cell steroidogenesis in EAO.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (age, 50–60 days) were kept at 22°C with a 14L:10D photoperiod and were fed standard food pellets and water ad libitum. The animals were killed according to protocols for animal use, in agreement with NIH guidelines for care and use of experimental animals and approved by the local ethical committee (IBYME-CONICET).

Immunization Schedule

Rats in the experimental (E) group were immunized with testicular homogenate (TH) prepared as previously described [20]. Briefly, rat testes were decapsulated, diluted in an equal volume of saline, and disrupted in an Omni mixer (I. Sorvall, Inc., Norwalk, CT) for 30 sec. The final concentration was 500 mg/ml wet weight. Rats in the E group were injected three times with 200 mg wet weight of TH/dose at 14-day intervals. Antigen (0.4 ml) emulsified with complete Freund adjuvant (CFA; 0.4 ml) was injected intradermally in footpads and at multiple sites near ganglion regions. The first two immunizations were followed by an i.v. injection of 0.5 ml of *Bordetella pertussis* (strain 10536; Instituto Malbrán, Buenos Aires, Argentina) containing 10^{10} microorganisms, and the third immunization was followed by an i.p. injection of 10^9 microorganisms. Rats in the control (C) group were injected with an emulsion of saline, CFA, and *B. pertussis* in the same conditions as the E group. The E, C, and normal (N) untreated rats were killed at different time periods (0–35, 50–60, 70–110, and 120–150 days) after the first immunization. Blood was collected and sera stored at −70°C until use. Testes were removed, weighed, fixed in Bouin solution, and embedded in paraffin or quickly frozen for cryostat sections to be used in histopathology or immunohistochemistry, respectively. The procedure to isolate testicular and peritoneal macrophages and Leydig cells is described below.

Histopathology

The histopathology of the testis was studied in sections obtained from three different levels and stained with hematoxylin-eosin.

Immunohistochemistry

A mouse monoclonal antibody, ED1, that recognizes a cytoplasmic antigen in rat monocytes, macrophages, and dendritic cells and a mouse monoclonal antibody, ED2, that recognizes a membrane antigen of tissue macrophages were used to identify circulating and resident macrophages, respectively. Serial cryostat testis sections fixed for 10 min in cold acetone were washed in PBS and incubated with 0.3% H_2O_2 in methanol for 30 min to block endogenous peroxidase. After incubation with 3% normal horse serum for 30 min at room temperature, sections were treated with Avidin/Biotin Blocking Solution (Vector Laboratories, Burlingame, CA) and then incubated with ED1 (10 μg/ml; 554954; BD Pharmingen, San Diego, CA), with ED2 (5 μg/ml; 550573; BD Pharmingen) in 0.1 M PBS with 0.03% Triton-X 100 and 5% normal rat serum, or with ED1 and ED2 for 1 h at room temperature in a humidified chamber. A biotinylated horse anti-mouse rat-adsorbed immunoglobulin (Ig) G (2 μg/ml; Vector Laboratories) was used as secondary antibody. The reaction was amplified with a Vectastain Elite ABC Kit (Vector Laboratories), and the reaction product was visualized by adding diaminobenzidine substrate (Vector Laboratories). For negative control, the first antibody was omitted. Sections were counterstained with hematoxylin. For each antibody, positively immunostained cells were counted using a 25× objective, and the ocular (12.5×) was fitted with a quadratic grid with a total area of 96 100 μm^2. Numerical density (number of macrophages per unit volume) was determined by counting positive macrophages (N_a) occurring within the total grid boundary. The total number of grid fields counted for each section was 30, and four animals per group were studied. Average nuclear diameter (D) was measured with a 100× Zeiss (Oberkochen, Germany) objective and an ocular micrometer calibrated with a stage micrometer [21]. The values of D (mean ± SEM) obtained from the N, C, and E groups were as follows for ED1: N group, 5.09 ± 0.20 μm; C group, 4.76 ± 0.31 μm; and E group: 5.25 ± 0.20 μm. The values of D (mean ± SEM) obtained from the N, C, and E groups were as follows for ED2: N group, 5.37 ± 0.20 μm; C group, 5.61 ± 0.07 μm; and E group, 5.89 ± 0.12 μm. The final numerical density or number of macrophages per unit volume testis (N_v) was then calculated by the Floderus equation [22]: $N_v = N_a/D + T - 2h$, where T is the section thickness (6 μm) and h is a correction factor to calculate nuclei diameters. The number of macrophages per testis was calculated by multiplying the final numerical density by the testis volume. The latter was calculated as previously described [23]. Some macrophages express ED1 and ED2 antigen. Incubating tissue sections with ED1 antibodies, we identified ED1-positive/ED2-negative and ED1-positive/ED2-positive subsets, and incubating tissue sections with ED2 antibodies, we detected ED2-positive/ED1-negative and ED1-positive/ED2-positive subsets. By combined ED1 and ED2 immunohistochemistry, we obtained the total number of macrophages.

