

Death receptor and mitochondrial pathways are involved in germ cell apoptosis in an experimental model of autoimmune orchitis

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BACKGROUND: Studies on experimental autoimmune orchitis (EAO) have helped to elucidate immunological mechanisms involved in testicular damage. We previously demonstrated that EAO is characterized by lymphomononuclear cell infiltrates and apoptosis of spermatocytes and spermatids expressing Fas and TNFR1. The aim of this work was to characterize the pathways involved in germ cell apoptosis in EAO and to determine the involvement of the Bcl-2 protein family in this process. **METHODS AND RESULTS:** EAO was induced in rats by immunization with testicular homogenate (TH) and adjuvants, whereas control (C) rats were injected with saline solution and adjuvants. Testis of EAO rats showed procaspase 8 cleavage products (western blot) with high caspase 8 activity. Cytochrome c content increased in the cytosol and decreased in the mitochondrial fraction of testis from EAO rats compared with C, concomitant with increased caspase 9 activity. Bax was mainly expressed in spermatocytes and spermatids and Bcl-2 in basal germ cells (immunohistochemistry). Bax β isoform content increased in EAO rat testis compared with C, whereas content of Bax α remained unchanged (western blot). However, Bax α content decreased in the cytosol and increased in the mitochondrial and endoplasmic reticulum (ER)-enriched fractions of testis from EAO rats compared with C (western blot). Bcl-2 content also increased in the testes of EAO rats. **CONCLUSIONS:** Our results demonstrated that extrinsic, mitochondrial and possibly ER pathways are inducers of germ cell apoptosis in EAO and that Bax and Bcl-2 proteins modulate this process.

Key words: apoptotic pathways/autoimmune orchitis/Bax/Bcl-2/germ cell apoptosis

Introduction

The most common cause of male infertility is a disturbance of spermatogenesis; however, in most cases, the cause of aspermatogenesis remains unknown. Previous studies demonstrated that, in some cases of idiopathic spermatogenesis alteration, lymphocytic infiltration and immune deposits were present in testis biopsies, indicating that some inflammatory or immunological factors are involved in tissue damage (Itoh *et al.*, 2005). Studies on experimental autoimmune orchitis (EAO) have helped to partially elucidate normal testicular immunoregulation as well as autoimmune disease mechanisms that induce testicular damage.

We developed an EAO in rats by active immunization with testicular homogenate (TH) and adjuvants (Doncel *et al.*, 1989). As we previously demonstrated (Lustig *et al.*, 1993; Theas *et al.*, 2003), EAO is characterized by an interstitial lymphomononuclear cell infiltrate and the damage of seminiferous tubules with germ cell apoptosis and sloughing. These phenomena were associated with an increased number of germ cells expressing Fas, Fas L and tumour necrosis factor receptor 1 (TNFR1) (Suescun *et al.*, 2003; Theas *et al.*, 2003).

Apoptotic cell death can be triggered from outside the cell by the activation of death receptors (TNFR1 and Fas) that upon binding their own ligands (TNF- α and Fas L, respectively) activate initiator caspases 8 and 10, which cleave and activate downstream effector procaspases (such as 3, 6 and 7) to kill the cell (Zimmermann *et al.*, 2001). Apoptosis may be triggered by signals that activate the mitochondrial pathway that regulates the release of cytochrome c from the mitochondria. Once released, cytochrome c promotes the activation of procaspase 9 within the apoptosome complex, with the subsequent proteolytic activation of effector caspases (Orrenius, 2004). The mitochondrial pathway is modulated by members of the Bcl-2 family proteins; anti-apoptotic proteins such as Bcl-2 and Bcl-xL inhibit, whereas proapoptotic members such as Bax, Bak and Bid stimulate cytochrome c release (Borner, 2003). Endoplasmic reticulum (ER) can also initiate apoptosis through the release of Ca⁺⁺, which sensitizes mitochondria to extrinsic and intrinsic death stimulus or directly activating death effectors. It has been demonstrated that Bcl-2, Bax and Bak modulate ER Ca⁺⁺ homeostasis, and ER-mediated apoptosis (Breckenridge *et al.*, 2003).

In the normal testis, apoptosis of germ cells occurs during spermatogenesis to balance the number of germ cells in relation to the supportive capacity of Sertoli cells. Germ cell apoptosis also occurs in response to different insults such as toxics, heat, hormonal deprivation and also in pathological models of testicular damage such as cryptorchidism and torsion in which programmed germ cell death is mediated by the Fas–Fas L system and the Bcl-2 family proteins (Koji and Hishikawa, 2003).

Although we demonstrated that in rats with EAO, apoptotic germ cells express Fas and TNFR1 and that caspase 3 is activated in germ cells with signs of degeneration (Suescun *et al.*, 2003, Theas *et al.*, 2003), the intracellular mechanisms that trigger apoptotic death in this model have not been studied. The aim of this work was to characterize the germ cell apoptotic pathways in EAO and the involvement of Bax and Bcl-2 proteins in the modulation of this process.

Materials and methods

Animals

Male Sprague–Dawley rats aged 50–60 days were kept at 22°C with 14 h light, 10 h dark schedule and fed standard food pellets and water *ad libitum*. Animal handling and experimentation were in accordance with the NIH Guide for the Care and Use of Experimental Animals.

