

Secretion of Bioactive Interleukin 1 by Rat Testicular Macrophages *In Vitro*

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ABSTRACT: The relative capacities for testicular macrophages and resident peritoneal macrophages to secrete the pro-inflammatory cytokine, interleukin 1 (IL-1), in response to stimulation by bacterial lipopolysaccharide (LPS), were compared *in vitro*. Macrophages were isolated from adult male rat testicular interstitial cells or peritoneal lavage by adherence to glass coverslips or plastic culture dishes. The macrophages were immediately cultured, with or without a maximal dose of LPS (1 µg/ml), over 24 hours at 32°C. Bioactive IL-1 production was measured by a sensitive thymocyte proliferation bioassay, employing recombinant human IL-1β as the reference standard. In comparison with the peritoneal macrophages, testicular macrophages displayed only a very small response to LPS, producing 2.8% of the amount of IL-1 per cell secreted by peritoneal macrophages cultured under identical conditions. Production of authentic

IL-1 was confirmed by inhibition of the bioassay response in the presence of human recombinant IL-1 receptor antagonist. A small molecular mass (<10 kDa based on ultrafiltration) inhibitor of IL-1 bioactivity was also present in the medium collected from both cultures, but this inhibitory activity did not account for the differences in activity observed. In cultures of total peritoneal cells under similar conditions, addition of testosterone (10–1,000 ng/ml) did not affect IL-1 production in response to LPS. These data indicate that testicular macrophages have a reduced ability to secrete bioactive IL-1, and they provide further evidence for an altered capacity for immune responses within the testis.

Key words: Cytokines, bioassay, immunoregulation, IL-1 receptor antagonist, testosterone.

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Macrophages have the capacity to promote immune responses by antigen presentation and costimulation of specific T cells, and by production of cytokines (Weaver and Unanue, 1990). Resident macrophages comprise a substantial cellular population of the testicular interstitial tissue of many species, including rodent species and humans (Miller, 1982; El-Demiry et al, 1987; Wang et al, 1994). The structural, phagocytic, and bactericidal functions of the testicular macrophages appear to be normal (Yee and Hutson, 1983; Miller et al, 1984; Wei et al, 1988), and these cells express the major histocompatibility complex class II (MHC II) molecules involved in immune recognition (Head and Billingham, 1985; Hedger and Eddy, 1987; Pöllänen and Niemi, 1987). Consequently, it is surprising that the testes of several laboratory rodents are “privileged” sites for tissue grafts (Ferguson and Scothorne, 1977; Bobzien et al, 1983; Head and Billingham, 1985).

Macrophages have the potential for dual regulation of

immune responses, secreting either pro-inflammatory cytokines, principally interleukin 1 (IL-1) and tumor necrosis factor-α (TNFα), or anti-inflammatory factors, which include transforming growth factor-β (TGFβ) and IL-1 receptor antagonist (IL-1ra) (Rutherford et al, 1993; Paul and Seder, 1994). Although isolated rat and mouse testicular macrophages were recently shown to synthesize and secrete bioactive TNFα in response to stimulation by bacterial lipopolysaccharide (LPS) *in vitro* (Hutson, 1993; Xiong and Hales, 1993; Moore and Hutson, 1994), in general, the secreted cytokine profile of the resident population of testicular macrophages has received relatively little attention.

IL-1 plays a central role in inflammation, promoting the influx of inflammatory cells into tissues and coordinating the proliferation and activation of T cells, B cells, and monocytes (Dinarello, 1994). There are two distinct forms of IL-1, both single-chain glycosylated 17-kDa proteins (IL-1α and IL-1β), with extensive homology and similar bioactivities. Although IL-1 is characteristically produced by stimulated monocytes and macrophages, which secrete principally IL-1β (March et al, 1985), both IL-1 forms are produced by a range of cell types. In the adult rat testis, IL-1α is secreted by Sertoli cells (Gérard et al, 1991), and it has been shown to have direct inhibitory actions on spermatogonial development and Leydig cell steroidogenesis *in vitro* (Calkins et al, 1990; Söder et

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al, 1991; Hales, 1992). In a previous study, macrophages collected from the testes of mice treated systemically with LPS, and isolated macrophages from untreated animals cultured for 24 hours prior to stimulation with LPS, were shown to produce IL-1 β mRNA (Hales et al, 1992). However, the activity of freshly isolated resident macrophages from untreated animals was not examined. Moreover, it has been shown that the ability to produce IL-1 mRNA does not necessarily correlate with the capacity to secrete biologically active IL-1 (Schindler et al, 1990; Herzyk et al, 1992). The principal aim of the present study was to explore the capacity of freshly isolated resident testicular macrophages to secrete bioactive IL-1 *in vitro*.