To detect TNFα and TNFR1, the immunoperoxidase technique described for ED1 and ED2 was applied to paraformaldehyde-fixed, isolated testicular macrophages and to paraffin testis sections. A rabbit polyclonal antibody anti-mouse TNFα that cross-reacts with rat TNFα (1:200; P-350, Endogen, Woburn, MA) and a goat polyclonal antibody anti-mouse that cross-reacts with rat TNFR1 (1:80; sc-1069; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used as primary antibodies. A rabbit anti-goat IgG (4 μg/ml; Vector Laboratories) and a goat anti-rabbit IgG (5 μg/ml; Vector Laboratories) were used as secondary antibodies, and normal goat or rabbit sera were used to avoid nonspecific staining for TNFα and TNFR1, respectively. As negative controls, the first antibodies were replaced by PBS or by respective rabbit or goat IgG isotypes. The TNFR1-positive germ cells were counted in 100 seminiferous tubules of three nonconsecutive sections from four to five animals per group using a 25× objective.

Localization of TNFR1 in Apoptotic Cells

The TNFR1 expression in apoptotic germ cells was studied by combining immunohistochemistry of TNFR1 and in situ end labeling of apoptotic DNA (ISEL) techniques. For the ISEL technique, DNA fragmentation in individual cells was visualized by indirect immunoenzyme detection of digoxigenin-labeled genomic DNA. To facilitate antigen retrieval, deparaffinized and hydrated tissue sections (thickness, <5 μm) were heated in a microwave (5 min at 370 W) in sodium citrate buffer (10 mM, pH 6.0) and quickly cooled in PBS. The procedure for the ISEL technique was performed as previously described [19].

To detect TNFR1 in apoptotic cells, we combined immunoperoxidase and ISEL techniques in testis serial sections as described above, and we also used ISEL/immunofluorescence (IF) techniques in the following procedure: testis sections were processed as indicated above for ISEL, with minor modifications. After incubation with the enzyme and digoxigenin-11-2′,3′-dideoxy-uridine-5′-triphosphate (dig-ddUTP), sections were washed and incubated with 1.5% normal sheep serum (NSS) in 2% BSA for 30 min at room temperature. Then, sections were incubated with anti-TNFR1 (1:30; Santa Cruz Biotechnology) for 1 h at room temperature. A fluorescein isothiocyanate-conjugated antidigoxigenin (1:8; Roche Molecular Biochemicals, Mannheim, Germany) and a rhodamine-conjugated anti-IgG (1:70; Sigma Chemical Co., St. Louis, MO) were used to detect ISEL-positive and TNFR1-positive cells, respectively. The antibodies were diluted in PBS containing 1% blocking reagent, 0.5% BSA and 1.5% NSS. The TNFR1-positive germ cells, ISEL-positive (apoptotic) germ cells, and double-positive germ cells were counted in 100 seminiferous tubules of two nonconsecutive testis sections from four rats with orchitis. The percentage of TNFR1 apoptotic germ cells was calculated as follows: the number of double-positive germ cells multiplied by 100 and then divided by the number of TNFR1-positive germ cells. The percentage of apoptotic cells expressing TNFR1 was calculated as the number of double-positive cells multiplied by 100 and then divided by the number of ISEL-positive cells.

Isolation and Culture of Testicular Macrophages

The isolation procedure was similar to the one described by Yee and Hutson [24], with minor modifications. Rats from the N, C, and E groups

(killed 70–150 days after the first immunization) were perfused with cold, sterile saline solution until tissues were pale. Interstitial cells were obtained by collagenase digestion or mechanical dispersion in PBS under sterile and low-endotoxin conditions as previously described [18]. Briefly, decapsulated testes were incubated with collagenase (0.25 mg/ml; Worthington Biochemical Corporation, Freehold, NJ) plus 0.1% BSA (fraction V; Sigma) in a Dubnoff shaking water bath at 34°C for 15 min. Tubes were filled with cool PBS and placed on ice for 3–4 min to allow the tubules to settle, then the supernatant was centrifuged at 300 × *g* for 5 min at 4°C to allow cell sedimentation. The pellet was resuspended in PBS, plated on polystyrene Petri dishes (2 ml/plate; plate diameter, 35 mm; Nunc, Inc., Naperville, IL) and on round coverslips and then incubated at 34°C for 6–10 min. The unattached cells were aspirated and the dishes vigorously rinsed three times with PBS. To obtain conditioned media, the adherent cells were cultured in Medium 199 (M199; Sigma) plus penicillin-streptomycin solution (1 ml/100 ml; Gibco-BRL, Life Technologies, Rockville, MD) for 24 h at 34°C in a humidified atmosphere of 95% air and 5% CO_2 in the absence or presence of lipopolysaccharide (LPS; 50 μg/ml; Sigma). Purity of macrophage preparations was evaluated in cell smears by counting the number of adherent cells that incorporated trypan blue or latex beads (diameter, 0.81 μm; Difco Laboratories, Detroit, MI) after 1-h incubation at 34°C. From 90% to 95% of adherent cells incorporated trypan blue or latex beads.