Immunization schedule

Rats of the EAO group were immunized with TH prepared as previously described (Doncel *et al.*, 1989). Briefly, rat testes were decapsulated, diluted in an equal volume of saline and disrupted in an Omni mixer for 30 s. The final concentration was 500 mg/ml wet weight. TH (0.4 ml) emulsified with 0.4 ml complete Freund's adjuvant (CFA) was injected intradermally in footpads and at multiple sites near ganglionar regions. EAO rats were injected three times with 200 mg of TH/dose/rat at 14-day intervals. The first two immunizations were followed by an i.v. injection of 0.5 ml of *Bordetella pertussis* (Bp) (strain 10536; Instituto Malbrán, Buenos Aires, Argentina) containing 10^{10} microorganisms and the third by an i.p. injection of 5×10^9 microorganisms. Control (C) group rats were injected with an emulsion of saline, CFA and Bp, in the same conditions as the EAO group. EAO and C rats were killed at different time periods (50–60 or 70–80 days) after the first immunization. Rats were killed and weighed, one testis was removed, weighed and quickly frozen for immunohistochemistry and western blot or processed for germ cell isolation. The other testis was fixed in Bouin for histopathology.

Histopathology

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

Activity of caspase 8 and 9 in isolated germ cells

To isolate germ cells, testes from C and EAO rats were decapsulated and digested with type I collagenase (0.3 mg/ml, Worthington Biochemicals, Lakewood, NJ, USA) in phosphate-buffered saline (PBS) with 0.1% bovine serum albumin (BSA) for 15 min at 34°C in a shaking water bath. Collagenase was inactivated by dilution with PBS, and seminiferous tubules were allowed to settle, then washed three times with PBS. Seminiferous tubules were mechanically dispersed, and cell debris were eliminated by pressing the cell suspension against a fine stainless steel screen. Contaminating red blood cells in the cell suspension were eliminated with lysing buffer (8.99 M NH_4Cl , 1 M KHCO_3 , 0.37 M tetrasodium EDTA, pH 7.3). Then 6×10^6 cells were

lysed in 150 μl of Tris–HCl buffer (50 mM Tris–HCl, 1 mM EDTA, 10 mM EGTA, 40 mM Digitonin, 1 mM PMSF, pH 7.4) for 30 min at 34°C. Lysed cells were centrifuged at $400 \times g$ for 20 min at 4°C and 150 μl of the supernatant incubated with 145 μl of assay buffer [100 mM HEPES, 1 mM EDTA, 10 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride (PMSF) in glycerol, pH 7.4] plus 5 μl of caspase 9-specific substrate (218805 Ac-LEDH-pNA, Calbiochem, Brand of EMD Biosciences, San Diego, CA, USA) for 3 h at 34°C. The enzyme-catalysed release of p-NA was read at 405 nm in a microplate reader. The detection of caspase 8 activity in germ cells obtained as described above was performed according to the instruction manual of the colorimetric caspase kit (BD ApoAlert K2029-1, BD Biosciences Clontech, Palo Alto, CA, USA).

Western blotting

To obtain total testicular lysate, decapsulated frozen testes were homogenized in a glass homogenizer with lysis buffer (1.5 ml buffer/g of tissue) [50 mM Tris, 150 mM NaCl, 1% sodium dodecyl sulphate (SDS), 2 mM EDTA, 1% sodium desoxycholate, 1% NP-40, pH 7.4] containing protease inhibitors (1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ pepstatin A and 10 $\mu\text{g}/\text{ml}$ aprotinin, Sigma Chemical, St Louis, MO, USA). Homogenates were centrifuged at $13\,000 \times g$ for 20 min at 4°C, and the supernatant was the total testicular lysate. To obtain cytosolic, mitochondrial and ER-enriched fractions, we used a procedure previously described (Zhou *et al.*, 2001; Vera *et al.*, 2004). Briefly, testes were homogenized in buffer A (6 ml buffer/g tissue) (0.25 M sucrose, 50 mM HEPES, 10 mM NaCl, 10 mM EDTA, 2 mM DTT) supplemented with protease inhibitors as indicated above. The crude homogenates were centrifuged at $1000 \times g$ for 10 min at 4°C and the supernatant centrifuged at $10\,000 \times g$ for 15 min at 4°C to sediment the low-speed fraction mainly containing mitochondria. The cytosolic and ER-enriched fractions were isolated following centrifugation of the $10\,000 \times g$ supernatant fraction at $100\,000 \times g$ for 60 min at 4°C. The supernatant was the cytosolic fraction and the pellet the microsomal fraction containing the ER. The mitochondrial and microsomal fractions were lysed in buffer (20 mM HEPES, 1.5 mM MgCl_2 , 10 mM KCl, 1 mM EDTA, 1 mM EGTA and 1 mM DTT) supplemented with protease inhibitors. To assess equal loading, protein concentration in the lysates was determined by the Lowry method (Bio-Rad DC Protein Assay; Bio-Rad, Hercules, CA, USA). Fifty micrograms of protein of total lysate and 20 μg of each fraction were resolved in SDS–polyacrylamide gel electrophoresis (PAGE), 14% for Bcl-2, Bax and cytochrome c and 12% for caspase 8 and electrotransferred as previously described (Diaz *et al.*, 2005). The molecular weight of the immunoreactive bands was determined by comparing them with a ladder of prestained protein standards with a molecular weight range of 108–21.2 kDa (161–0305 Bio-Rad) or 150–10 kDa (161–073 Bio-Rad) applied to a line in each gel. Protein transference and equal loading were monitored by staining membranes with Ponceau red. Then, membranes were blocked with blocking solution [5% (w/v) of non-fat dry milk in tris-buffered saline tween-20 (TBST) buffer: 10 mM Tris, 154 mM NaCl, 0.1% Tween-20 (v/v), pH 7.5] for 90 min. Blots were probed overnight with mouse monoclonal antibodies against Bcl-2 (1:300) (sc-7382, Santa Cruz Biotechnology, CA, USA), Bax (1:200) (sc-7480, Santa Cruz Biotechnology) directed to the NH_3 terminal of the protein that recognizes Bax α and Bax β , with a rabbit polyclonal antibody against caspase 8 (1:250) (sc-7890, Santa Cruz Biotechnology) and goat polyclonal anti-cytochrome c (1:2500) (sc-8385, Santa Cruz Biotechnology). As internal loading controls a rabbit polyclonal antibody anti- β -actin (1:6000) (A-2066, Sigma Chemical), a goat polyclonal antibody anti-nicotinamide adenine dinucleotide (NADH)-ubiquinone oxidoreductase chain 4 (ND4) 1:1000 (sc-20499, Santa Cruz Biotechnology) and a rabbit polyclonal antibody anti-calnexin

