Cytokine profiles in the testes of rats treated with lipopolysaccharide reveal localized suppression of inflammatory responses

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Cytokine profiles in the testes of rats treated with lipopolysaccharide reveal localized suppression of inflammatory responses. Am J Physiol Regul Integr Comp Physiol 288: R1744–R1755, 2005. First published January 20, 2005; doi:10.1152/ajpregu.00651.2004.—Evidence indicates that the testis possesses a reduced capacity to mount inflammatory and rejection responses, which undoubtedly contributes to the ongoing survival of the highly immunogenic germ cells. The contribution of local cytokine expression to this condition was investigated in adult male rats treated with lipopolysaccharide to induce inflammation. Cytokine mRNA and protein expression were determined in tissue extracts and fluids by Northern blot analysis, quantitative PCR, or RNase protection assay and specific ELISAs. Testicular expression of the proinflammatory cytokines, interleukin (IL)-1β and tumor necrosis factor-α (TNF-α), was considerably attenuated compared with the liver (control tissue); in contrast, the testicular IL-6 response was enhanced. Expression of IL-10, a type 2 immunoregulatory cytokine, was similar in both testis and liver, whereas the immunoregulatory/anti-inflammatory cytokines transforming growth factor-β1 and activin A were constitutively elevated in both normal and inflamed testes. The IL-1β and transforming growth factor-β1 proteins were present principally in their latent (inactive) forms, indicating that enzymatic processing is an important control mechanism for these two cytokines within the testis. These data indicate that inflammatory and regulatory cytokine activity is regulated at both transcriptional and posttranslational levels in a testis-specific manner. It is concluded that a novel pattern of suppression of proinflammatory cytokine responses and normal or elevated expression of immunoregulatory cytokines may be responsible for reduced inflammatory responses and enhanced graft survival in the testis. These data have important implications for the understanding and treatment of male autoimmune infertility, testicular inflammation, and carcinogenesis.

immunoregulation; interleukins; fertility; transforming growth factor-β; tumor necrosis factor-α; lipopolysaccharide; viability; spermatogenesis

Survival of the meiotic and postmeiotic male germ cells, which first appear at the time of puberty and consequently express a broad range of spermatogenesis-specific autoantigens, has long been attributed to local immunoregulation (65). Local immunoregulatory mechanisms also have been implicated in the extended survival of transplants to the testicular interstitial tissue in experimental rodents (17, 26) and in the ability of testicular cells to confer protection to grafts at various external sites (36, 58). The mechanisms of this immunoregulation remain poorly defined. It has been suggested that this unique immunological property of the testis may be due to selective destruction of antigen-specific T cells through expression of Fas ligand on the testicular Sertoli cells (2), but this hypothesis has been challenged by subsequent studies (1, 13). More recently, we have proposed an alternative hypothesis that inflammatory responses in the testis are modified by deviation from the production of key proinflammatory cytokines, such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α, toward immunoregulatory, immunosuppressive, or anti-inflammatory cytokine production (27). This possibility was first suggested by observations that isolated rat testicular macrophages have a reduced capacity to produce IL-1β and TNF-α in vitro compared with macrophages from other tissues (25, 32, 33) and exhibit immunosuppressive characteristics in vivo (3, 48).

A number of in vitro studies have shown that the somatic (Sertoli, Leydig, or peritubular) cells and germ cells are also a source of cytokine production in the testis. Leydig cells express macrophage inhibitory factor constitutively (43), and IL-1β, IL-1α, and IL-6 on stimulation with the inflammatory stimulus lipopolysaccharide (LPS) in vitro (39, 51, 67). Isolated Sertoli cells produce IL-1α constitutively and IL-6 after stimulation with either LPS or exogenous IL-1 (12, 18, 51). Spermatocytes and spermatids express TNF-α (14). Expression of immunosuppressive cytokines of the transforming growth factor (TGF)-β family, TGF-β1 and activin A, has been observed in isolated peritubular cells and Sertoli cells (15, 47). These last two cytokines inhibit a number of proinflammatory activities of macrophages and lymphocytes, including the production and/or actions of IL-1, TNF-α, and IL-6 (37, 53). Although a precise biological role for many of these cytokines in normal spermatogenesis remains to be defined, there is no doubt they all may play a role in the response of the testis to immunological and pathogenic challenges. It is important to recognize, however, that evidence for the involvement of most of these cytokines in testicular inflammation is based largely on studies in vitro. The use of isolated cells for such studies can lead to cryptic upregulation of expression due to the isolation procedures or culture conditions themselves. A small number of studies have examined IL-1β and TNF-α responses to inflammation in the intact testis in vivo (21, 24, 31, 45, 62), but a direct quantitative comparison between cytokines in the testis and those of other tissues has never been carried out before.

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With these facts in mind, we felt it was vital to obtain quantitative data on cytokine mRNA expression and protein production in the intact adult testis both before and during inflammation in vivo. In the case of IL-1β, TGF-β1, and activin A in particular, assessment of mRNA and the resulting protein product is essential: IL-1β and TGF-β1 are produced as inactive precursors that require activation by local proteases (4, 42), whereas the monomeric subunit of activin A is able to dimerize with the α subunit of inhibin to form a functional antagonist of activin bioactivity (54). Moreover, production of the type 2 immunoregulatory cytokines IL-10 and IL-4, which are important regulators of cellular immune responses (52), has never been investigated in the testis previously.