Peritoneal Macrophage Isolation

Macrophages were obtained by lavage of the peritoneal cavity with 15 ml of sterile PBS. The fluid collected in conical tubes was incubated with trypsin (2.5 mg/ml; Worthington) in a Dubnoff shaking bath at 34°C for 10–15 min. Then, 10 ml of PBS were added, and the rest of the procedure was the same as that described for testicular macrophages.

Macrophage-Conditioned Media

Macrophage-conditioned media (MCM) was collected from cultures of testicular or peritoneal macrophages with a Pasteur pipette in sterile conditions, centrifuged at 3000 rpm for 10 min, and stored at −70°C before TNFα determination by ELISA. The total number of adherent cells per plate was calculated by a cell adhesion colorimetric microassay using 0.5% crystal violet [18].

Immunoassay for Rat TNFα (ELISA)

A solid-phase sandwich ELISA for rat TNFα (KRC 3011; Biosource International, Camarillo, CA) was used to quantify TNFα in rat serum and in testicular MCM (TMCM) and peritoneal MCM (PMCM) from the N, C, and E groups. All samples were centrifuged before immunoassay. Briefly, samples including standards of known TNFα content were incubated in 96-well plates coated with an antiserum to TNFα (90 min). After removal of excess second antibody, streptavidin-peroxidase was added. After incubation (45 min) and washing to remove all unbound enzyme, a substrate solution (tetramethyl benzidine) was added for 30 min in the dark. After the addition of stop solution, plates were read within 2 h in an ELISA plate reader at 450 nm. The minimum detectable dose of rat TNFα with this cytoscreen kit is less than 4 pg/ml. Samples from the N, C, and E groups obtained in several immunization protocols were run in the same assay, and each sample was measured three times.

In Vitro Testosterone Production by Leydig Cells

Purified Leydig cell preparations were obtained from rats of the N, C, and E groups as previously described [25, 26]. Briefly, testes were removed, decapsulated, and digested with collagenase (0.25 mg/ml; Worthington). The digestion procedure was stopped by dilution with fresh medium. Two successive washes and sedimentations were then performed. The supernatants were pooled and interstitial cells collected by centrifugation at 220 × *g* for 10 min. Crude cell preparations were purified on a discontinuous five-layer Percoll (Pharmacia, Uppsala, Sweden) density gradient (21%, 26%, 34%, 40%, and 60%). The gradient was centrifuged at 800 × *g* for 30 min, and the interphase between 40% and 60% was collected and washed with medium to remove Percoll [26]. In all experiments, cell viability, as assessed by trypan blue exclusion method, was approximately 95%. Purity of cell preparation, as determined by a positive cytochemical reaction for 3β-hydroxysteroid dehydrogenase was 85%. Leydig cells (10^5 cells/tube) were incubated in M199 with or without hCG (10 ng/ml; CR-127 recombinant hCG; NIH, Bethesda, MD) in a Dubnoff shaking water bath for 3 h at 34°C in an atmosphere of 95% O_2 and 5% CO_2. The effect of TNFα on Leydig cell steroidogenesis was evaluated by adding recombinant rat TNFα (150 pg/ml; Biosource International) to Leydig cells. Cell viability was approximately 95% before and after TNFα incubation. Media were collected and centrifuged, and the supernatants were stored at −20°C. Testosterone was measured by RIA in the incubation media using testosterone (1,2-^{3}H(N); 60 Ci/mmol; New England Nuclear, Boston, MA) and a specific antibody from Immunotech Diagnostic (Montreal, Canada) as previously described and validated [27]. The sensitivity of the assay was 12.5 pg/ml. The within-assay and interassay coefficients of variation were 7% and 11%, respectively.

Statistical Data Analysis

Statistical evaluations were done using one-way or two-way ANOVA and the Tukey test for testosterone results, TNFα analysis of conditioned media, and morphometric studies. The nonparametric Mann-Whitney rank test was used to analyze quantitative data of TNFR1-positive cells. A value of $P < 0.05$ was considered to be significant.

RESULTS

Seventy-five percent of rats from the E group (average percentage obtained from rats killed 50–150 days after the first immunization) developed EAO characterized by an interstitial mononuclear cell infiltrate, sloughing of the germinal epithelium, and seminiferous tubule atrophy, as previously described [20].

A dark-brown reaction product was observed in the cytoplasm of ED1-positive and ED2-positive macrophages present in the interstitium of the testis of all groups of rats studied (Fig. 1). Some ED2-positive macrophages exhibited a more intense reactivity in the periphery of cell cytoplasm.

In normal rats, most testicular macrophages expressed ED2 antigen, whereas a minor proportion expressed ED1 antigen only (Table 1). An increase in the total number of macrophages was observed in rats from the E and C groups compared to rats from the N group. Compared to rats in the N group, the increase in the total number of macrophages of rats in the C group mainly corresponds to the arrival of monocytes-macrophages from the circulation (ED1-positive cells). In contrast, rats with orchitis showed a significant increase in both populations of macrophages (ED1-positive/ED2-negative and ED2-positive/ED1-negative). It has been suggested [2] that ED1-positive/ED2-positive cells represent a transient subset of macrophages.

