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Journal of Reproductive Immunology
xxx (2005) xxx–xxx

JOURNAL OF REPRODUCTIVE IMMUNOLOGY

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Interleukin-6 and IL-6 receptor cell expression in testis of rats with autoimmune orchitis

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Received 6 April 2005; received in revised form 30 August 2005; accepted 18 October 2005

Abstract

Experimental autoimmune orchitis (EAO) is an organ-specific model of autoimmunity characterized by an interstitial lymphomononuclear cell infiltrate as well as sloughing and apoptosis of germ cells. EAO was induced in adult male Sprague–Dawley rats by active immunization with testicular homogenate and adjuvants. Rats injected with saline solution and adjuvants were used as control group. The aim of this work was to study the expression of interleukin-6 (IL-6) and its receptor (IL-6R) in the testis of rats with EAO and analyze whether IL-6 could be involved in germ cell apoptosis. By immunohistochemistry, we detected IL-6 expression was detected in testicular macrophages and Leydig cells of control and EAO rats. Sertoli cells showed IL-6 immunoreactivity in most of the seminiferous tubules of control rats, while a few IL-6^+ Sertoli cells were found in the testis of rats with EAO. IL-6R immunoreactivity was observed in macrophages, Leydig and germ cells. A significant increase was noted in the number of IL-$6R^+$ germ cells in rats with EAO compared to control rats. The content of IL-6 (ELISA) in the conditioned media obtained from testicular macrophages of rats with orchitis was significantly higher than in the control group. By immunofluorescence performed on isolated testicular macrophages, IL-6 was shown to be expressed by monocytes recently arrived from circulation ($ED1^+$ cells), while resident macrophages ($ED2^+$ cells) were negative. In vitro experiments (trypan blue and MTS assays) showed that IL-6 (50 ng/ml) reduced germ cell viability. We demonstrated also using the TUNEL technique that IL-6 added to cultures of seminiferous tubule segments induced apoptosis of germ cells. Our results suggest that IL-6 and IL-6R may be involved in the pathogenesis of autoimmune orchitis by promoting testicular inflammation and germ cell apoptosis.

Keywords: IL-6; IL-6R; Autoimmune orchitis; Germ cell apoptosis; Testicular macrophages

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0165-0378/$ – see front matter © 2005 Published by Elsevier Ireland Ltd.
doi:10.1016/j.jri.2005.10.006

1. Introduction

Experimental autoimmune orchitis (EAO) is an organ-specific model of autoimmunity characterized by a testicular interstitial lymphomononuclear infiltrate, apoptosis and sloughing of germ cells from damaged seminiferous tubules (Doncel et al., 1989; Theas et al., 2003). In this model, we have shown previously an increased number of $ED1^+$ cells (monocytes recently arrived to the testis from circulation) and $ED2^+$ cells (testicular resident macrophages) and high content of tumor necrosis factor-alpha (TNF-α) in testicular macrophage-conditioned media (Suescun et al., 2003).

Interleukin-6 (IL-6) is one of the most potent cytokines that promote inflammatory events through expansion and activation of T cells, differentiation of B cells and induction of the acute phase response (Kamimura et al., 2003). This cytokine binds to its receptor (IL-6R or gp80) leading to dimerization of gp130/IL-6R and activation of signal transduction pathways, which generate functionally distinct and sometimes opposite responses such as cell growth and differentiation or growth arrest and apoptosis (Oritani et al., 1999; Kamimura et al., 2003).

In normal rat testis, IL-6 is produced by interstitial macrophages (Kern et al., 1995; Bryniarski et al., 2005), Leydig (Boockfor et al., 1994) and Sertoli cells (Cudicini et al., 1997). Recently, Potashnik et al. (2005) detected IL-6 expression also in germ and peritubular cells. Several cytokines (TNF-α, interleukin-1, nerve growth factor-β) and lipopolysaccharide (LPS) stimulate the synthesis of IL-6 by Leydig and Sertoli cells (Syed et al., 1993; Okuda et al., 1995a; Stéphan et al., 1997). Recently, Elhija et al. (2005) reported a significant increase in testicular IL-6 production by systemic inflammation induced by LPS. IL-6 has been proposed to act as an autocrine/paracrine factor regulating spermatogenesis and steroidogenesis (Hedger and Meinhardt, 2003). It has been demonstrated that IL-6 inhibits DNA synthesis in spermatocytes and spermatogonia (Hakovirta et al., 1995).

Several pro-inflammatory cytokines including IL-6, are involved in development of clinical and experimental autoimmune diseases (Barak and Shoenfeld, 1999). In murine EAO, the exogenous administration of IL-6 prevents development of disease (Li et al., 2002). However, the local effects of IL-6 within the testis have not been explored. The aim of the present work was to study the expression of IL-6 and IL-6R in testicular cells and their role in germ cell apoptosis in rats with autoimmune orchitis.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats aged 50–60 days were kept at 22 °C with a 14-h light/10-h dark schedule and fed standard food pellets and water ad libitum. Rats were killed according to protocols for animal use, in agreement with NIH guidelines for care and use of experimental animals.