The aim of the present study was to examine both proinflammatory and regulatory cytokine production profiles in the whole rat testis in vivo by using our well-characterized LPS-induced inflammation model (19, 20, 48, 49). This model mimics the human response to inflammation and infection of the male reproductive tract, leading to suppression of the hypothalamic-pituitary axis, reduction in androgen production by the Leydig cells, and disruption of the developing germ cells that correlates with the severity of the inflammation (49). We previously have shown that this inhibition of testis function is accompanied by a transcriptional upregulation of the inflammatory mediators IL-1β (21), monocyte chemoattractant protein-1 (20), and inducible nitric oxide synthase (48). However, the resident macrophages of the testis appear not to participate in the inflammatory response, and the result is a minor, transient infiltration of circulating monocytes with little or no neutrophil or lymphocyte recruitment (49). Indeed, the data are indicative of a restrained inflammatory response, which has implications for understanding the ability of antigens to avoid detection in this organ, as well as the response to various infections and tumors in the testis. The hypothesis to be tested was as follows: that the testis displays a unique basal and inflammatory cytokine profile compared with other tissues and, more specifically, that production of proinflammatory cytokines is suppressed while anti-inflammatory/immunoregulatory cytokines are maintained or upregulated. This study also represents the first detailed quantitative investigation of a broad range of cytokine responses to inflammation in the intact testis.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (100–120 days old) and adult C3H/HeJ mice (50–70 days old) were obtained from the Monash University Central Animal Services or from Charles River Laboratories (Kislegg, Germany). Experimental procedures were approved by the Monash University Standing Committee on Ethics in Animal Experimentation and the Regionungs Praesidium, Giessen, and conform to the respective Codes of Practice for the Care and Use of Animals for Experimental Purposes and the National Research Council’s Guide for Care and Use of Laboratory Animals. For all experiments, the liver was chosen as a control tissue because this organ contains a substantial resident macrophage population comparable to that of the testis.

Experimental Procedures

Experiment 1: LPS dose and extended time-course comparison (0–72 h). Systemic inflammation was induced in the rats by an injection (ip) of pyrogen-free saline (1 ml/kg body wt) containing 0.1 mg/kg (low dose) or 5 mg/kg (high dose) of LPS from Escherichia coli, serotype 0127:B8 (Sigma, St. Louis, MO). The effects of these doses of LPS on the pituitary and testicular axis are induction of a mild inflammatory event and severe endotoxemia, respectively (49). For the control group (0 h), rats were injected (ip) with pyrogen-free saline only (1.0 ml/kg body wt). At 0, 3, 6, 12, 18, 24, and 72 h postinjection, the rats (3 animals per time point and LPS dose) were killed by CO2 overdose, and one testis and a fragment of liver were snap-frozen for subsequent RNA extraction.

Experiment 2: acute inflammation time course (0–6 h). Rats were injected (ip) with pyrogen-free saline (control group; 3 animals/time point) or saline containing 0.1 mg LPS/kg body wt (5 animals/time point). At 1, 2, 3, 4, 5, and 6 h postinjection, the rats were killed by decapitation, and trunk blood was collected into tubes without anticoagulant. The blood was allowed to clot, and serum was obtained by centrifugation (1,500 g, 10 min, 4°C) and stored at −20°C until assay. One testis was used for collection of testicular interstitial fluid (IF) by drainage under unit gravity via a small incision at the caudal end of the capsule (16 h, 4°C), as previously described (28). The collected fluid was centrifuged (800 × g, 10 min, 4°C) to remove cellular debris and stored at −20°C. The remaining testis was snap-frozen and stored at −70°C.

Experiment 3: tissue sampling study. Rats were injected (ip) with pyrogen-free saline (control group) or 0.1 mg LPS/kg body wt (treatment group). Six rats from each group were killed 3 h postinjection by CO2 overdose, and blood was obtained by cardiac puncture for both serum (as already described) and plasma by collection into tubes containing 100 U heparin, followed by immediate centrifugation (800 g, 10 min, 4°C) to remove blood cells and platelets. The testes were removed, and one testis from each animal was perfused for 1 min with sterile saline via the testicular artery to clear the vascular bed (64). The contralateral testis was not perfused. Testicular IF was collected from both the perfused and nonperfused testes, as described above, and stored at −20°C. A further six animals from each group were anesthetized with ether, and the thorax and abdomen were exposed. The testicular artery on one side was clamped, and the ipsilateral testis was collected, snap-frozen, and stored at −70°C for protein extraction. The remaining testis was perfused with saline by whole-body perfusion via the descending aorta, followed by peridate-lysine-parafomaldehyde fixative (10–15 min), as previously described (68). The perfused testis was collected, postfixed in peridate-lysine-parafomaldehyde solution for a further 3 h, followed by immersion at 4°C in 7% sucrose for 36 h, then snap-frozen in liquid nitrogen, and embedded in Optimal Cutting Temperature (OCT) compound (Tissue-TEK, Elkhart, IN). The fixed, frozen testes were cut at 10-μm thickness using a Leica cryostat (Leica Instruments, Heidelberg, Germany), mounted on Superfrost Plus glass slides (Menzel, Braunschwig, Germany), air dried for 24 min, and then stored at −20°C.

Experiment 4: inflammation after Leydig cell ablation. Rats (12/treatment group) were injected (ip) with ethane dimethane sulfonate (EDS; 75 mg/kg body wt) dissolved in dimethyl sulfoxide (DMSO)/water (1:3) to ablate the Leydig cells or DMSO/water alone (carrier control) as previously described (34, 68). Ten days later, one-half of the rats in each group were injected (ip) with pyrogen-free saline (controls), and the remaining animals were injected with 0.1 mg LPS/kg body wt. All rats were killed 3 h after this second injection by CO2 overdose. One testis and a fragment of liver (control) were collected into liquid N2 for subsequent RNA extraction, and the remaining testis was used for collection of IF as described above.

RNA Extraction and Northern Blot Analysis

Total RNA was extracted from testis and liver samples using the acid-guanidinium thiocyanate-phenol chloroform extraction method (11). Northern blotting was carried out as described previously (20). The following plasmids were used: 1,264-bp rat IL1β cDNA...
in pMosBlue-T (Amersham Biosciences, Buckinghamshire, UK); 526-bp rat IL10 cDNA in pBlueScript (Stratagene, La Jolla, CA); 793-bp rat TNFa cDNA in pBlueScript; and 950-bp rat TGFβ cDNA in pMosBlue-T. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) cDNA was as described previously (20). β-actin cDNA was amplified from testicular mRNA using the primers 5'-CTCTTCAGGCTTCCTCTCCTCTC-3' and 5'-AAAGGCTATGCAAAATGCTCTC-3', followed by subcloning into PCR II dual promoter plasmid (Promega, Southampton, UK) using standard methods. cDNA sequences were confirmed using dye terminator reactions at the Monash Medical Centre Wellcome Trust Sequencing Facility.