The procedure to isolate testicular macrophages from rats injected with trypan blue 48 h before death showed that 90–95% of adherent cells incorporated trypan blue

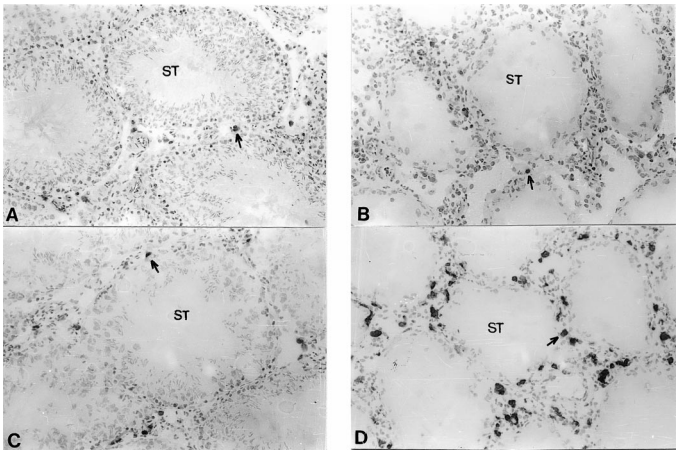

FIG. 1. Immunoperoxidase technique. ED1-positive (**A** and **B**) and ED2-positive (**C** and **D**) macrophages (arrow) in testis sections of rats from control (**A** and **C**) and experimental groups (**B** and **D**). ST, Seminiferous tubules. Magnification ×150.

TABLE 1. Number of ED1+ and ED2+ macrophages/testis (× 10^6) in each group.[a]

	ED1 + ED2 Ab	ED1 Ab	ED2 Ab	
	Total	ED1-positive/ ED2-negative	ED1-positive/ ED2-positive	ED2-positive/ ED1-negative
Normal	7.46 ± 1.13	0.64 ± 0.32[d]	3.76 ± 0.26	3.56 ± 0.34
Control	14.49 ± 1.14[b]	3.28 ± 0.42[bf]	7.00 ± 0.54[b]	4.21 ± 0.33[f]
Experimental	26.10 ± 3.32[bc]	8.74 ± 0.58[bce]	5.49 ± 0.71[be]	11.87 ± 0.70[bc]

[a] Numbers of labeled macrophages were estimated by ED1, ED2, and combined ED1/ED2 immunohistochemistry as follows: number of ED1-positive/ED2-negative cells = total cells − ED2-positive cells; number of ED1-positive/ED2-positive cells = ED1-positive cells − ED1-positive/ED2-negative cells; number of ED2-positive/ED1-negative cells = ED2-positive cells − ED1-positive/ED2-positive cells. Brackets represent the antibodies (Ab) employed to identify macrophage subpopulations. Values are mean ± SEM.
[b] $P < 0.01$ vs. respective normal.
[c] $P < 0.01$ vs. respective control.
[d] $P < 0.05$ vs. ED2-positive/ED1-negative cells of the same group.
[e] $P < 0.01$ vs. ED2-positive/ED1-negative cells of the same group.
[f] $P < 0.01$ vs. ED1-positive/ED2-positive cells of the same group.

(Fig. 2A). The TNFα was observed in the cytoplasm of testicular macrophages (Fig. 2B). The percentages of TNFα-positive testicular macrophages were 18% and 77.5% in the C and E groups, respectively.

As shown in Figure 3A, a significant increase ($P < 0.05$) in TNFα content of TMCM from rats with EAO was observed compared to the N or C groups in the absence and in the presence of LPS. In addition, a significant difference was observed between TNFα content in TMCM of the C and N groups. Similar results were obtained in TMCM of macrophages isolated by mechanical dispersion or by collagenase digestion. No significant differences in TNFα content of PMCM was observed among the N, C, and E groups (Fig. 3B). As shown in Figure 3, only peritoneal macrophages of the N, C, and E groups responded to LPS stimulation by an increase in TNFα secretion. We were unable to detect TNFα by ELISA in the sera of rats from the N, C, and E groups.