(1:1000) (sc-11397, Santa Cruz Biotechnology) were used. After six washes (5 min each) in TBST buffer, membranes were incubated with an anti-mouse, anti-rabbit or anti-goat biotinylated antibody (2.5 µg/ml, Vector Laboratories, Burlingame, CA, USA) depending on the primary antibody used. Primary and secondary antibodies were diluted in blocking buffer solution. The reaction was enhanced with horseradish-streptavidin-peroxidase conjugates (Chemicon International, Temecula, CA, USA), and chemiluminescence was used to detect the horseradish-peroxidase-labelled protein onto Kodak BioMax MS Film (NEN™ Life Science products, Boston, MA, USA). The western blot films were scanned and bands were quantified by a densitometric analytical system.

Immunohistochemistry

To detect Bax and Bcl-2 expression in the testis, we used an amplified immunoperoxidase technique and the tyramide signal amplification (TSA) detection kit (NEL-700, PerkinElmer Life and Analytical Sciences, Boston, MA, USA) in cryostat testis sections fixed in cold acetone and methanol, respectively.

Testis sections were washed in PBS and incubated with 5% normal horse serum (NHS) and 3% BSA for 30 min at room temperature (RT). Endogenous biotin reaction was prevented with Avidin/Biotin Blocking Solution (SP-2001, Vector Laboratories). Sections were reacted overnight with mouse monoclonal antibodies anti-Bcl-2 (10 µg/ml) (Santa Cruz Biotechnology) or anti-Bax (2 µg/ml) (Santa Cruz Biotechnology) in PBS with 2% NHS at 4°C in a humidified chamber. A biotinylated horse anti-mouse rat adsorbed (4 µg/ml) (Vector Laboratories) diluted in PBS with 5% normal rat serum was used as secondary antibody. Endogenous peroxidase was blocked with 0.3% H₂O₂ in methanol for 30 min. For the immunoperoxidase technique, the reaction was amplified with a Vectastain Elite ABC Kit (PK-6200, Vector Laboratories), and the reaction product was visualized by adding diaminobenzidine substrate (SK-4100, Vector Laboratories). In the TSA technique, sections were incubated with horseradish-streptavidin-peroxidase conjugates (1:100) (Chemicon International) for 30 min at RT and the signal amplified using biotinylated tyramide (1:100) (NEL-700, PerkinElmer Life and Analytical Sciences) for 6 min at RT. To detect biotinylated tyramide, sections were incubated with streptavidin-Cy3 conjugated (1:400) (Zymed Laboratories, San Francisco, CA, USA) for 30 min at RT. For negative controls, the first antibody was omitted.

Statistical analysis

Statistical evaluations were done with the Student's *t*-test. The Dunnett test was employed to analyse differences in caspase 8 activity and the non-parametric Welch test to analyse differences in cytochrome c and Bax content in the fractions. Differences were considered significant if $P < 0.05$.

Results

Histopathology

Testicular histopathology of EAO rats was characterized by mild interstitial infiltration of lymphomononuclear cells and focal damage of seminiferous tubules 50 days after the first immunization (Figure 1B). From 80 days on, a higher degree of interstitial cell infiltration and more severe and extended seminiferous tubule damage with a high degree of germ cell sloughing was observed. Basal germ cells remained attached to the seminiferous tubule wall (Figure 1C). None of the C group rats showed testicular lesion (Figure 1A).

Testicular weight

Rats from the EAO group killed 50–60 and 70–80 days after the first immunization showed a significant decrease in testicular weight associated with testicular damage compared with C group rats (50–60 days C: 1.67 ± 0.05 g, EAO: 1.16 ± 0.05 g^{***}, $n = 18$; 70–80 days C: 1.56 ± 0.05 g, EAO: 0.87 ± 0.04 g^{***}, $n = 8$, $***P < 0.001$; mean \pm SEM). Body weight of C and EAO rats killed at the time periods studied did not differ significantly (50–60 days C: 411.20 ± 31.81 g, EAO: 455.67 ± 18.06 g; 70–80 days C: 430.73 ± 26.50 g, EAO: 438.15 ± 19.03 g; mean \pm SEM).

Western blot of caspase 8 and caspase 8 activity

Because we previously demonstrated that TNFR1 and Fas are expressed in apoptotic germ cells, we decided to study death receptor activation by evaluating caspase 8 activity. We observed that procaspase 8 is processed in the testis of C and EAO rats, but a higher content of procaspase 8 and its cleaved forms were present in the testis of EAO rats compared with C rats, 50–60 and 70–80 days after the first immunization (Figure 2). Also, caspase 8 activity was significantly higher in germ cells isolated from rats with EAO compared with C at the two time periods studied (Figure 3). Activity and processing of caspase 8 was higher in testis of EAO rats at 70–80 compared with 50–60 days. These results confirm that the processing of procaspase 8 generates a functional caspase 8 heterodimer.