2.2. Immunization schedule

Rats in the experimental group were immunized with testicular homogenate (TH) prepared as previously described (Doncel et al., 1989). Briefly, rats from experimental (E) group were injected three times with 200 mg (w/w) of TH/dose/rat at 14-day intervals. TH (0.4 ml) emulsified with complete Freund's adjuvant (CFA) (0.4 ml; F-5881, Sigma Chemical Co., St. Louis, MO, USA) was injected intradermally in footpads and at multiple sites near ganglionar regions (popliteal and neck nodes). The first two immunizations were followed by an i.v. injection of 0.5 ml *Bordetella pertussis* (Bp) (strain 10536; Instituto Malbrán, Buenos Aires, Argentina) containing 10^{10} microorganisms and the third one by an i.p. injection of 5×10^9 microorganisms. Control group rats were injected with an emulsion of saline solution and CFA, and Bp was used as co-adjuvant following the experimental group schedule. Rats were killed at different time periods (7–35, 50–60, 70–110, 120–150 days) after the first immunization. Testes were removed, weighed, fixed in Bouin's solution and embedded in paraffin for histopathology and immunohistochemistry or processed for macrophages, germ cells and seminiferous tubules isolation and culture. Blood was collected and sera stored at $-70\,^{\circ}C$ until use.

2.3. Histopathology

The histopathology of the testis was studied in paraffin-fixed sections obtained from three different levels and stained with hematoxylin–eosin (H&E).

2.4. IL-6 and IL-6R immunohistochemistry

Testis sections were deparaffinized and hydrated. Endogenous peroxidase was quenched with 0.3% H_2O_2 in methanol for 30 min. To avoid non-specific staining, sections were incubated with 1.5% normal goat serum (NGS) for 30 min at room temperature and then treated with avidin–biotin blocking solution (SP-2001; Vector Laboratories, Burlingame, CA, USA). In order to detect IL-6, testis sections were incubated with rabbit polyclonal antibody anti-rat IL-6 (1:150; 500-P73, PeproTech, Rocky Hill, NJ, USA) or with an anti-rat IL-6 antibody horseradish peroxidase-conjugated from an IL-6 ELISA kit (undiluted; Part 890,142, R6000, Quantikine M Immunoassay, R&D Systems, MN, USA). IL-6R expression was detected with a rabbit polyclonal anti-mouse IL-6R that cross-reacts with rat IL-6R (1:100; sc-660, Santa Cruz Biotechnology Inc., CA, USA). A biotinylated goat anti-rabbit IgG (4 μg/ml, BA-5000; Vector Lab.) was used as secondary antibody for IL-6 (PeproTech) and IL-6R antibodies, while a universal biotinylated secondary antibody (Vectastain Elite ABC kit, PK-6200, Vector Lab) was used for IL-6 antibody (R&D Systems). The reaction was amplified with the Vectastain Elite ABC kit (PK-6200, Vector Lab.) and the reaction product visualized by adding diaminobenzidine substrate (SK-4100, Vector Lab.). As negative controls, the first antibodies were replaced by PBS or by rabbit IgG isotype. Specificity of IL-6 antibody (Peprotech) was confirmed by pre-absorption of the antibody with 50-fold recombinant human IL-6 cytokine (G5541, Promega Corporation, Madison, WI, USA). Sections were counterstained with hematoxylin. IL-6 immunohistochemistry (with each Peprotech and R&D Systems antibody) was performed in 4–7 rats/group killed

50 or 80 days after the first immunization. IL-6R-positive germ cells were counted using a 25× objective in 100 seminiferous tubules of three non-consecutive testis sections from 4–5 animals/group killed at different time periods after the first immunization.

2.5. ED1/ED2 and IL-6 double immunofluorescence

In order to detect IL-6 in $ED1^+$ or $ED2^+$ testicular macrophages, a double immunofluorescent technique was performed. 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 monocytes and resident macrophages, respectively (Dijkstra et al., 1985). Isolated testicular macrophages obtained as described in the following section were permeabilized with 0.1% Triton-X 100 in PBS. Non-specific binding sites were blocked with 5% NGS and 3% BSA (A-4503, fraction V, Sigma Chemical Co.) in PBS for 30 min at room temperature. Cells were then reacted with a rabbit polyclonal antibody anti-rat IL-6 (1:50, Peprotech) followed by an anti-goat rhodamine-conjugated IgG (1:100; AP307R, Chemicon International Inc., Temecula, CA, USA) in 5% normal rat serum. Then, for macrophage subset identification, cells were incubated with ED1 (10 μg/ml; 554,954, BD Pharmingen, San Diego, CA) or ED2 (5 μg/ml; 550,573, BD Pharmingen) antibodies, followed by an anti-mouse fluorescein isothiocyanate (FITC)-conjugated IgG (1:50; FI-2001, Vector Lab.). For negative controls, the first antibodies were replaced with PBS or IgG isotype. Cells were observed using epifluorescence optics with an Axiophot microscope. The double immunofluorescence technique was performed in three different experiments with testicular macrophages obtained from 2 rats/experiment/group.