By immunohistochemistry, we demonstrated that TNFR1 is expressed in macrophages, lymphocytes, and Leydig cells in all groups of rats. We also observed TNFR1 immunoreactivity in a scarce number of spermatocytes in testis sections from the C group and in numerous degenerating spermatocytes and spermatids in rats with EAO (Fig. 4). In these rats, the number of TNFR1-positive cells significantly increased from Day 70 onward compared to rats in the C group (Fig. 5). By combining ISEL and im-

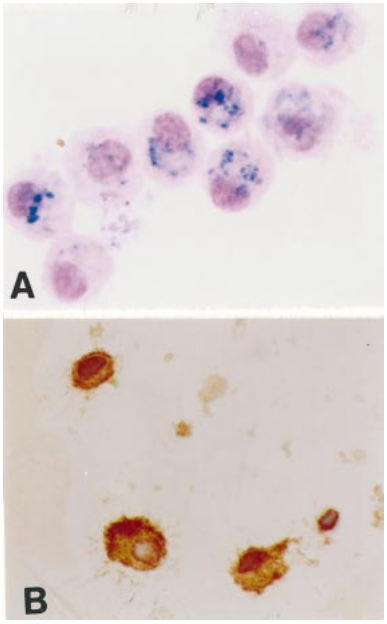

FIG. 2. **A**) Isolated testicular macrophages identified by endocytosis of trypan blue. **B**) Testicular macrophages isolated from a rat with orchitis show TNFα immunoreactivity. Magnification ×750.

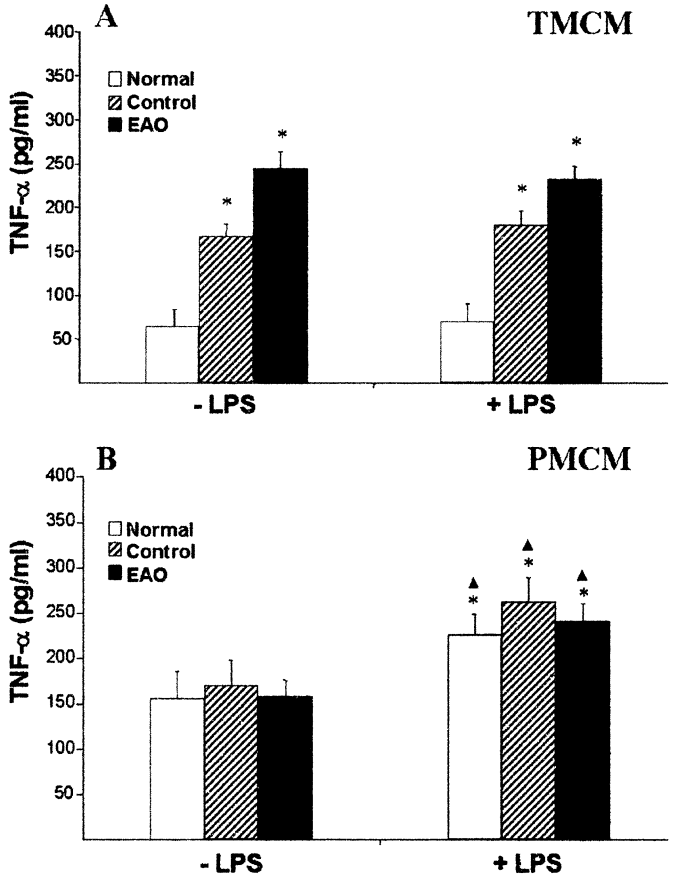

FIG. 3. ELISA for analysis of TNFα concentrations in TMCM (**A**) and PMCM (**B**) after 24-h culture, in the presence or absence of LPS (50 μg/ml). Values represent the mean ± SEM. *$P < 0.05$ vs. control and/or normal groups in each condition, ▲$P < 0.05$ vs. respective group without LPS.

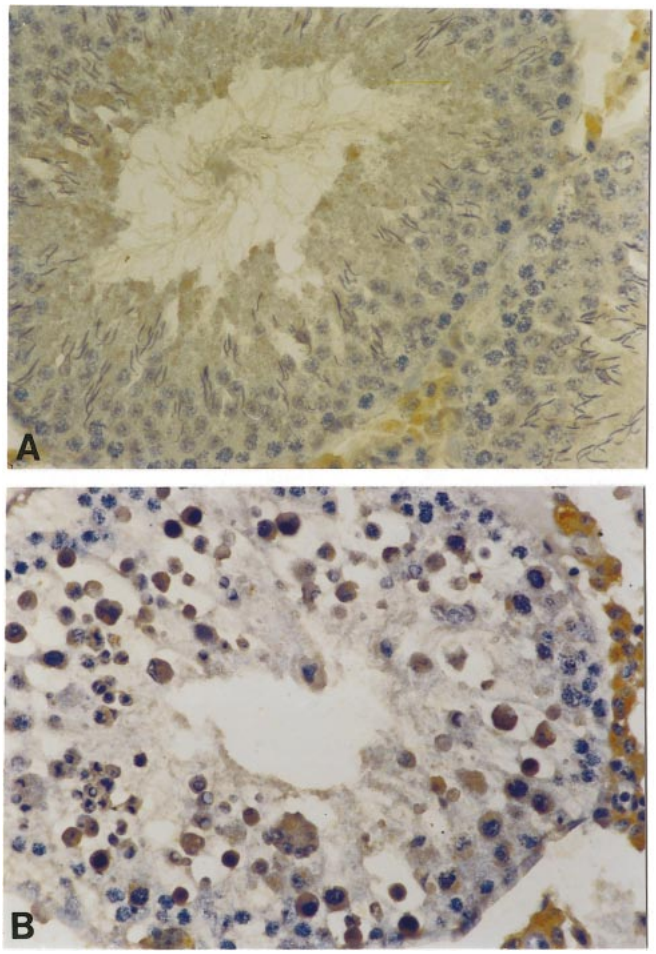

FIG. 4. Localization of TNFR1 by immunoperoxidase technique in testis sections from rats of control (**A**) and experimental (**B**) group killed 80 days after the first immunization. **A**) Some interstitial cells are TNFR1-positive, whereas no positive reaction is observed in seminiferous tubules. **B**) Numerous TNFR1-positive spermatocytes and spermatids are seen in a damaged seminiferous tubule. Magnification ×270.