The membranes from both low-dose (0.1 mg/kg) and high-dose (5 mg/kg) LPS-treated testes and liver were exposed at the same time overnight to a phospho-imaging screen (Fuji Photo Film, Tokyo, Japan). After the intensity of signal was read, the membranes were exposed to BioMax film (Eastman Kodak, Rochester, NY) in the same X-ray cassette in the presence of an intensifying screen (Kodak). Normalization of the variations in RNA loading between samples was performed by probing the same RNA blot with cDNAs encoding GAPDH and β-actin mRNA (housekeeping controls), and all Northern blot data were normalized against each control. The signal intensity of radioactive molecules hybridized to the RNA blot was quantified using phospho-image analysis. After exposure to the phospho-imaging screen, the image was scanned in a Fuji BAS1000 MacBas Bio-Imaging Analyzer and analyzed using MacBas Version 2.5 software (Fuji). After background membrane intensity was subtracted from cytokine signal intensity, the real cytokine intensity was corrected against the intensity of GAPDH and β-actin separately in each sample. Repeat experiments (n = 3 per animal and time point) were done in parallel and placed on the same phospho-imaging screen and used the same exposure time for each cytokine, so it was possible to compare cytokine expression in the testis and liver. The liver control (0 h) signal included on each membrane was assigned an arbitrary value of 1.0. For IL6 and IL10, where there was no detectable band in the normal liver (0 h) samples, relative absorbance values were expressed compared with the background intensity in the control lane. All experiments included a minimum of three animals per treatment group and time point. Very similar expression profiles were obtained using either GAPDH or β-actin as a loading control, as both housekeeping genes were unchanged in testis and liver after LPS-treatment. Whereas GAPDH mRNA levels in testis and liver were comparable, testis extracts contained considerably higher levels of β-actin mRNA transcripts than did the liver samples. Consequently, all Northern data are presented relative to GAPDH.

### Real-Time Quantitative PCR Using the LightCycler

Quantitative analysis of IL-1β, IL-10, and IL-4 mRNA expression was performed using standard methods in a LightCycler (Roche Diagnostic, Mannheim, Germany). Total RNA (1–2 μg) obtained from testis and liver was reverse transcribed using Omniscript (Qiagen, Hilden, Germany). The primers used for amplification of the cDNAs are listed in Table 1 (Sigma-Genosys, Castle Hill, Australia).

Amplified PCR products were sequenced using the dye terminator method to confirm amplification of the correct product (data not shown). Standard curves using the recombinant plasmids of known concentrations (0.01 pg/μl to 1 ng/μl) and samples containing RNA without reverse transcriptase were included for every run. All samples were normalized against β-actin expression and subsequently corrected for the tissue-specific difference in β-actin expression, which was between 7- and 10-fold higher in the testis compared with the liver (data not shown). Confirmation of expression levels was performed using normalization to total RNA in each extract, which gave almost identical results. The corrected data were then standardized against liver from control animals (assigned the arbitrary value of 1.0) to allow quantitative comparisons between liver and testis expression and direct comparison with the Northern blot analysis data. Each quantitative PCR experiment included a minimum of three animals per treatment group and time point.

### RNase Protection Assay for IL-1β and IL-1α

Ribonuclease protection analysis of cytokine expression was performed using a commercially available kit (RiboQuant Multi-Probe, BD Biosciences, Heidelberg, Germany) according to the manufacturer’s instructions. The custom template set contained DNA templates for both IL1β and IL1α, as well as GAPDH and another housekeeping gene, L32. Although there is an alternate transcript of IL-1α in the rat testis that lacks exon 5 (23, 61), the IL-1α probe used in the present study hybridized to a region of mRNA from exon 1 of the gene and would pick up both testicular transcripts.

Total RNA was isolated with the single-step acid phenolguanidinium thiocyanate-chloroform extraction method (11) using Trizol reagent (Invitrogen, Karlsruhe, Germany). Briefly, 32P-radiolabeled antisense riboprobes were generated by multiplex in vitro transcription using [α-32P]UTP (Amersham, Braunschweig, Germany) and T7 RNA polymerase. Radiolabeled probes were hybridized with 5 μg of total testicular or liver RNA samples at 36°C overnight. After RNase A/T1 treatment for digestion of single-stranded RNA, samples together with undigested probes and in vitro transcribed 100-bp RNA markers (Century Marker, Ambion, Huntingdon, UK) were resolved on a 50 × 30 cm 5–6% polyacrylamide/7 M urea gel. The gel was dried for 1 h at 80°C under vacuum (Bio-Rad, Munich, Germany), exposed to a phosphorimage screen overnight and analyzed on a Fuji PhosphorImager FLA3000G (Raytest, Straubenhardt, Germany). Subsequently, RNase-protected bands were quantified using Fiji Image Gauge software. For each treatment group analyzed, RNA was prepared from four randomly selected animals. The probe set was labeled three or four separate times for analyses of samples from each testis examined. Liver RNA (with or without LPS) was included as control for cytokine expression. All cytokine data was normalized for loading against both GAPDH and L32, with similar results obtained.

### Preparation of Tissue Extracts for Protein and Cytokine Measurements

Samples of frozen testes were homogenized (1:3, wt/vol) in 5 mM phosphate-buffered saline (PBS), pH 7.5, 0.1% monodet P-40, 0.5% Tween 20, and 0.1% SDS, containing Complete protease inhibitor cocktail (Roche) at 4°C. For detection of IL-1β protein only, testes were homogenized (1:2, wt/vol) in 5 mM PBS, pH 7.5, containing a protease inhibitor cocktail [in mM: 2 4-(2-aminoethyl)-benzenesulfonyl fluoride, 1 EDTA, 0.13 bestatin, 0.014 E-64, 1 leupeptin, 0.0003 aprotinin; Sigma]. Lysates were centrifuged twice at 15,000 g for 30 min at 4°C. Supernatants were collected and stored at −20°C until analyzed. Protein concentrations of supernatants of homogenized testes were determined by using DC Protein Assay Kit (Bio-Rad) by comparison to a bovine serum albumin standard.