2.6. Isolation and culture of testicular macrophages

The isolation procedure was performed under sterile and low endotoxin conditions, as previously described (Yee and Hutson, 1983). Briefly, rats from control and experimental groups were perfused with cold sterile saline solution until tissues were pale. Decapsulated testes were incubated with type I collagenase (0.3 mg/ml; Worthington Biochemical Corporation, Freehold, NJ, USA) in PBS containing 0.1% BSA for 15 min at 34 °C in a Dubnoff shaking water bath. After adding PBS to inactivate collagenase, the seminiferous tubules were allowed to settle at 4 °C and the supernatant centrifuged at 300 × *g* for 5 min at 4 °C. The pellet was resuspended in PBS (2 ml), plated on 35 mm diameter polystyrene Petri dishes (Nunc Inc., Naperville, IL, USA) and on round coverslips placed on 24-well plates and incubated for 6–10 min at 34 °C in a humidified atmosphere with 5% CO_2. Dishes and plates were rinsed several times with PBS to remove unattached cells. Cells attached to coverslips were fixed with 2% paraformaldehyde for the immunofluorescent technique. In order to obtain the testicular macrophage-conditioned media (TMCM), cells attached to the dishes were cultured in Medium 199 (31100-027, Sigma Chemical Co.) plus antibiotic–antimycotic solution (1×; 15240-096, Gibco, Grand Island, NY, USA) for 20 h at 34 °C in a humidified atmosphere with 5% CO_2. TMCM was collected, centrifuged and stored at −70 °C until measurement of IL-6 by ELISA. Adherent cells were harvested and

counted with trypan blue to determine the number of cells/dish and cell viability. Purity of macrophage preparations was 90–95% as evaluated by latex bead phagocytosis and ED1 plus ED2 immunohistochemistry performed on cells attached to coverslips.

2.7. Immunoassay for rat IL-6

A commercial rat-specific IL-6 ELISA kit (R6000, Quantikine M Immunoassay, R&D Systems, MN, USA) was used to quantify IL-6 in TMCM and sera of rats from control and experimental groups killed 130 days after the first immunization. Samples from 4 rats/group were measured by triplicate. The minimum detectable concentration of rat IL-6 was less than 10 pg/ml. All procedures followed the manufacturer's instructions.

2.8. Culture of isolated seminiferous tubule segments

Testes removed from normal untreated rats (aged 50–60 days) were decapsulated and seminiferous tubule segments (STS) microdissected under a transillumination stereomicroscope in a Petri dish containing Dulbecco's Modified Eagle's Medium-nutrient mixture F-12 (1:1; D-MEM/F12, 12500-039, Gibco), as previously described (Parvinen and Ruokonen, 1982). The isolated STS (~2 mm in length) were transferred to a 96-well culture plate in 90 μl of D-MEM/F12 supplemented with L-glutamine (2 mM; G-8540, Sigma Chemical Co.), insulin-transferrin-selenium-A supplement (1×; ITS-A, 51300-044, Gibco), sodium DL-lactic acid (1 mM; L-4263, Sigma Chemical Co.) and antibiotic–antimycotic solution (1×; 5240-096, Gibco) (DMEM/F12 Sup). To each well with two STS, 90 μl of DMEM/F12 supernatent alone or containing recombinant human IL-6 (rhIL-6; final concentration: 50 ng/ml, G5541, Promega Corporation) were added. The plates were incubated for 18 h at 34 °C in a humidified atmosphere with 5% CO_2. In order to obtain germ cells, the STS were squashed (Erkkila et al., 1997). Germ cell viability was evaluated by the trypan blue exclusion method and apoptosis by the TdT-mediated dUTP nick end labeling (TUNEL) technique as indicated below.

2.9. Assessment of apoptosis

STS were obtained as described above from dark zones of seminiferous tubules (VII–VIII stages). After the incubation period, STS were squashed and fixed (Erkkila et al., 1997). The squash preparations were irradiated in a microwave oven (370 W for 5 min) in 10 mM sodium citrate buffer, pH 6 and permeabilized with 0.1% Triton-X 100 in 0.1% sodium citrate for 5 min at 4 °C. Non-specific labeling was prevented by incubating the preparations with blocking solution (5% blocking reagent; 1,096,176, Roche Molecular Biochemicals GmbH, Mannheim, Germany, in 150 mM NaCl and 100 mM maleic acid, pH 7.5) for 30 min at room temperature. After 10 min incubation with terminal deoxynucleotidyl transferase (TdT) buffer (1× TdT reaction buffer, 1× $CoCl_2$, Roche; and 1.25 mg/ml BSA, Sigma Chemical Co.), the apoptotic DNA was 3′-end labeled with digoxigenin-11-dideoxy-uridine triphosphate (4 μM Dig-ddUTP; 1,363,905, Roche) by the TdT reaction (0.17 U/ml TdT; 220,585, Roche) in TdT buffer for 1 h at 37 °C. In negative controls, TdT enzyme was replaced with the same volume of distilled water. The preparations were then incubated

with blocking solution (2% blocking reagent in 150 mM NaCl and 100 mM maleic acid, pH 7.5) for 30 min at room temperature, followed by the detection of the Dig-dd-UTP with alkaline phosphatase-conjugated anti-digoxigenin antibody (1:2000; 1,093,274, Roche) for 2 h at room temperature. Squash preparations were rinsed and equilibrated in alkaline phosphatase buffer (100 mM Tris–HCl, 100 mM NaCl, 50 mM $MgSO_4$, pH 9.5) containing 1 mM levamisole (L-9756, Sigma Chemical Co.). Then, alkaline phosphatase substrates, nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP, 1,697,471, Roche) were added for 60 min. The reaction was stopped by washing preparations with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). Squashes were light counterstained with eosin and hematoxylin, dehydrated and mounted. TUNEL data were obtained from two experiments. In each experiment, three wells (containing two seminiferous tubule segments) were individually squashed and analyzed. Finally, TUNEL was quantified in 5–10 fields/squash.