Western blot of cytochrome c and caspase 9 activity

Cytochrome c levels significantly increased in the cytosolic fraction and decreased in the mitochondrial fraction of testis lysates from EAO rats compared with C rats (Figure 4). The

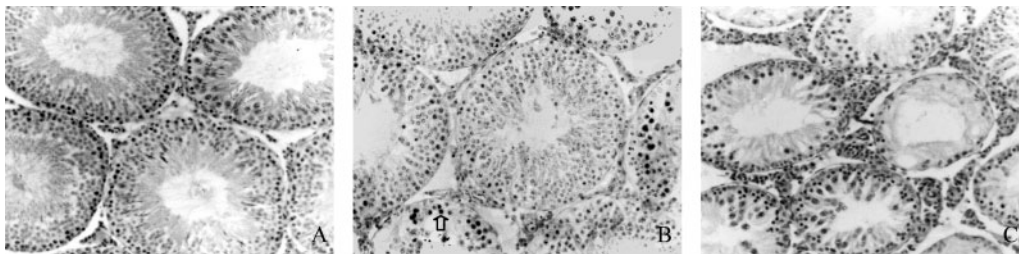


Figure 1. Testis sections of rats from control (A) and experimental autoimmune orchitis (EAO) groups killed 50 days (B) and 80 days (C) after the first immunization. **A:** normal histology of seminiferous tubules and interstitium. **B:** focal damage mainly involving three seminiferous tubules with germ cell degeneration (arrow). **C:** severe germ cell sloughing of several seminiferous tubules and abundant interstitial cell infiltrate. HE (original magnification $\times 100$).

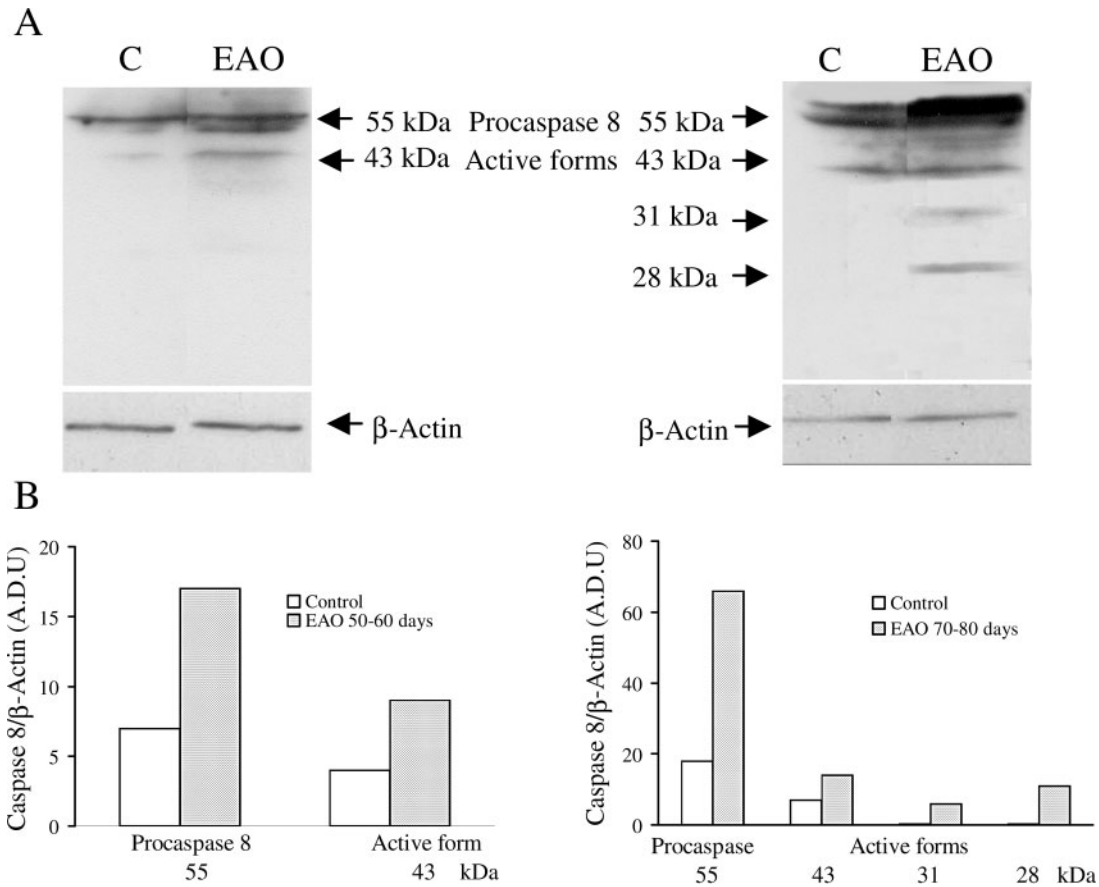


Figure 2. Caspase 8 activation. **A:** representative western blot of caspase 8 processing in total testicular lysates from control (C) and experimental autoimmune orchitis (EAO) rats killed 50–60 days (left) and 70–80 days (right) after the first immunization. **B:** semiquantitative results of caspase 8 content obtained by densitometric analysis of western blots. Data are representative of three rats per group at each time period. ADU, arbitrary densitometric units.

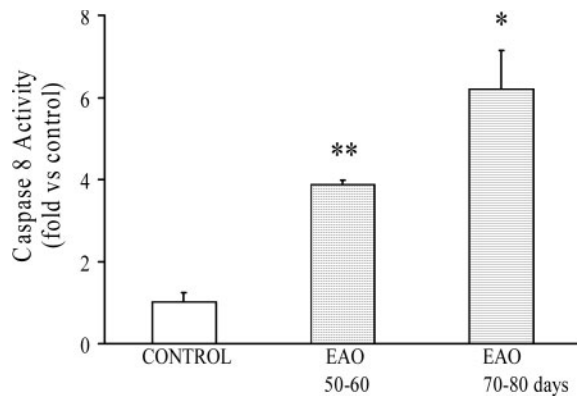


Figure 3. Caspase 8 activity determined by colorimetric method in germ cells obtained from testes of control and experimental autoimmune orchitis (EAO) rats killed 50–60 days and 70–80 days after the first immunization. Data represent mean \pm SEM of three rats per group. * $P < 0.05$, ** $P < 0.01$ versus control.

release of mitochondrial cytochrome c to the cytosol of germ cells from EAO rats might explain this behaviour.