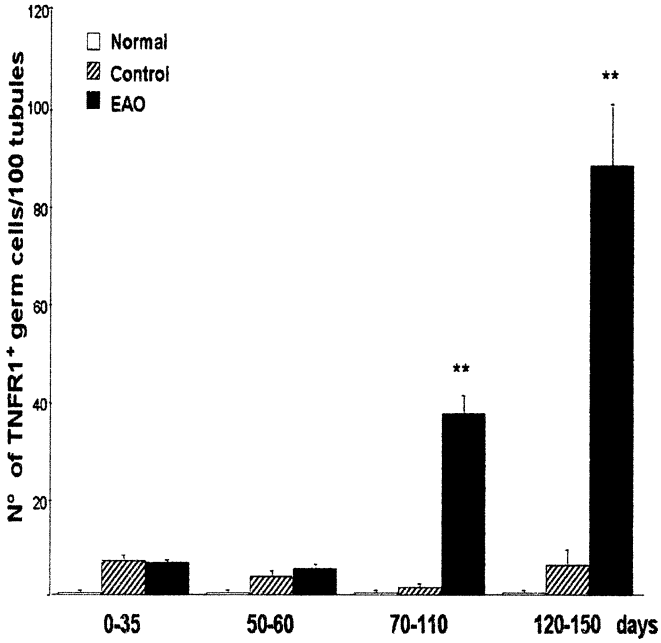

FIG. 5. The number (N°) of germ cells immunoreactive for TNFR1 (TNFR1$^+$) was quantified in testis sections. Values represent the mean ± SEM. $^{**}P < 0.01$ vs. respective control.

munohistochemistry in serial testis sections, we detected TNFR1 expression in apoptotic ISEL-positive cells (Fig. 6). Moreover, by IF, we observed apoptotic germ cells expressing TNFR1 (Fig. 7). We observed that 40.1% ± 11.2% of apoptotic germ cells expressed TNFR1, whereas 64.0% ± 15.0% of TNFR1-positive germ cells were also ISEL-positive.

Leydig cells from rats with EAO showed an enhanced in vitro testosterone production in basal and hCG-stimulated conditions (Fig. 8) compared to those from rats in the N and C groups ($P < 0.01$), confirming previous results [25]. Figure 8 shows that TNFα inhibited testosterone production in basal and hCG-stimulated Leydig cells from the N, C, and E groups. However, the percentage of inhibition of TNFα on testosterone production by hCG-stimulated Leydig cells from the E group was significantly higher (70%) compared to the N and C groups (50%).

DISCUSSION

Previous results [16] showed an increase of MHC II-positive mononuclear cells, mainly macrophages, in the testicular interstitium of rats with EAO. Accordingly, the present data show an increase in the number of testicular macrophage subsets in rats with severe EAO. This phenomenon is caused by the increase of macrophages recently arrived in the testis from circulation (ED1-positive/ED2-negative cells) and to the increase of ED2-positive/ED1-negative resident macrophages, probably by mitosis and/or differentiation of recently arrived macrophages. In rats from the C group, an increase in testicular macrophages (mainly ED1-positive/ED2-negative cells) in relation to rats from the N group was also observed. The nonspecific inflammatory effect of adjuvants could explain this phenomenon. Similar data were reported by Gerdprasert et al. [28] in rat testis in an LPS-inflammatory experimental model. In the present study, we detected TNFα in the cytoplasm of testicular macrophages and observed an increase in the percentage of TNFα-positive macrophages in rats with orchitis compared to controls. Recent data obtained by reverse transcription-polymerase chain reaction in human testicular biopsy specimens of pathologies involving inflammation also showed an increase in the number of interstitial macrophages and TNFα expression in testicular macrophages [29]. We also observed that testicular macrophages are able to secrete TNFα in vitro, confirming previous results [30]. In rats with EAO, we detected a significant increase of TNFα content in the TMCM of the E group compared to the C group. Also, rats injected with adjuvants exhibited a higher TNFα content in the TMCM compared to normal rats, probably because of the inflammatory effect induced by adjuvants.