2.10. Cell viability on cultured germ cells (MTS)

Testes from normal untreated rats (aged 50–60 days) were decapsulated and digested with type II collagenase (0.3 mg/ml, Worthington Biochemicals Corp.) in PBS with 0.1% BSA for 15 min at 34 °C in a Dubnoff shaking water bath. After adding PBS, seminiferous tubules were allowed to settle and then washed three times with DMEM/F12 medium. After mechanical dispersion of the seminiferous tubules with a Pasteur pipette, cell debris was eliminated by pressing the cell suspension against a fine stainless steel screen. Isolated germ cells (100,000 cells/50 μl/well) were plated into a 96-well culture plate in DMEM/F12 supplement. To each well, 50 μl of DMEM/F12 supplement alone or containing rhIL-6 (final concentration: 50 ng/ml) was added. The plates were incubated for 18 h at 34 °C in a humidified atmosphere with 5% CO_2. Cell viability was then evaluated using the MTS Cell Titer Cell Proliferation assay (G5421, Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, this assay is a colorimetric method for determining the number of viable cells. The reagent contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt; MTS] and an electron-coupling reagent (phenazine methosulfate). MTS is bioreduced by dehydrogenase enzymes found in metabolically active cells into a formazan product soluble in tissue culture medium. The quantity of formazan product measured by the amount of 490 nm absorbance is directly proportional to the number of living cells in culture. Optical density (OD) was read at 490 nm in a microplate reader. Assays were performed by adding 20 μl of the reagent to each 96-well plate containing 100 μl of cell suspension.

2.11. Statistical analysis

Differences in the number of IL-$6R^+$ germ cells were evaluated by the non-parametric median test, while Student's *t*-test was used to evaluate differences in IL-6 content in TMCM (ELISA) and in the percentage of $TUNEL^+$ cells. Two-way ANOVA was performed to analyze differences in cell viability (trypan blue exclusion method and MTS assay). Data represent media ± S.E.M. Differences were considered significant at the $p < 0.05$ level.

3. Results

3.1. Histopathology

As previously described (Doncel et al., 1989), 50 days after the first immunization EAO was characterized by a mild infiltration of lymphomononuclear cells and several foci of damaged seminiferous tubules intermingled with normal tissue (focal EAO; Fig. 1B). From 70 days on, an increase of the interstitial cell infiltrate and extended damage of seminiferous tubules with different degrees of germ cell sloughing and presence of degenerating spermatocytes and spermatids were observed (severe EAO; Figs. 1C and 2B and D). Leukocytes were never observed inside the seminiferous tubules (Fig. 1B and C). None of the rats from the experimental group killed before 50 days after the first immunization or from the control group (Fig. 1A) showed pathological alterations of the testis.

3.2. Immunohistochemical expression of IL-6 and IL-6R

Testicular macrophages and Leydig cells of rats from control and EAO groups showed IL-6 immunoreactivity with the antibodies employed: R&D Systems (Fig. 2A and B) and Peprotech (data not shown). We observed also IL-6^+ Sertoli cells in several seminiferous tubules of control rats, while only a few Sertoli cells showed faint IL-6 immunoreactivity in rats with EAO (R&D System antibody; Fig. 2B). Using the Peprotech antibody, we detected IL-6 expression also in peritubular cells of rats with severe orchitis only (data not shown). IL-6R expression was detected in testicular macrophages, Leydig and germ cells of rats from control and EAO groups (Fig. 2C and D), while IL-$6R^+$ peritubular cells were found only in rats with orchitis. A few IL-$6R^+$ germ cells were observed in testis of control rats while many seminiferous tubules showed a large number of IL-$6R^+$ germ cells in testis of rats with severe orchitis (Fig. 2C and D). Some IL-$6R^+$ germ cells from testes of the experimental group showed signs of degeneration judged by nuclear condensation. Fig. 3 shows quantification of IL-$6R^+$ germ cells with a significant increase in the number of IL-$6R^+$ germ cells from day 70 onwards in rats with EAO compared to control rats. Negative controls (Fig. 2E) and pre-absrobed IL-6 antibody (Peprotech, Fig. 2F) showed no staining confirming specificity of the immune reaction.

3.3. Co-localization of ED1 or ED2 and IL-6 in testicular macrophages

As shown by double immunofluorescent staining technique performed on isolated testicular macrophages from rats with severe orchitis, most of the recently arrived monocytes-macrophages ($ED1^+$) expressed IL-6 while resident macrophages ($ED2^+$) did not express this cytokine (Fig. 4).

3.4. Determination of IL-6 by ELISA

ELISA results showed that testicular macrophages obtained from control group and from rats with severe EAO released IL-6. A significant increase in IL-6 content was observed in