Because it is known that cytochrome c released from the mitochondria to the cytosol triggers caspase 9 activation within the apoptosome, we evaluated caspase 9 activity. Caspase 9 activity was significantly greater in germ cells

isolated from the testis of rats with EAO compared with C (Figure 5). We did not study the proteolytic cleavage of caspase 9 because it has been demonstrated that procaspase 9 can be activated without processing and removal of its pro-domain (Stennicke *et al.*, 1999).

Immunohistochemical localization of Bcl-2 and Bax

Both immunohistochemical techniques employed showed similar cell expression of Bcl-2 and Bax proteins. Because the reaction product was more intense with the TSA detection kit, we included only microphotographs of testis sections processed with this technique.

Bcl-2 was detected only in spermatozoa present in the lumen of the seminiferous tubules of testis from C rats (Figure 6A). In contrast, in EAO rats, this protein was mainly expressed in the basal cells of seminiferous tubules (Figure 6B). In testis sections from C rats, only basal cells were immunoreactive for Bax (Figure 6C), whereas in EAO rats, Bax was highly expressed in germ cells, mainly spermatocytes and spermatids, some of them sloughed in the tubular lumen (Figure 6D). None of the interstitial cells showed immunoreactivity for Bcl-2 and Bax. No positive cells were detected when the first antibody was omitted (Figure 6E).

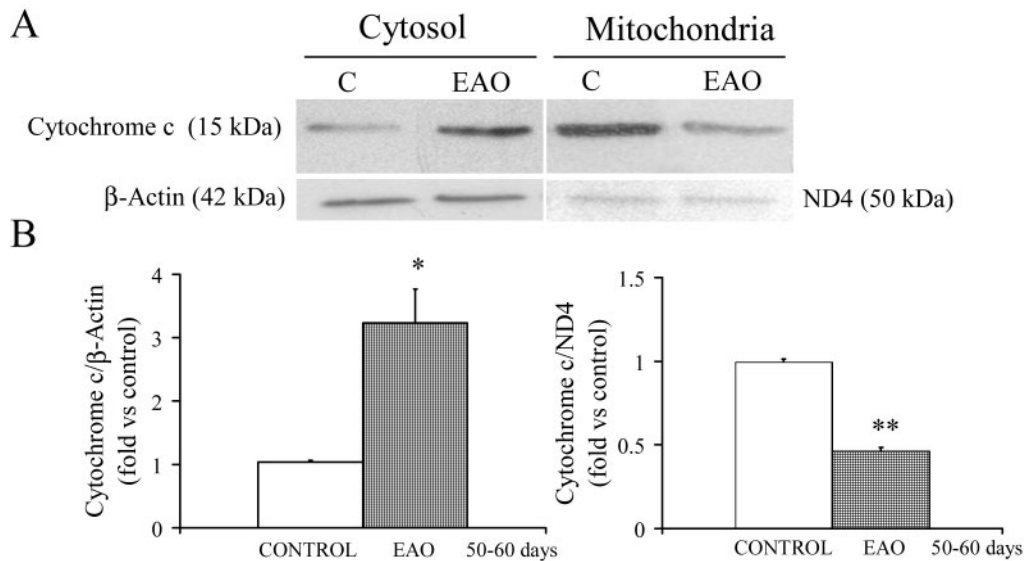


Figure 4. Cytochrome c translocation from mitochondria to cytosol. **A:** representative western blot of cytochrome c content in cytosolic and mitochondria lysates obtained from testes of control (C) and experimental autoimmune orchitis (EAO) rats killed 50–60 days after the first immunization. **B:** semiquantitative results of cytochrome c content obtained by densitometric analysis of western blots. Data represent mean \pm SEM of four rats per group. * $P < 0.05$, ** $P < 0.01$ versus respective control.

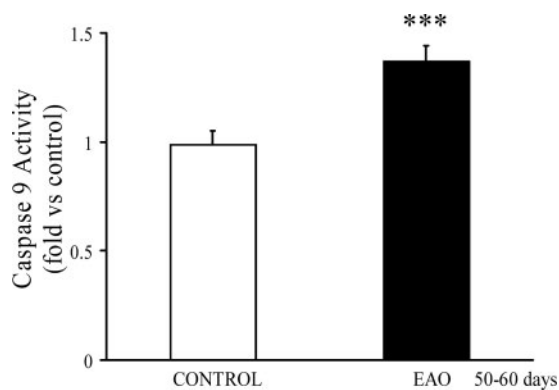


Figure 5. Caspase 9 activity determined by a colorimetric method in germ cells obtained from testes of control and experimental autoimmune orchitis (EAO) rats killed 50–60 days after the first immunization. Data represent mean \pm SEM of eight rats per group. *** $P < 0.001$ versus control.

Western blot analysis of Bcl-2 and Bax

Bcl-2 content increased 2.0- and 10.1-fold in the testes of EAO rats from 50–60 and 70–80 days after the first immunization compared with the respective C rats (Figure 7). We showed that testes from C and EAO rats expressed two isoforms of Bax: Bax α of 21 kDa and Bax β of 24 kDa. Bax α content in the testis of C compared with EAO rats did not differ (Figure 8), whereas the levels of Bax β significantly increased, by 3.4- and 4.7-fold in the testis of EAO versus C rats both 50–60 and 70–80 days after the first immunization, respectively (Figure 8). Because Bax α may be translocated to different organella membranes in apoptotic cells, we analysed Bax content in the cytosol-, mitochondria- and ER-enriched fractions obtained from C and EAO rats killed 50–60 days after the first immunization. We observed that Bax α levels decreased in the cytosol and increased in the mitochondrial and ER fractions of testicular lysates obtained from EAO rats (Figure 9).