Kern et al. [5] reported a poor immunoaccessory function of testicular macrophages compared to peritoneal macrophages. Concordantly, we observed a response to LPS stimulation by peritoneal, but not by testicular, macrophages. However, we speculate that a change in the subpopulations and in the immune function of testicular macrophages occurs in EAO when the immune system encounters testis-specific autoantigens and macrophages have been primed by lymphokines. In fact, in rats with EAO, we observed an activation of testicular macrophages and an increase of the TNFα released by these cells and not by peritoneal macrophages. We also detected an increase of IL-6

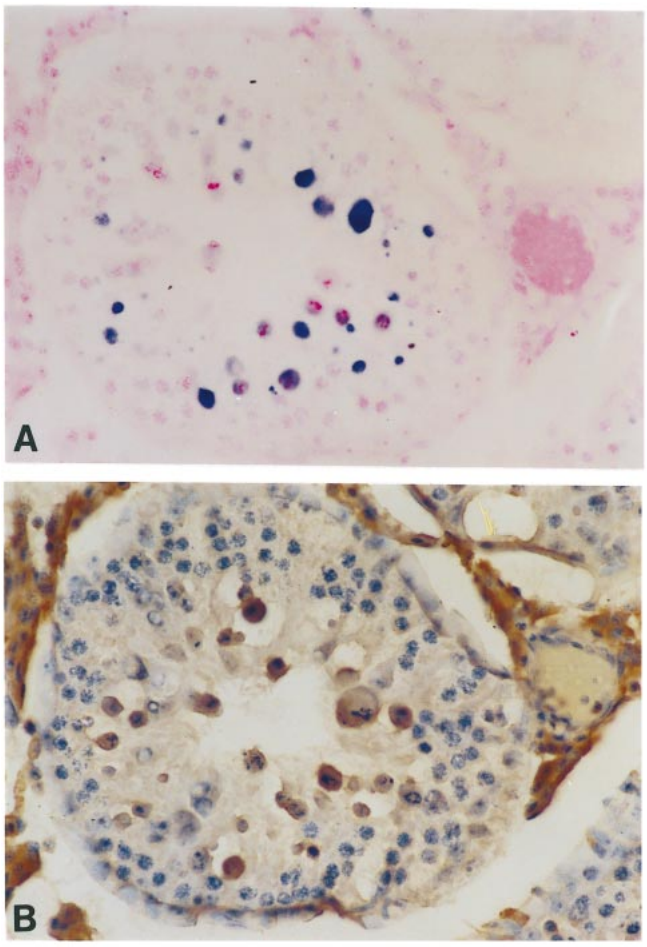

FIG. 6. Colocalization of TNFR1 in apoptotic germ cells. The ISEL (**A**) and immunoperoxidase (**B**) techniques were performed in serial testis sections from a rat with orchitis killed 50 days after the first immunization. Some of the ISEL-positive apoptotic spermatocytes and spermatids show TNFR1 immunoreactivity. Magnification ×270.

production by testicular macrophages [31] and an increase of Fas-FasL and cell adhesion molecule expression [19, 32] that could be explained largely by the effects of TNFα and other proinflammatory cytokines. In accordance with these data and those reported using another experimental model [33], we postulate that in rats with EAO, TNFα is probably involved in the progression of the disease because of its ability to increase endothelial cell permeability, to facilitate lymphomonocyte extravasation in the testis, and to activate T cells and macrophages through paracrine and autocrine mechanisms.

In autoimmune orchitis, a severe damage of seminiferous tubules characterized by degeneration, death, and sloughing of germ cells, mainly spermatids and spermatocytes, occurs. We recently demonstrated that in EAO, germ cell death occurs by apoptosis and the Fas-FasL system is involved in this process [19]. We reported that approximately 44% of apoptotic germ cells express Fas, suggesting that other death pathways could be involved, as has been demonstrated by Richburg et al. [34] in gld mice (which lack functional Fas). In the present study, we observed that with the increase of TNFα secretion by testicular macrophages, a simultaneous increase of TNFR1-positive germ cells occurs in rats with EAO. The observation that approximately 40%–50% of apoptotic germ cells expressed TNFR1 suggests that the TNFα-TNFR1 system is involved together