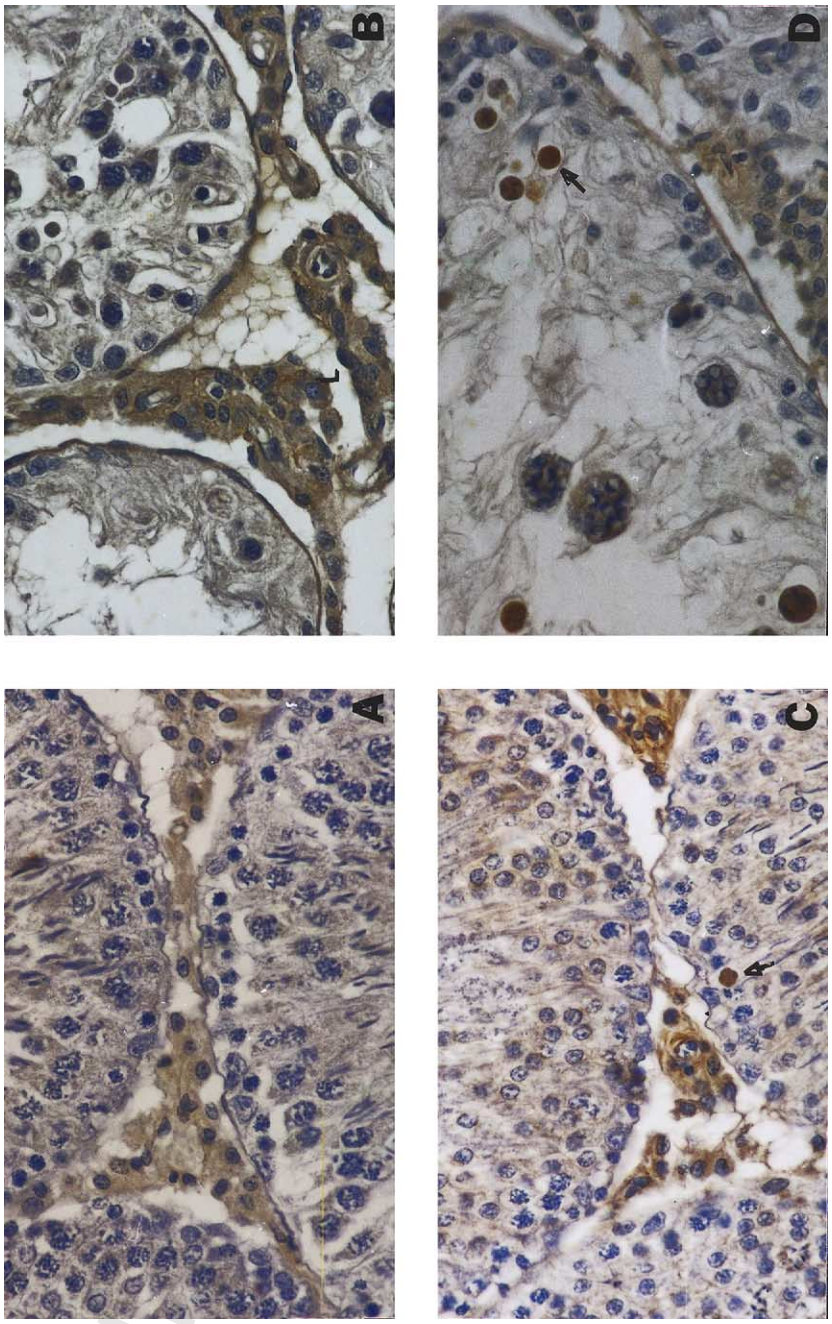

Fig. 1. Testis sections of rats from control group (A) and experimental group immunized with testicular homogenate and adjuvants killed 50 days (B) and 80 days (C) after the first immunization. (A) The normal histology of seminiferous tubules and interstitium. (B) The focal damage mainly involving two seminiferous tubules with germ cell sloughing and germ cell degeneration (arrow). (C) The severe germ cell sloughing of several seminiferous tubules and abundant interstitial cell infiltrate. H&E; note the severe tubular atrophy showing decreased diameter of seminiferous tubules (D). Magnification 180×.