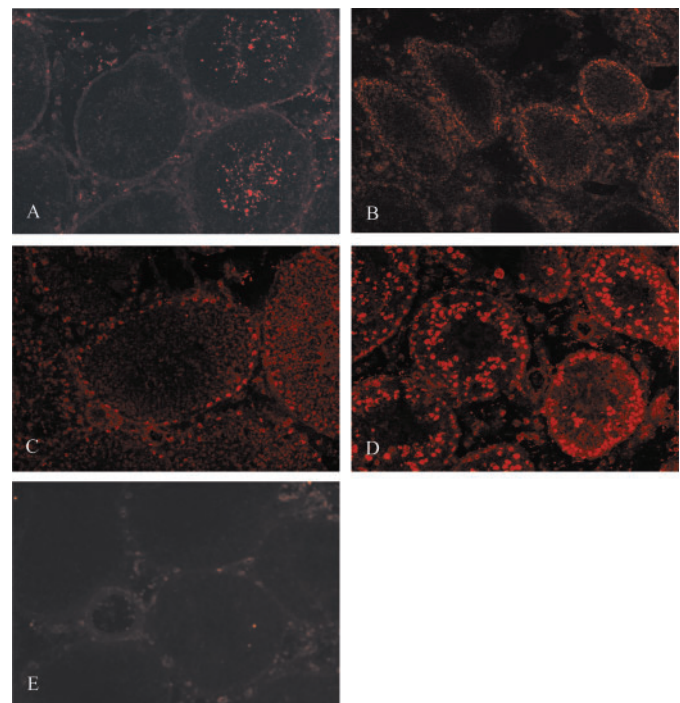


Figure 6. Immunohistochemical localization of Bcl-2 (**A** and **B**) and Bax (**C** and **D**) in cryostat testis sections from control rats (**A** and **C**) and experimental autoimmune orchitis (EAO) rats (**B** and **D**) killed 55 and 77 days after the first immunization, respectively. In the negative control (**E**) the first antibody was omitted (original magnification $\times 90$).

Discussion

Apoptosis of spermatocytes and spermatids is one of the main characteristics of seminiferous tubule damage in EAO. As we demonstrated previously, apoptosis precedes germ cell sloughing. No signs of apoptosis were observed in Sertoli and Leydig cells (Theas *et al.*, 2003). In this model, the number of apoptotic

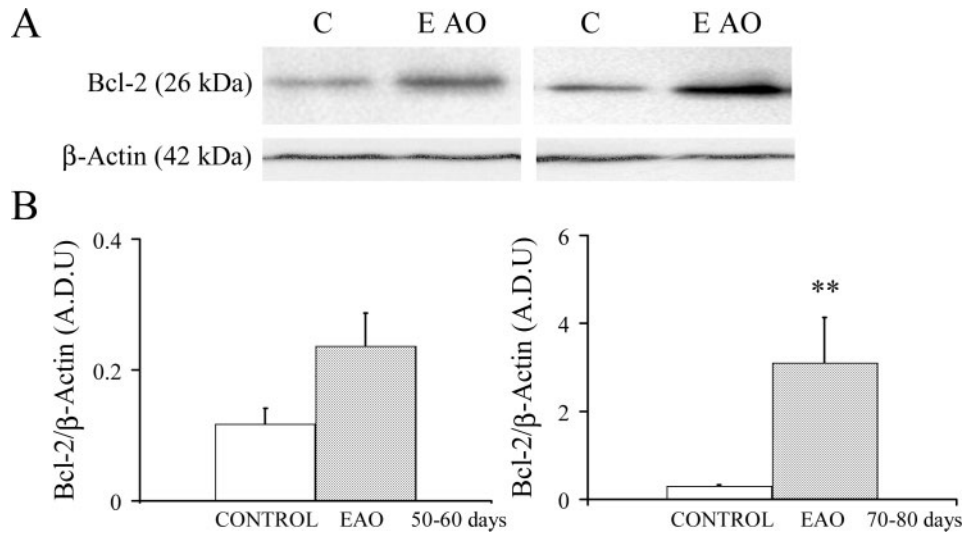


Figure 7. Bcl-2 content in the testis. **A:** representative western blot of Bcl-2 content in total testicular lysates from control (C) and experimental autoimmune orchitis (EAO) rats killed 50–60 days (left) and 70–80 days (right) after the first immunization. **B:** semiquantitative results of Bcl-2 content obtained by densitometric analysis of western blots. Data represent mean \pm SEM of four rats per group at each time period. ** $P < 0.01$ versus respective control. ADU, arbitrary densitometric units.