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**Table 1. Sequence of primers for RT-PCR of rat IL1β, IL4, IL10, and β-actin cDNA**

<table>
<thead>
<tr>
<th>Gene Target</th>
<th>GeneBank Accession No.</th>
<th>Product Size (Base Pairs)</th>
<th>5′ → 3′ Sequences (Forward Primer)</th>
<th>(Reverse Primer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL1β</td>
<td>E05490</td>
<td>519</td>
<td>CGAGATGAGGAGCCAGGAG</td>
<td>TCCGCAACATTCTGCCTCC</td>
</tr>
<tr>
<td>IL4</td>
<td>X16058</td>
<td>277</td>
<td>TGACGGGATTCTGATTTTGAG</td>
<td>GATCCAGTCTCTGAGCTT</td>
</tr>
<tr>
<td>IL10</td>
<td>X60675</td>
<td>181</td>
<td>GCGAGCTTTAAGGTTCTTAA</td>
<td>GGGTGAAGATACTGAGACG</td>
</tr>
<tr>
<td>β-actin</td>
<td>V01217</td>
<td>202</td>
<td>GATATCGCTGCTGCTGCTG</td>
<td>TGCGGTTACCTGATGCTAGG</td>
</tr>
</tbody>
</table>
**ELISA**

**IL-1β ELISA.** IL-1β in testicular IF, testicular extracts, serum, and plasma was measured using a Quantikine M Rat IL-1β Immunoassay (R & D Systems, Minneapolis, MN) (66), according to the manufacturer’s protocol. All samples were assayed at two to three serial dilutions in freshly prepared assay buffer, and samples of rat testicular extract, IF, blood, and serum were diluted in parallel with the standard across the effective assay dose-response range. The assay had a sensitivity of 50 pg/ml and an intra-assay coefficient of variation of 7.6%.

**IL-10 ELISA.** IL-10 was measured in testicular IF, testicular extracts, serum, and plasma using a Biosource rat IL-10 assay kit (Biosource, Camarillo, CA), according to the manufacturer’s protocol. The assay had a sensitivity of 60 pg/ml and an intra-assay coefficient of variation of 10.2%.

**Activin A ELISA.** Activin A was measured in testicular IF, testicular extracts, serum, and plasma using a specific ELISA (Oxford Bio-Innovations, Oxfordshire, UK), according to the manufacturer’s instructions with some modifications, and using human recombinant activin A as standard, as previously described (8). The assay had a sensitivity of 5 pg/ml and an intra-assay coefficient of variation of 4.2%.

**TGF-β1 ELISA.** TGF-β1 was assayed using a two-site ELISA kit (R & D Systems) with recombinant human TGF-β1 as standard (40), according to the manufacturer’s instructions. This assay shows 57% cross-reactivity with human TGF-β1.0. 0.15% with TGF-β2, and 0.96% with TGF-β3. Rat TGF-β1 possesses 91% homology with human TGF-β1 at the precursor protein level (56). All samples were assayed at two to three serial dilutions against the standard. To measure total TGF-β1 levels, latent TGF-β1 in the samples was acid-activated by addition of 2.5 M acetic acid/10 M urea for 15 min, followed by neutralization with 2.7 M NaOH/M HEPES. Samples of acid-activated rat testicular extract, IF, plasma, and serum showed significant parallelism with the human TGF-β1 standard across the effective assay dose-response range. The assay had a sensitivity of 150 pg/ml and an intra-assay coefficient of variation of 10.9%.

All assay plates were read on a Multiskan RC plate reader (Labsystems, Helsinki, Finland) at the appropriate wavelength, and data were processed using the Genesis Lite enzyme immunoassay software (Labsystems). All samples were assayed in duplicate and assessed against the standard using routine parallel-line assay statistics. Values for testis extracts and blood are presented as means ± SE of replicate samples. Due to the small volumes of IF obtained per animal relative to the volume required for assay, cytokine concentrations in testicular IF were obtained from pools of equal volume samples from all animals in each experimental group, unless otherwise indicated. The pooled samples were assayed at serial dilutions against the standard, and data are expressed as mean activity with 95% confidence intervals, as appropriate.

**Testosterone Assay**

Testicular IF was assayed for testosterone without extraction using a direct 125I-testosterone radioimmunoassay (44).

**Immunohistochemistry**

Frozen sections were washed twice with PBS. Endogenous peroxidase activity was quenched by incubation in 5 mM periodate solution for 5 min and subsequently immersed in 0.03 M sodium borohydride for 30 min to reduce aldehyde groups formed by the periodic acid (7). Nonspecific binding was minimized by incubation of the sections in CAS-Block (Zymed, San Francisco, CA) with 10% normal goat serum for 30 min. Endogenous biotin was blocked by incubation of sections with an avidin-biotin blocking solution (Vector, Houston, TX) for 30 min. IL-1β was detected using a rabbit anti-rat IL-1β antiserum (Endogen, Woburn, MA) diluted 1:200 in 1% bovine serum albumin in PBS and incubated overnight at 4°C in a humid chamber. The antibody was raised against recombinant rat IL-1β and does not cross-react with IL-1α. Antibody localization was detected using a biotinylated goat anti-rabbit IgG (DakoCytomation, Glostrup, Denmark), amplification with ABC-horseradish peroxidase complex (Vectastain Elite ABC kit, Vector), and visualized using diaminobenzidine tetrahydrochloride as substrate. Slides were counterstained with 0.1% Mayer’s hematoxylin. The specificity of staining was determined by replacing the primary antibody with 5% rabbit serum.