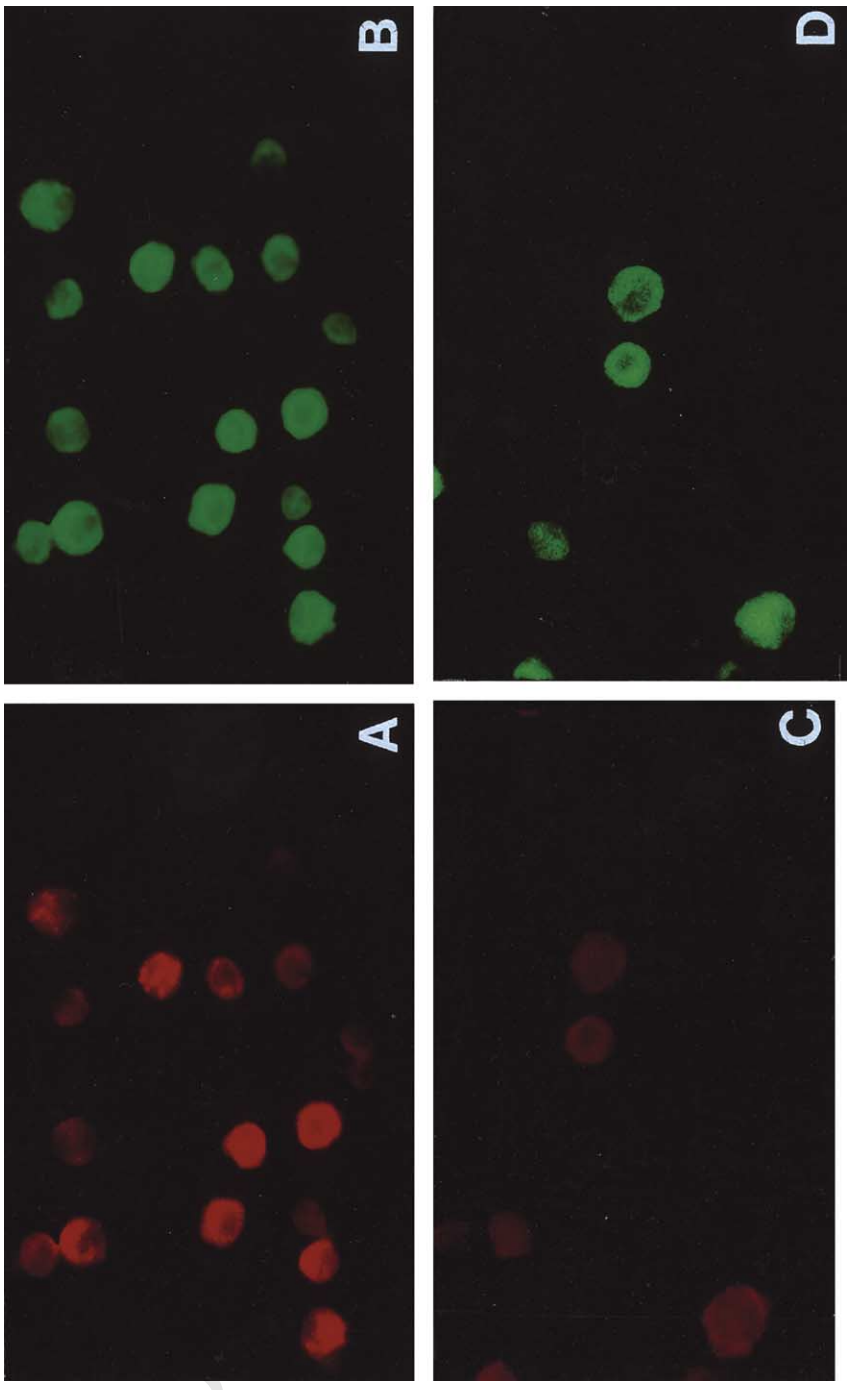

Fig. 2. Immunolocalization of IL-6 (A and B) and IL-6R (C and D) in testis sections from control rats (A and C) and rats with severe EAO (B and D) killed 80 days after the first immunization. IL-6 and IL-6R$^+$ cells are observed in the interstitium of rats from control and EAO groups (A–D) while IL-6$^+$ Sertoli cells are shown in control rats (A). Several IL-6R$^+$ germ cells (arrow) are observed in the damaged seminiferous tubules of rats with EAO (D) while a unique IL-6R$^+$ germ cell (arrow) is observed in a control rat (C). Omission of primary antibodies (E) or pre-absorption of IL-6 antibody (Peprotech) with IL-6 (F) showed negative staining. Magnification 300×.

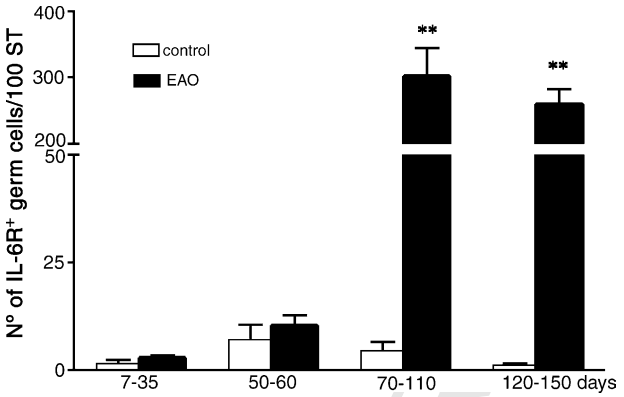

Fig. 3. Quantification of IL-6R$^+$ germ cells (GC) in testis sections of control and EAO rats killed at different periods of time after the first immunization. The number of IL-6R$^+$ GC was quantified in 100 seminiferous tubules (ST) of three non-consecutive testis sections. Values represent mean ± S.E.M. of 4–5 rats/group/period of time. $^{**}p < 0.01$ vs. respective control. Days: days after immunization; testicular histopathology: (+/−) focal damage; (+) severe damage; (++) severe and extended damage. Control rats and experimental rats killed 7–35 days after the first immunization showed normal testicular histology (−).

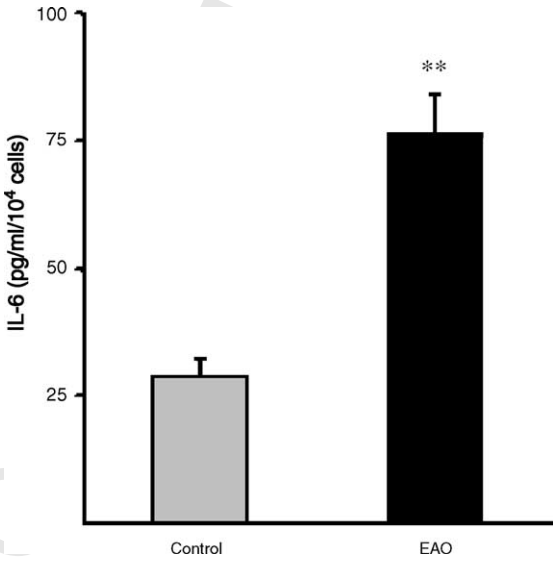

Fig. 4. IL-6 expression in ED1$^+$ and ED2$^+$ testicular macrophages isolated from a rat with severe EAO. Secondary antibodies conjugated with rhodamine or FITC were used to detect IL-6 or ED1/ED2, respectively. Most ED1$^+$ macrophages (B) expressed IL-6 (A), while ED2$^+$ macrophages (D) were negative for IL-6 expression (C). Magnification 750×.