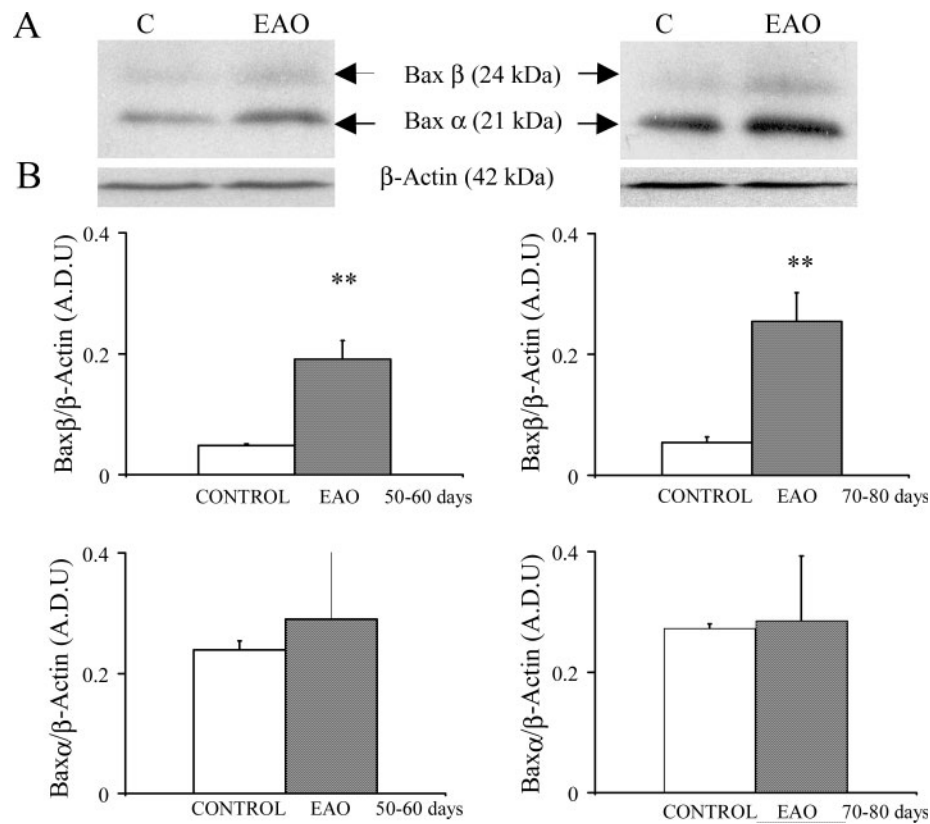


Figure 8. Bax content in the testis. **A:** representative western blot of Bax content in total testicular lysates from control (C) and experimental autoimmune orchitis (EAO) rats killed 50–60 days (left) and 70–80 days (right) after the first immunization. **B:** semiquantitative results of Bax content obtained by densitometric analysis of western blots. Data represent mean \pm SEM of four rats per group at each time period. ** $P < 0.01$ versus respective control. ADU, arbitrary densitometric units.

germ cells increased from day 50 after the first immunization and was associated with the degree of testicular damage. Apoptotic germ cells in EAO expressed the death receptors Fas and TNFR1, and spermatocytes of damaged seminiferous tubules showed

immunoreactivity for active caspase 3 (Suescun *et al.*, 2003; Theas *et al.*, 2003). The presence of the active forms of caspase 8 and its increased activity indicates that in EAO, apoptosis may be triggered by death receptor activation. Up-regulation of

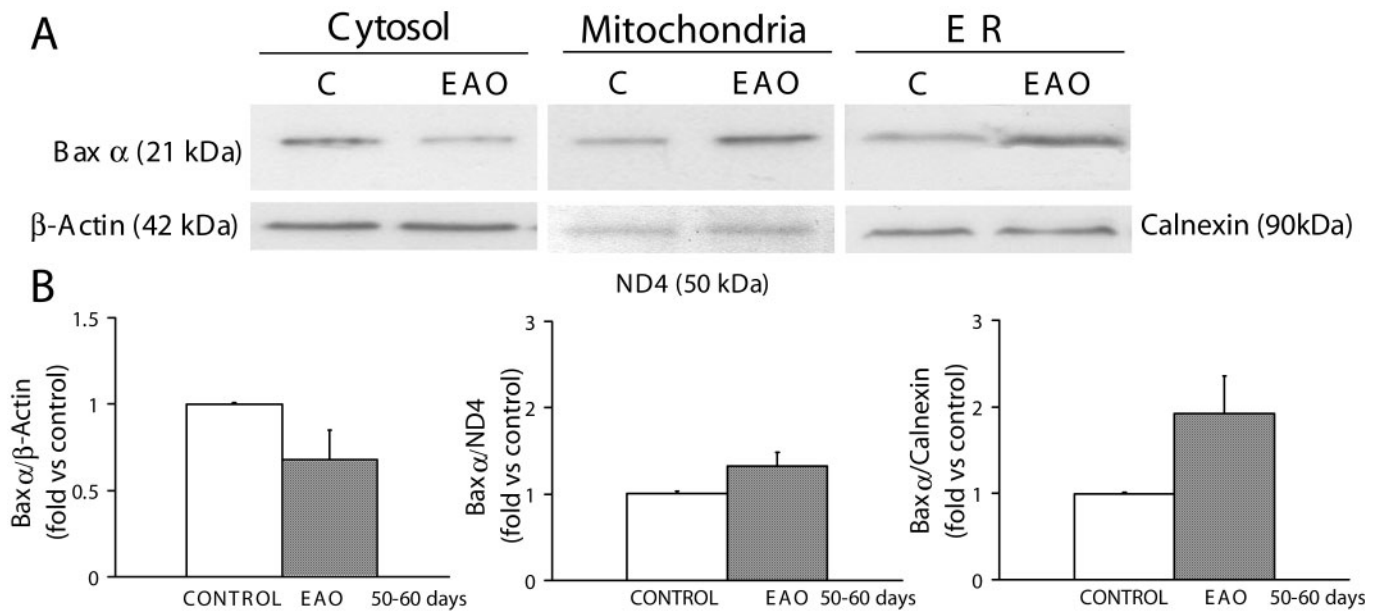


Figure 9. Bax α translocation from cytosol to mitochondria and endoplasmic reticulum (ER). **A:** representative western blot of Bax content in cytosolic, mitochondrial and ER lysates obtained from testes of control (C) and experimental autoimmune orchitis (EAO) rats killed 50–60 days after the first immunization. **B:** semiquantitative results of Bax content obtained by densitometric analysis of western blots. Data represent mean \pm SEM of three rats per group.

procaspase 8 may contribute to the increased apoptotic response observed in EAO. The higher content of cytochrome c in the cytosol and the concomitant decrease of this protein in the mitochondria of testes from rats with orchitis suggest that mitochondria become activated and that the cytochrome c released to the cytoplasm is able to initiate the mitochondrial pathway. The high caspase 9 activity observed in the germ cells from rats with EAO confirms that the mitochondrial pathway is also activated. We might speculate that caspase 8 and 9 are responsible for the activation of the executioner caspase 3 and subsequent germ cell apoptosis.