**IL-1 Bioassay**

Bioactive IL-1 was measured by an in vitro lymphocyte proliferation assay, using thymocytes from LPS-resistant C3H/HeJ mice, as previously described (25, 29). Briefly, the thymocytes were cultured in a 96-well plate (1.2 × 10^6 cells/250-μl well; 37°C) in Dulbecco’s modified Eagle’s medium with 2.5% fetal calf serum and 1 μg/ml phytohaemagglutinin, alone or with various dilutions of testicular IF. After 72 h of culture, [3H]thymidine (0.5 μCi/μl) was added to each well, and the cells were cultured for a further 16 h, after which the cells were harvested using a Packard Micromat 196 automated cell harvester (Packard Instrument, Downers Grove, IL) and counted in a Packard 1900 TR scintillation counter.

**Size Exclusion High-Performance Liquid Chromatography Fractionation of Testicular IF**

Two pools (200 μl each) were prepared by combining equal volumes of testicular IF collected from six rats injected with 0.1 mg/kg LPS 3 h previously, and from six saline-treated control rats as described in experiment 2 above. The control IF pool was spiked with 800 pg of recombinant rat Mr 17010-β1 to mark the elution position of processed native IL-1β within testicular IF. Both pools were fractionated by size exclusion chromatography on a Phenomenex (Torrance, CA) BioSep SEC-S2000 column (300 × 7.8 mm) in 10 mM PBS, pH 7.25, at a flow rate of 0.5 ml/min. Fractions of 0.5 min (0.25 ml) were collected for assay by IL-1β ELISA. The column was calibrated with gel filtration standards from Bio-Rad.

**Statistical Analysis**

All statistical analyses were performed using SigmaStat version 1.0 software (Jandel Scientific Software, San Rafael, CA). Data were analyzed by one- or two-way ANOVA after appropriate transformation to equalize the variance between experimental groups, in conjunction with Student Newman-Keuls multiple range tests. Comparisons between experimental groups were considered statistically significant at the P < 0.05 level.

**RESULTS**

**Cytokine mRNA Expression Time Course During Inflammation (Experiment 1)**

Using Northern blot analysis, appropriate size transcripts were detected for IL1β, TNFα, IL6, and TGFβ1 in testes from normal and LPS-treated rats (Fig. 1, A–D). A signal of the appropriate size for IL10 was also observed in the testis after LPS-treatment (Fig. 1E), but levels of expression were too low for comparative quantification. The data indicated the presence of multiple transcripts of IL6 in the rat testis, corresponding to identical multiple transcripts in the liver (Fig. 1C), as previously reported (51). For simplicity of the data presentation, only quantification of the upper band is shown, although both bands displayed similar expression patterns. In addition to the 2.5-kb TGFβ1 signal usually seen, the TGFβ1 probe also hybridized with a smaller band (1.5 kb) in the testis (Fig. 1D). This smaller transcript has also been observed in the rat prostate (59).
IL-1β, TNF-α, and IL-6 were all expressed at low levels in the control testis and were upregulated by LPS over a time frame similar to that seen for the liver controls (3–6 h). Testicular expression of both IL1β and TNFα was maximally stimulated, even at the lower dose of LPS, and showed significantly (~5-fold in both cases) lower levels of expression compared with the liver controls. In contrast, testicular IL6 expression was stimulated by LPS in a dose-dependent manner and to a much greater level than that seen in the liver. Testicular TGFβ1 was constitutively expressed at very high
levels (~10-fold higher) compared with the liver and was unaffected by LPS. Because both doses of LPS caused similar effects on the main cytokines of interest, all subsequent studies used the lower dose of LPS only. It should be noted that the physiological response to this lower dose more closely approximates that experienced during mild systemic inflammation.

Real-time quantitative PCR was used to quantify IL10 and IL4 expression, because this provides a more sensitive method of detection. IL1β expression was also reevaluated by this method to determine whether results were directly comparable between the two mRNA detection methods (Fig. 2). There was a very good correspondence between the results obtained for IL1β using quantitative PCR and Northern blot analysis, with IL1β expression in the testis consistently much lower (~5- to 7-fold lower) than the liver in control and LPS-treated samples (Fig. 2A). IL10 was expressed at similar levels in both the testis and liver before and after LPS-treatment, although expression levels were quite variable between animals. The peak of IL10 mRNA expression appeared to be slightly later than that of IL1β, i.e., 6 h compared with 3 h for both testis and liver (Fig. 2B). IL4 was detectable in all liver samples by real-time PCR, but was only detectable in ~30% of all testis samples, and did not appear to be upregulated by LPS treatment (data not shown).

**Cytokine Protein Time Course During Inflammation (Experiment 2)**

Activin A is formed from a dimer of two identical βA subunits. Because these subunits can also bind to the inhibin α-subunit to form inhibin A, production of activin A can only be measured in any meaningful way at the level of the mature protein (22). Consequently, the production of activin A after LPS-treatment was measured by ELISA and compared with IL-1β expression over the first 6 h after treatment. Activin A levels in testicular IF were found to be 5- to 10-fold higher than in blood (serum) under normal conditions (Fig. 3A). After LPS treatment, activin A showed a gradual increase in the circulation, with concentrations peaking at 2–4 h later, but there was no increase of activin A in testicular IF (Fig. 3A). Activin A was detectable in all liver samples by real-time PCR, but only detectable in ~30% of all testis samples, and did not appear to be upregulated by LPS treatment (data not shown).

***Fig. 3. Profile of activin A and total IL-1β protein levels over 0–6 h in testis and blood of rats injected with a low dose (0.1 mg/kg) of LPS.***

A: activin A in testicular interstitial fluid (IF) and serum. Values are mean ± SE of 5 animals at each time point. Cytokine levels in saline-treated control animals did not change significantly across the experimental period and have been omitted for clarity. Statistical notation indicates comparison with the relevant 0-h value. *Significantly different from 0 h (P < 0.05); lack of superscript indicates no significant difference from 0 h (P > 0.05). B: IL-1β in a single pool of testicular IF collected from the 5 animals at each time point assayed at serial dilutions. Values are mean activity relative to the IL-1β ELISA standard.