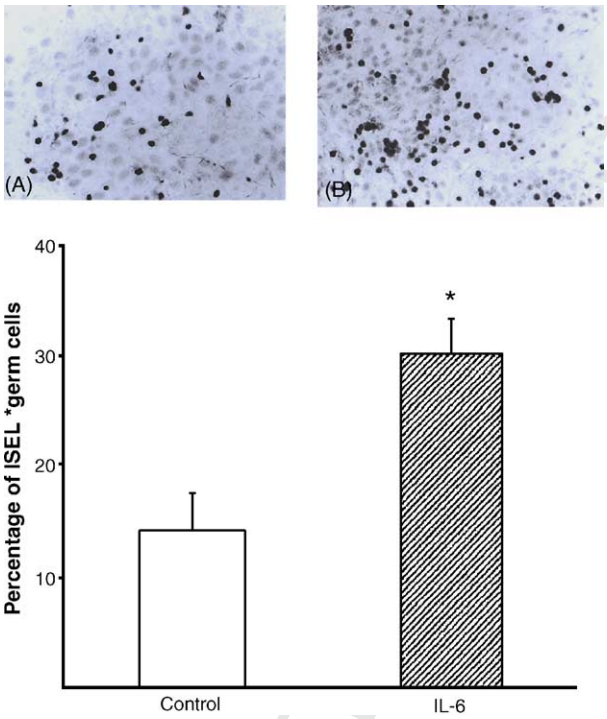

Fig. 5. IL-6 production by testicular macrophages. IL-6 was measured by ELISA in the testicular macrophage-conditioned media obtained from control and EAO rats sacrificed 130 days after immunization. Values are mean ± S.E.M. of 4 rats/group. $^{**}p < 0.01$ vs. control.

the TMCM of rats with orchitis compared to TMCM of control group (Fig. 5). IL-6 was undetectable in sera of rats from both groups.

3.5. Effect of IL-6 on germ cell viability and apoptosis

In order to study the involvement of IL-6 in germ cell viability and apoptosis, STS and isolated germ cells from normal untreated rats were incubated with or without IL-6

Table 1
In vitro effect of IL-6 on germ cell viability

	IL-6 (ng/ml)	
	0	50
Trypan blue (% of dead cells)	22.01 ± 4.43	37.12 ± 6.38*
MTS assay (OD × 10^{-3})	81.00 ± 5.70	54.30 ± 6.30**

Seminiferous tubule segments and isolated germ cells from normal rats were incubated with IL-6 for 18 h. Germ cell viability was evaluated by trypan blue exclusion method and MTS assay, respectively. Data represent mean ± S.E.M. of four independent experiments.

* $p < 0.05$.

** $p < 0.01$.

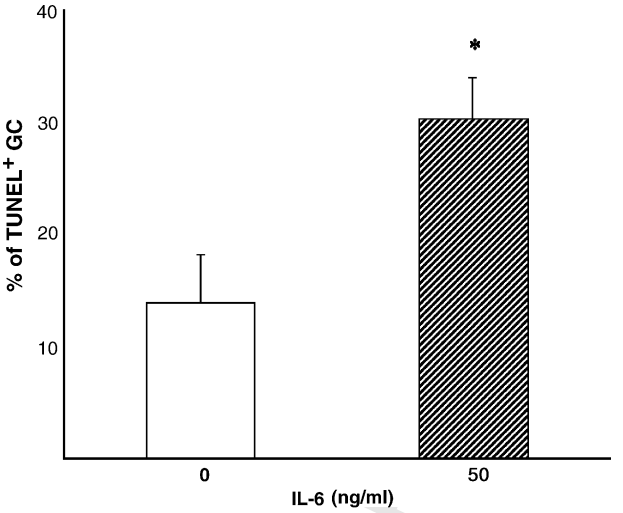

Fig. 6. In vitro effect of IL-6 on germ cell (GC) apoptosis. TUNEL technique was performed on GC obtained from squashed seminiferous tubule segments (STS) of normal rats previously incubated with IL-6 for 18 h. Data are expressed as mean ± S.E.M. of two independent experiments. * $p < 0.05$. In the upper panel, microphotographs of the TUNEL technique: (A) untreated STS and (B) IL-6-treated STS. Magnification 137×.

(50 ng/ml) for 18 h. IL-6 significantly increased the number of dead germ cells (Table 1) and the percentage of apoptotic TUNEL$^+$ cells (Fig. 5) on squashes obtained from STS. In isolated germ cells, IL-6 induced a significant reduction in cell viability (Table 1). Also, the percentage of caspase-3$^+$ germ cells increased after IL-6 treatment (% of caspase-3$^+$ cells: untreated, 30.2%; IL-6-treated, 46.9%). All together, these data showed that IL-6 increased the percentage of germ cell death by about 15% regardless of the methodology employed (Fig. 6).

4. Discussion

By immunohistochemistry, testicular macrophages and Leydig cells of rats from control and EAO groups were expressed to express IL-6 and IL-6R. Several seminiferous tubules from testes of control rats showed IL-6 immunoreactivity in Sertoli cells. The observation of IL-6$^+$ Sertoli cells in some of the seminiferous tubules could be explained by the different IL-6 production at selective stages of the seminiferous epithelial cycle (Hakovirta et al., 1995; Syed et al., 1993). Few IL-6$^+$ Sertoli cells with faint immunostaining were observed in rats with focal and severe orchitis, suggesting downregulation of expression of this cytokine in Sertoli cells in EAO. We speculate that this downregulation is another indicator of Sertoli cell alteration. We reported previously morphological alterations in Sertoli cell cytoplasm (Doncel et al., 1989) as well as reduction in inhibin production in rats with severe EAO (Suescun et al., 2001). In contrast to control rats, we detected IL-6 and IL-6R immunoreactivity in peritubular cells of rats with EAO. Due to interactions between per-

itubular and Sertoli cells, we may speculate that upregulation of IL-6 and IL-6R expression in peritubular cells of rats with EAO is related to Sertoli cells alterations mentioned above. The increased number of Il-6R$^+$ germ cells in rats with severe orchitis occurs simultaneously with a higher degree of testicular damage and increased number of apoptotic germ cells (Theas et al., 2003).