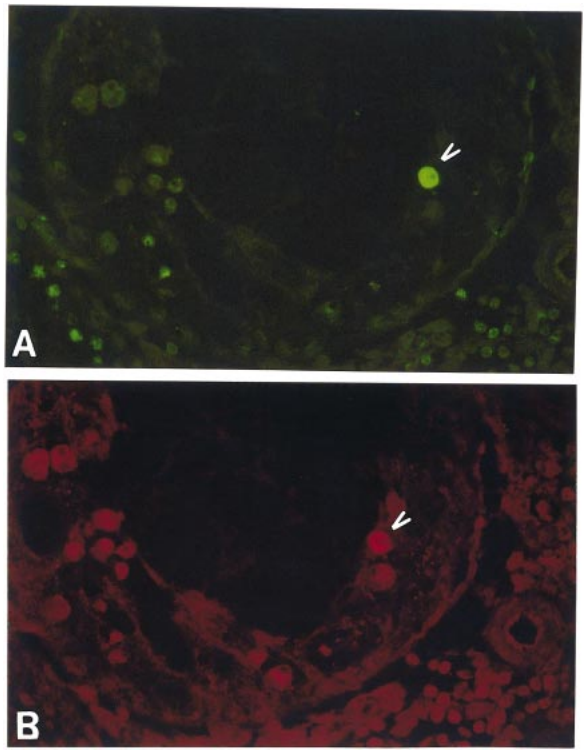

FIG. 7. Immunofluorescent technique using fluorescein isothiocyanate (**A**) and rhodamine (**B**) conjugates. Testis sections from a rat with orchitis killed 80 days after the first immunization shows a damaged seminiferous tubule with one ISEL-positive apoptotic germ cell (**A**; arrow) expressing TNFR1 (**B**; arrow). Magnification ×270.

with the Fas-FasL system in germ cell apoptosis. It is possible that TNFα released by interstitial macrophages could enter the seminiferous tubules, reaching the adluminal compartment, as has been demonstrated for IL-1α [35, 36]. Moreover, Hellani et al [37] reported that TNFα may favor the opening of Sertoli cell tight junctions, inhibiting oligodendrocyte-specific protein (OSP)/claudin 11 cell expression. It has been demonstrated that OSP has the ability to form tight-junction strands [38], and Gow et al [39], studying OSP/claudin 11-null mice, suggested a key role for OSP in formation of the blood-testis barrier (BTB). We can spec-

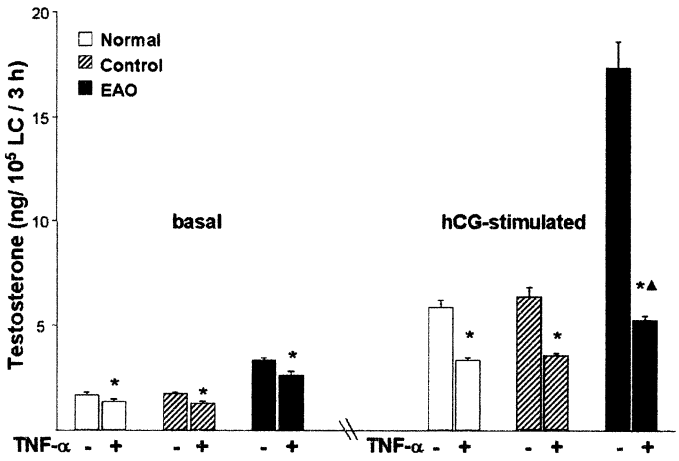

FIG. 8. Effect of TNFα (150 pg/ml) on testosterone production by Leydig cells (LC) after 3-h incubation in basal (−) or hCG (+; 10 ng/ml)-stimulated conditions. *$P < 0.01$ in each group of rats comparing TNFα treatments, ▲$P < 0.01$ vs. N and C groups with TNFα.

ulate that the high production of TNFα by testicular macrophages in EAO could eventually alter BTB permeability by inhibiting OSP expression. In addition, alterations of BTB have been reported in guinea pigs treated with CFA [40]. In EAO, sloughing of germ cells could also alter this barrier.

Previous work [23] showed low or normal serum testosterone levels and signs of hypogonadism in rats with EAO. However, we observed [25; present study] increased in vitro testosterone production by purified Leydig cells obtained from rats with EAO. This apparent paradox has been reported in several experimental models, with increased in vitro testosterone production being associated with severely damaged seminiferous tubules [41]. Alterations in blood flow or decrease in capillary permeability in damaged testes or in the metabolic clearance of testosterone could explain this fact.

Expression of TNFR1 by Leydig cells observed in the present report suggests, as has been reported in the literature [4, 42], that TNFα can modulate Leydig cell steroidogenesis. Numerous studies described an inhibitory effect of TNFα on testosterone production by Leydig cells [9, 11, 12]. Most authors suggested that TNFα appears to act as a transcriptional repressor of steroidogenic enzyme gene expression [4, 42] and through a decrease in steroidogenic acute regulatory gene expression, probably mediated via TNFR1 [12]. Most of these studies were performed in normal Leydig cells. In a previous study [25] and in the present one, we observed that in contrast to normal rats, Leydig cells from rats with orchitis show in vitro hyperresponsiveness to hCG and the highest sensitivity to TNFα under stimulated conditions. We can speculate that in contrast to controls, the inflammatory microenvironment caused by a complex network of cytokines and other factors in the testis of rats with EAO could induce an increase in the in vitro steroidogenic response and in the number or the avidity of TNF receptors in Leydig cells. These data extend our previous work, in which we reported that TMCM of rats with EAO induced a bimodal effect on normal Leydig cell steroidogenesis, depending on the TMCM concentration [18]. The present results suggest that TNFα could be one of the molecules responsible for the inhibitory effect.

In conclusion, we postulate that in an in vivo model of autoimmune orchitis, TNFα is involved in the perpetuation of the inflammatory state, in the induction of germ cell apoptosis, and in the regulation of Leydig steroidogenesis.

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