Although cytokine concentrations in IF reflect secretion into the interstitial space of the testis, these concentrations are potentially affected by cytokine levels in the circulation and by changes in IF volume, such as that known to occur during LPS-induced inflammation (49). Measurement of cytokine levels in whole testis extracts provides the real testicular content (both intracellular and secreted) but cannot discriminate differences in distribution between testicular cells and compartments. Consequently, the levels of several cytokines were measured and compared in both IF and total testis extracts (experiment 3) (Table 2). Neither IL-10 nor the active TGF-β1

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**Fig. 2.** Real-time quantitative PCR measurement of IL1β (A) and IL10 (B) mRNA expression in testis and liver of rats injected with a low dose (0.1 mg/kg) of LPS. Values in histograms are means ± SE of measurements from 3–5 separate animals collected at 0, 3, 6, and 12 h after LPS administration. Statistical notation indicates comparison between testis and liver value at each time point. *Significantly different (P < 0.05); lack of superscript indicates no significant difference (P > 0.05).
protein were detectable in either IF or testis extracts from either normal or LPS-treated animals, indicating endogenous levels of <50 pg/μl and <150 pg/μl, respectively, in the adult testis even after LPS treatment. Acid activation of the testis samples, however, revealed the presence of relatively high levels of latent TGF-β1 in these samples (Table 2).

It was noted that, at 3 h after LPS treatment, IF volume was reduced by >2 SD below control levels (i.e., <85 μl/testis) in 5 of the 12 animals, although the average IF volume (94 ± 12 μl/testis; mean ± SE) in these animals remained not significantly different from normal controls (118 ± 8 μl/testis; P > 0.05). These changes in volume in a minority of the testes had negligible or marginal effects on mean TGF-β1 or activin A concentrations in IF at this time point. It is possible, however, that the reduction in IF volume may have had a concentrating effect on the IL-1β levels. Similar levels of all three cytokines were found in the IF from both nonperfused and perfused testes (data not shown), indicating that the presence of blood cells during IF collection had little or no effect on measurable cytokine levels in the samples. The levels of both IL-1β or activin A were similar in plasma and serum (data not shown). However, serum contained much higher levels of latent TGF-β1 than did plasma (23 ± 8 vs. 3.9 ± 0.9 ng/ml; mean ± SE; n = 6 rats) due to platelet activation after collection.

The data confirm the presence of IL-1β, activin A, and TGF-β1 proteins in the normal testis (Table 2), with only IL-1β showing a large increase after LPS administration, consistent with the mRNA data (Figs. 1 and 2). Although activin A displayed a very slight elevation (25%) in testicular IF after LPS treatment in this experiment, this was not reflected by changes in the whole testis, whereas total TGF-β1 was unchanged in either compartment. The concentration of IL-1β in both normal and LPS-treated testes was considerably higher than that observed in the blood, and IL-1β levels in whole testis extracts were also significantly greater than in IF in the normal controls. After LPS treatment, there was an increase in IL-1β in all compartments, but testicular levels remained considerably higher than circulating levels. Activin A levels were significantly higher in the IF than in either the total testis or in blood, whereas TGF-β1 levels were highest in the whole testis extracts. The concentrations of TGF-β1 in plasma and IF were almost identical. These data indicate that there are substantial differences in the compartmentalization, production, secretion, and/or clearance of all three cytokines in the normal and inflamed testis.

Characterization of Factors Determining IL-1β Activity in Testis

The relative upregulation of total IL-1β protein in the inflamed testis compared with correspondingly low levels of mRNA expression levels led us to examine the production of this cytokine in the testis in more detail. Immunoreactive IL-1β was localized to the cytoplasm of small numbers of macrophages and the majority of Leydig cells after LPS-treatment but was not detectable in the normal testis using immunohistochemistry (Fig. 4). The immunopositive macrophages and Leydig cells were readily identified by their highly characteristic nuclear morphologies, as previously described (16). The seminiferous epithelium was negative for IL-1β immunoreactivity in both control and inflamed testes, with the exception of some staining over residual bodies in the LPS-treated testes (data not shown).

Ablation of the Leydig cells by administration of EDS (experiment 4) reduced intratesticular testosterone levels from 473 ± 115 ng/ml in control animals (mean ± SE; n = 6) to 12 ± 6 ng/ml in the Leydig cell-depleted group. This treatment did not significantly affect testicular IL1β mRNA expression or IF protein levels in normal or LPS-treated animals (Fig. 5, A and C). In contrast, EDS-treatment caused a significant decline in IL1α mRNA expression (Fig. 5B). LPS treatment had no effect on IL1α mRNA expression (Fig. 5B). These data implicate a subset of testicular macrophages as the principal source of IL-1β in the inflamed testis and suggest that the contribution from the Leydig cells is comparatively minor.

Measurement of total IL-1 bioactivity in testicular IF, i.e., the secretion of both the α and β forms of IL-1 (Fig. 6), confirmed an earlier study showing that the IF contains significant bioactive IL-1 that can be specifically and completely inhibited by the native IL-1 receptor antagonist (29), but that higher doses of IF become inhibitory in the bioassay (25). However, in apparent contradiction to the small but significant increase in testicular IL1β mRNA after LPS treatment and the relatively large increase in IL1β protein in the IF, there was no corresponding change in the IL-1 bioactivity levels in testis IF in these animals.

Fractionation of testicular IF from LPS-treated rats by size-exclusion high-performance liquid chromatography confirmed that most, if not all, of the IL-1β immunoreactivity in this fluid was associated with a peak that eluted earlier than the processed IL-1β protein and corresponded to the size of the biologically inactive IL-1β precursor (Mr of 30 × 10^3) (Fig. 7B). These data indicate that the large increase in IL-1β immunoreactivity in testicular IF after LPS treatment is due to production and secretion of the precursor molecule, which is not converted subsequently to active IL-1β within the testis.