We have demonstrated that testicular macrophages express and secrete IL-6 and that in EAO, testicular macrophages upregulate IL-6 production suggesting the activation of these cells. ELISA showed a 2.5-fold increase in IL-6 production per macrophage in rats with severe EAO compared to controls. Since we reported also an increased number of ED1$^+$ cells in EAO (Suescun et al., 2003), a greater increase in final testicular IL-6 content is expected to occur in rats with orchitis. The high level of TNF-α produced by testicular macrophages in EAO (Suescun et al., 2003) could stimulate IL-6 synthesis by testicular macrophages in an autocrine manner. The autoregulation of IL-6 synthesis has been reported for other cell types (Kozawa et al., 1997).

Immunofluorescent results show that only ED1$^+$ monocytes recently arrived to testis from circulation produce IL-6, while ED2$^+$ resident macrophages do not. It is possible that different subsets of macrophages play different functional roles within the testis in EAO. As suggested by Gerdprasert et al. (2002) in a testicular inflammatory model, ED1$^+$ macrophages could preserve the pro-inflammatory profile of circulating monocytes while ED2$^+$ cells, sensitive to the testicular microenvironment, exhibit an anti-inflammatory profile.

Our in vitro experiments demonstrated IL-6 to be able to induce germ cell death through apoptosis. In vivo, we observed a simultaneous increase in the number of IL-6R$^+$ germ cells and apoptotic germ cells in rats with EAO (Theas et al., 2003). In vitro and in vivo results suggest that IL-6-induced apoptosis of germ cells expressing IL-6R. Since IL-6 expression in Sertoli cells is reduced in EAO, we consider that apoptosis of the numerous IL-6R$^+$ germ cells could be triggered by IL-6 produced by the increased number of activated ED1$^+$ testicular macrophages, although we cannot exclude involvement of IL-6 secreted by Leydig cells in this process. In contrast, in control rats IL-6 secreted by Sertoli cells could induce apoptosis of the few IL-6R$^+$ germ cells, thus collaborating with maintenance of an adequate germ/Sertoli cell ratio. The apoptotic effect of IL-6 may be mediated through the modulated expression of pro- or anti-apoptotic factors (Usuda et al., 2001; Minami et al., 2000; Oritani et al., 1999; Choi and Hwang, 2003; Boer et al., 2003) or through germ cell cycle arrest. In fact, Hakovirta et al. (1995) demonstrated that IL-6 inhibits DNA synthesis in spermatocytes and to a lesser extent, in spermatogonia and this inhibition could possibly induce cell arrest and subsequent apoptosis as demonstrated for other factors (Selva et al., 2000; Salazar et al., 2003; Wolgemuth et al., 2004).

We speculate that IL-6 produced by interstitial testicular cells could reach the adluminal compartment of seminiferous tubules, as shown for other cytokines of similar molecular weight (Banks and Kastin, 1992; McLay et al., 1997). Moreover, in EAO, the blood–testis barrier may be altered (Pelletier, 2001), facilitating the passage of IL-6 and other factors to the adluminal compartment.

Besides the possible role of IL-6 in germ cell apoptosis in EAO, this cytokine could also play an important role in recruiting immune cells to the testis during inflammation, as shown in others models (Kamimura et al., 2003). In fact, it has been shown that IL-6 is a

potent inducer of the monocyte chemoattractant protein-1 (MCP-1) (Biswas et al., 1998) and we have described recently an increase in testicular MCP-1 content in rats with EAO (Guazzone et al., 2003).

A pathogenic role of IL-6 has been demonstrated in several autoimmune diseases (Samoilova et al., 1998; Okuda et al., 1999b; Yamamoto et al., 2000; Boe et al., 1999). However, in murine EAO induced by injection of testicular germ cells without adjuvants, Li et al. (2002) showed that IL-6 reduced the incidence and severity of orchitis. Since IL-6 was exogenously administrated, the authors highlighted the fact that this cytokine may not necessarily play a pathogenic role in EAO. The discrepancy between our results and those of Li et al. (2002) could be due to use of different experimental models and the fact that we studied the endogenous testicular behaviour of IL-6. Also, it is possible that different doses of IL-6 could be responsible for its pro- or anti-inflammatory effects. Further studies blocking endogenous IL-6 are needed in order to clarify the pathogenic role of this cytokine in testicular inflammation.

In conclusion, the high production of IL-6 by interstitial $ED1^+$ macrophages, the increased expression of IL-6R in germ cells of rats with EAO and the involvement of this cytokine in germ cell apoptosis suggest a pathogenic role of IL-6 in autoimmune orchitis.

Acknowledgments

This PROJECT was funded by grants from The Universidad de Buenos Aires (UBA), Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT) and Fundación Antorchas. We thank the Instituto Nacional de Microbiología “A. Malbrán”, División Vacunas Bacterianas for the generous gift of *Bordetella pertussis*. The authors are Research Members (M.S.T., L.L.) or Fellows (C.R.) of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and of the UBA (V.A.G.).

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