In other experimental models of testicular damage, it has been demonstrated that the activation of caspases 8 and 9 occurs together with Fas and Fas L up-regulation (Eid *et al.*, 2002; Nair and Shaha, 2003). In EAO, we observed that apoptosis of germ cells also occurs together with an increased number of Fas and Fas L-positive germ cells (Theas *et al.*, 2003) and with caspase 8 and 9 activation.

The role of mitochondria in germ cell apoptosis has also been demonstrated in different experimental models of testicular damage (Zhou *et al.*, 2001; Li *et al.*, 2003; Nair and Shaha, 2003; Vera *et al.*, 2004). The apoptotic mitochondrial pathway may be activated by death receptors, nitric oxide (NO) and proapoptotic members of the Bcl-2 family protein such as Bid and Bax (Borner, 2003; Vieira and Kroemer, 2003; Blaise *et al.*, 2005). As we did not study Bid cleavage and insertion in the mitochondria, we cannot exclude that death receptors could also activate this pathway in EAO. We hypothesized that NO may participate as a potential inducer of the mitochondrial pathway in this model because we observed a higher content of nitrite in conditioned media of testicular macrophages from rats with EAO compared with C rats (Theas and Lustig, unpublished data).

The two isoforms of Bax protein, Bax β (24 kDa) and Bax α (21 kDa), are products of alternative mRNA splicing. Bax β , lacking the C terminal transmembrane domain, represents the cytosolic forms of Bax, and Bax α the membrane-bound forms (Oltvai *et al.*, 1993). In relation to Bax isoforms present in normal rat testis, the expression of 21 kDa Bax protein and not the 24 kDa isoform has been reported (Yan *et al.*, 2000; Yu *et al.*, 2001). However, we were able to detect the expression of the two Bax isoforms, showing that the amount of testicular Bax β protein increases in the testes of rats with testicular damage. It has been suggested that Bax could homodimerize or heterodimerize with Bax and Bcl-2, providing an additional level of regulation of apoptosis (Oltvai *et al.*, 1993). The higher expression of Bax may counter the antiapoptotic activity of Bcl-2 as proposed by Oltvai *et al.* (1993). In fact, we also noticed that Bcl-2 content in the mitochondrial fraction from testis of rats with EAO was lower compared with C (data not shown). We speculate that Bax β up-regulation may be involved in the modulation of germ cell apoptosis through the disruption of the proapoptotic/antiapoptotic protein balance.

Bax α has a conserved C-terminal hydrophobic domain that is unleashed after an apoptotic stimulus allowing its insertion in the mitochondria, ER and nuclear membranes (Schinzel *et al.*, 2004). Bax α content was similar in total testis lysates from C and EAO rats; however, a role for this protein in germ cell apoptosis through the induction of the apoptotic mitochondrial pathway is presumed, because in the testes of rats with EAO, translocation of Bax α from cytosol to mitochondria plus a concomitant release of cytochrome c to the cytoplasm occurs. Also the increased content of Bax α in the ER suggests that Bax α may also regulate the ER apoptosis pathway. Accordingly, it has been reported that following different apoptotic stimuli,

relocalization of Bax from cytosol to subcellular compartments such as mitochondria, ER and the nuclear envelope occurs (Sinah Hikim *et al.*, 2003; Zhang *et al.*, 2003; Gajkowska *et al.*, 2004). The redistribution of Bax may take place without changing the expression levels of this protein (Zhang *et al.*, 2003).

At 70–80 days, when severe testicular damage occurs, the intensity of the apoptotic signal (caspase 8 activity and Bax content) and the number of apoptotic germ cells are maximal (Theas *et al.*, 2003) as well as Bcl-2 content. The up-regulation of Bcl-2 and its immunolocalization in basal germ cells suggest a protective role of Bcl-2 counteracting apoptotic stimuli to avoid the sloughing of spermatogonia that remained attached to the seminiferous tubules in severe orchitis. This hypothesis is supported by data indicating that overexpression of Bcl-2 increases cell viability under adverse circumstances such as loss of cell adhesion (Sorenson, 2004). Recently (Toruner *et al.*, in press) studying the genes up-regulated by NF- κ B that inhibit anoikis of intestinal epithelial cells demonstrated through under-expression/over-expression experiments the functional importance of Bcl-2 in the inhibition of this special type of apoptosis. An increase in Bcl-2 germ cell expression has also been reported in other models of testicular damage such as cryptorchidism or testosterone withdrawal (Woolveridge *et al.*, 1998; Zhang *et al.*, 2003). It has been postulated that the rise in Bcl-2 protein levels could be very important to maintain germ cells in the testis to guarantee the damaged tissue responding to normal situation (Zhang *et al.*, 2003).

In other pathologies of tissue damage such as steatohepatitis, apoptosis of hepatocytes also occurs with high Bcl-2 levels (Ramalho *et al.*, 2006). The authors suggested that the increased Bcl-2 expression could prevent a worse situation that could compromise the functionality of the liver.

It has been demonstrated that cytokines could regulate Bcl-2 family protein expression (Spanaus *et al.*, 1998; Kim *et al.*, 2002; Simonart and Van Vooren, 2002). We speculate that this regulation may occur in EAO because we previously demonstrated an increase in proinflammatory cytokines, TNF- α and IL-6, produced by interstitial cells (Suescun *et al.*, 2003; Rival *et al.*, in press).

Our results showed that in EAO, several apoptotic pathways (death receptor, mitochondria and possibly ER) are activated and are responsible for germ cell apoptosis. We speculate that in EAO, inflammation and the autoimmune response alter the normal testicular immunosuppressor microenvironment, allowing cytokines and soluble factors to enter the seminiferous tubules leading to germ cell apoptosis and infertility. More data about immunoregulation of the testis are needed to understand human testicular pathology and idiopathic infertility associated with immunological mechanisms.

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