**DISCUSSION**

This study describes the first quantitative and comparative investigation of both mRNA and protein responses of a panel

### Table 2. Measurement of cytokines by ELISA in the testis and in blood 3 h after injection of saline or LPS

<table>
<thead>
<tr>
<th>Sample</th>
<th>n</th>
<th>IL-1β, ng/ml</th>
<th>Activin A, ng/testis</th>
<th>Total TGF-β1, ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testis IF 0.005</td>
<td>0.063:0.09</td>
<td>9.2 (3.9:4.41)</td>
<td>0.48 (0.44:0.52)</td>
<td>6.4 (0.59:0.69)</td>
</tr>
<tr>
<td>Testis extract</td>
<td>6</td>
<td>0.348±0.100</td>
<td>2.79±0.808**</td>
<td>0.118±0.017</td>
</tr>
<tr>
<td>Plasma</td>
<td>6</td>
<td>0.024±0.007</td>
<td>0.12±0.035**</td>
<td>0.091±0.005</td>
</tr>
</tbody>
</table>

Values are means ± 95% CI (IF only) or means ± SE (testis extract and plasma). Statistical comparisons are between unstimulated (−LPS) and LPS-stimulated (+LPS) values. *P < 0.05; **P < 0.01.
of pro- and anti-inflammatory cytokines to an inflammatory stimulus in the rat testis. The data in the present study establish that, compared with the liver, the adult rat testis constitutively expresses anti-inflammatory cytokines of the TGF-β family (i.e., TGF-β1 and activin A) at elevated levels, even under normal conditions, but has a diminished capacity for production of the two key proinflammatory cytokines, IL-1β and TNF-α. In the case of IL-1β, moreover, there appears to be reduced posttranslational processing of the inactive precursor, which normally occurs at the time of its secretion from the cell (4). This is an entirely novel observation. In contrast, IL-6, a cytokine with both proinflammatory and anti-inflammatory properties, displayed elevated responsiveness to LPS in the testis. Basal and inflammatory expression of the type 2 immunoregulatory cytokine IL-10 mRNA in the testis appears to be comparable to the liver. Expression of the other type 2 cytokine, IL-4 was detectable only in a minor proportion of testicular extracts. Production of these two immunoregulatory cytokines have not been investigated in the testis previously either under normal or inflammatory conditions. Altogether, these data confirm our hypothesis that there is effective immunological deviation of cytokine production in the rat testis, represented by a diminished capacity to produce key proinflammatory cytokines and normal or constitutive expression of several...
immunoregulatory cytokines. This pattern of cytokine production in response to an inflammatory stimulus is consistent both with the relatively limited cellular inflammatory responses observed in the LPS-treated rat testis in vivo (3, 19, 48) and the ability of the rat testis to support grafts for extended periods (17, 26).

Most previous studies on cytokine expression in the testis have been either nonquantitative or semiquantitative. A direct comparison between the levels of expression in the testis with control tissues and serum using both mRNA and protein was undertaken in the present study to assess both synthesis and the biologically relevant concentrations of the key cytokines produced. In the case of IL-1β, it was necessary to measure the bioactivity of the protein as well because the precursor protein is biologically inactive (4). Measurement of cytokine protein levels in the testicular IF provides a more direct indication of their physiological significance relative to receptor binding affinity, which mRNA measurements alone cannot provide. By assessment of the differences in concentrations in whole testis, interstitial fluid, and blood, it is clear that some differential compartmentalization of cytokines exists, consistent with different sites of production and action. Moreover, the relative stability of TGF-β1 and activin A concentrations, even in the face of dramatically changing IF volume in some testis samples, indicates that an effective feedback regulation exists for these two cytokines and that intratesticular levels of these constitutively produced cytokines seem to be tightly controlled even when IF volume changes during inflammation.

Despite obvious structural and functional differences between the testis and liver, the use of the liver as a comparative control in the present study can be justified by the fact that both tissues possess a large resident macrophage population. The volume densities of macrophages in the rat liver and testis, as determined by stereological and other analysis, are similar (6, 35, 68). Although there is some evidence that the liver itself possesses a degree of immunological protection against graft rejection, based on the relative success rates for transplantation of this organ (9), it is nonetheless clear that the proinflammatory response of the testis is severely compromised relative to the liver. Unlike the testis, the macrophages of the liver possesses normal inflammatory activity and can respond to LPS with a strong proinflammatory response (41), a response consistent with other organs such as lung, kidney, and skin.

Fig. 6. IL-1 bioactivity in dilutions of testicular IF from saline-injected control and LPS-treated rats 3 h after treatment. Values are means ± SE of 4 replicate wells. Values with same letter subscript are not significantly different (P > 0.05).

Fig. 7. Fractionation of testicular IF from LPS-treated rats 3 h after treatment and IF from saline-injected control rats spiked with rat recombinant IL-1β by size-exclusion high-performance liquid chromatography. A: ultraviolet profile (A280) of rat IF. Absorbance profiles were identical for both IF samples. Molecular weight marker elution points are indicated: thyroglobulin (Thy; 670 × 10^3), bovine γ-globulin (bGG; 158 × 10^3), ovalbumin (Ova: 44 × 10^3), myoglobin (Myo; 17 × 10^3), and vitamin B12 (VitB; 1,350). B: IL-1β immunoactivity of low-dose LPS stimulated testicular IF and normal rat IF spiked with native IL-1β. Fraction numbers in B are positioned to align with the corresponding time axis in A (as indicated by the vertical broken lines).
Previous in vitro studies have shown that macrophages, which are usually the principal source of IL-1β and TNF-α in other tissues, are deficient in production of these cytokines in the rat testes (25, 32, 33). Data suggest that this deficiency is related to heterogeneity of the testicular macrophage population, which comprises a large, poorly inflammatory resident population and a much smaller population of macrophages with relatively normal inflammatory functions (19, 27, 48, 68). The reduced production of these cytokines in whole testis extracts, as observed in the present study, also may be attributed largely to this deficiency.

Other cellular sources of these cytokines have been reported in the testis previously, most notably the Leydig cells in the case of IL-1β and the developing germ cells for TNF-α (14, 39). In the present study, we observed IL-1β protein expression in a subset of macrophages after LPS treatment. Although IL-1β was also observed in the Leydig cells, the fact that ablation of Leydig cells by EDS did not reduce IL-1β expression in the whole testis clearly indicates macrophages, most likely a subset with normal inflammatory functions (48), as the major source. Using a different antibody, Jonsson and colleagues (31) also detected expression of IL-1β in a subset of testicular macrophages in rat testis, although not in the Leydig cells. A recent study of IL1 family member mRNA expression in the human testis also indicated that IL1β expression is largely confined to the interstitial tissue, at least under normal conditions (57). In contrast, Huleihel and colleagues (30) examined the mouse testis and reported observing IL-1β staining in interstitial cells, including the Leydig cells, but also in spermatogonia, spermatocytes, and Sertoli cells. Whether these observations suggest that the mouse testis is substantially different from the rat and human or the differences are the result of different methods or reagents used is not clear. However, localization of immunoreactive IL-1β in residual bodies was observed in the present study, which may be indicative of very low-level expression in the germ cells, since cytoplasmic proteins tend to become concentrated in the residual bodies. This still requires confirmation, as residual bodies also are a common site of nonspecific binding of antibodies. Ablation of Leydig cells had no effect on IL-1β but did cause a reduction in IL-1α expression. This is a novel observation establishing the importance of Leydig cells or their hormones to maintaining expression of IL-1α, but not IL-1β, in the normal testis.

Despite the apparently low level of IL1β mRNA expression in the testis, physiologically relevant concentrations of IL-1β protein were detected in testicular extracts and IF. Moreover, production and secretion of IL-1β into the testicular IF increased after LPS treatment but occurred without any change in either total IL-1 bioactivity or IL1α mRNA expression. A very similar result has been reported previously (31), and the authors suggested that the lack of alteration in bioactivity might be due to a compensatory increase in antagonists of IL-1, such as the IL-1 receptor antagonist, in the LPS-treated testis. The data in the present study, however, support an alternative explanation. Clearly, LPS induces production of IL-1β precursor and its subsequent secretion into the IF. This precursor, however, is not substantially cleaved to its biologically active form, a process that in other tissues occurs at the time of secretion by the action of IL-1 converting enzyme/caspase-1 (63). Because the precursor is not bioactive, this would account for the elevated levels of IL-1β protein without corresponding changes in bioactivity after LPS-treatment. Thus the data implicate downregulation of both IL1β transcription and the activity of its specific processing enzyme as two distinct mechanisms for reducing the proinflammatory actions of this cytokine in the testis. This is an entirely novel finding and opens up a separate line of inquiry regarding the processing of cytokines as a regulatory mechanism operating in the rat testis. A similar regulation of TGF-β1 also appears to operate in the testis, since large amounts of this cytokine are produced but are not immediately processed into the mature bioactive form.

In contrast to IL1β and TNFα, production of IL6 in the testis during inflammation does not appear to be downregulated. This may be due to the fact that contributions from cells other than macrophages, including both Leydig and Sertoli cells (5, 12, 51, 60), are more important for this cytokine. In fact, we have found that testicular macrophages are relatively poor producers of IL-6 in vitro (Hedger MP, unpublished data). More significant for immunoregulation in the testis, however, is the fact that IL-6 is not a classical proinflammatory cytokine like IL-1 and TNF-α. Although IL-6 displays some proinflammatory actions, it also plays an important immunoregulatory role by inducing acute phase protein production and type 2 T-cell responses and regulating antigen-presenting cell development (10, 46). Most significantly for testis immunoregulation, administration of IL-6 has been shown to be able to reduce the development and severity of autoimmune orchitis in C3H/HeJ mice (38).

Although a role for TGF-β1 in testicular immunoregulation has been suggested previously (29, 55), this is the first demonstration that this cytokine is expressed at constitutively high levels in the normal adult testis. Similar to IL-1β, most TGF-β1 in the testis is present as the latent, inactive precursor form, and active TGF-β1 in the testis was below the detection limit of the assay in the present study. However, it should be noted that TGF-β1 is a very potent immunosuppressive cytokine even at very low picogram per milliliter levels (29, 42). Moreover, in contrast to IL-1β, which is usually processed at the time of its secretion, TGF-β1 is normally secreted as the precursor and is activated at its site of action by local proteases (42). Despite the evidence for a very high level of local TGF-β1 production in the testis, the evidence of a comparatively higher levels of protein in the whole testis reflecting the contribution from the peritubular cells and Sertoli cells, the fact that the levels of the TGF-β1 precursor in the testicular IF were almost identical to circulating levels also suggest a close relationship between concentrations of this cytokine in the testis and the blood.

Activin A has profound inhibitory effects on T- and B-cell function and both IL-1- and IL-6-mediated inflammatory responses (53). The increase of activin A levels in the circulation after LPS treatment in adult rats confirms earlier observations in mice and sheep (53). Unlike TGF-β1, activin A is produced by the Sertoli cells and possibly the peritubular cells in the adult testis (15). Its presence at levels in the IF in excess of those found in the blood, even during inflammation, suggests a relatively high constitutive level of expression in the normal testis. Together, the high levels of production of activin A and TGF-β1 in the adult testis strongly suggest a role for both of these cytokines in local immunoregulation. This hypothesis is given support by several of the observations of the present study, most notably because inhibition of IL-1 converting...
enzyme/caspase-1 activity is one of the known anti-inflammatory actions of activin A (50). The fact that both cytokines failed to show significant upregulation after LPS treatment in the testis might be due to the possibility that expression of these cytokines is at a maximum level even under normal conditions. The relative distribution of TGF-β1 and activin A in the testicular IF relative to the whole testis also suggests that there is some differential compartmentalization of the activity and functions of the two cytokines.

In summary, it appears that the on-going suppression of inflammatory and immune responses in the testis contributes to reduced proinflammatory cytokine production and constitutive expression of immunoregulatory cytokines of the TGF-β family. On the other hand, production of IL-10 appears normal and also may contribute substantially to testicular immunoregulation. IL-4 was not detectable, although highly localized interactions between immune cells in the interstitium mediated by this cytokine cannot be excluded. It remains to be determined which factors within the testicular environment are responsible for directing this unique pattern of cytokine responses in the adult testis. These cytokine responses not only have implications for understanding extended graft survival and protection of the developing germ cells from autoimmune attack but also may play a role in the susceptibility to, and progression of, infections and tumors in the testis.

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