Materials and Methods

Animals

Adult (70- to 110-day-old) male outbred Sprague-Dawley rats, and adult (50- to 80-day-old) inbred C3H/HeJ mice were obtained from the Monash Central Animal House, Clayton, Victoria, Australia.

Macrophage Isolation and Culture

Testicular interstitial cells were isolated from decapsulated rat testes, by collagenase dispersal (0.25 mg/ml) in a shaking water bath (20 minutes, 80 cycles/minute, 34°C) (Hedger et al, 1990), or by gentle forceps dissection of the interstitial tissue from the tubules (Hedger and Eddy, 1986), in the absence of enzymes. Peritoneal cells were collected by lavage (Meltzer, 1981). In one experiment, the peritoneal lavage was separated into two equivalent fractions, and one fraction was incubated with collagenase at a final concentration of 0.25 mg/ml in a shaking water bath, as described above for testicular interstitial cells.

Testicular and peritoneal cells were diluted in Dulbecco's modified Eagle's medium (DMEM): Ham's F12 (1:1) to provide optimal cell attachment conditions (5.0×10^6 interstitial cells/ml and 2.0×10^6 peritoneal cells/ml, respectively). Macrophages were isolated by differential adherence to either 14-mm siliconized glass coverslips or 15-mm or 35-mm plastic culture wells (Costar, Cambridge, Massachusetts) in a final volume of 250–300 μ l/cm² at 32°C or 37°C over 30 minutes, followed by washing with DMEM:F12 (five times) to remove nonadherent cells, as previously described (He et al, 1991). The glass coverslips with attached cells were inserted into 15-mm culture wells prior to culture.

Testicular and peritoneal macrophages were cultured (32°C, 24 hours, 5% CO₂/95% air) in DMEM:F12 containing 0.01% "low-endotoxin" bovine serum albumin (BSA; Sigma Chemical Company, St. Louis, Missouri or ScimaR, Melbourne, Australia) with or without LPS (1 μ g/ml; Sigma). The final incubation volumes were 1.0 ml (15-mm well) and 3.0 ml (35-mm wells), respectively. At the end of the culture, medium samples were collected and, unless otherwise indicated, dialyzed (molecular weight cut-off 3,500) against three changes of DMEM alone (4°C, 24 hours). In some experiments, the culture medium was char-

coal-extracted (10 mg/ml, 4°C), as previously described (Hedger et al, 1990). Pyrogen-free water (Modulab LS pyrogen-free water system, Liquipure, Oxon, U.K.) and glassware was used in all macrophage cultures.

In each experiment, adherent cells were identified by Giemsa-May-Grünwald staining of at least two additional coverslips or culture wells prior to culture and/or after culture. Macrophages and Leydig cells were identified by combined esterase/3 β -hydroxysteroid dehydrogenase (3 β -HSD) staining of cells without air-drying, as previously described (He et al, 1991). The numbers of total cells, esterase-positive, and 3 β -HSD-positive cells per coverslip or culture well were calculated from the average number of cells per field observed in 10 randomly selected fields of view in each sample well, at a magnification of 400 \times , using an Olympus Microscope (Sun et al, 1993).

Leydig Cell Culture

Conditioned Leydig cell culture medium was collected from cultures (24 hours, 32°C) of adult rat Leydig cells (50,000 cells/0.5 ml in 15-mm culture wells) without hormonal stimulation or lipoproteins, as previously described (Hedger et al, 1990).

Peritoneal Cell Culture

Total peritoneal cells were cultured with LPS (1 μ g/ml) in 15-mm plastic culture wells (100,000 cells/well) for 24 hours at 32°C, in the absence or presence of testosterone (10–1,000 ng/ml). At the end of the culture, the medium was collected, centrifuged (1,000 \times g, 10 minutes) to remove cells, and dialyzed overnight as above.

Phagocytosis Assay

Testicular and peritoneal macrophages were cultured (0–60 minutes) at 37°C or at 0°C (on ice) in DMEM:F12 containing 0.005% 0.8- μ m-diameter polystyrene beads (Sigma) and 0.1% BSA in a final volume of 500 μ l (5% CO₂/95% air). At several time points (5, 10, 15, 30, and 60 minutes), the incubation medium was removed (three replicates at each incubation temperature and time point), and the wells were rinsed with DMEM:F12 (three times) and 0.9% saline (two times). The cells were counterstained with toluidine blue, and the number of beads found over each cell profile at each time point was determined from a random sample of 20 cells per well using an Olympus microscope. An individual cell was determined to be actively phagocytic when the cell profile at 60 minutes of culture (37°C) displayed more than the average number of beads found at the same time in cultures at 0°C (i.e., due to nonspecific adherence to the cell surface). The rate of bead incorporation by both macrophage populations was approximately linear over 0–30 minutes at 37°C, and this rate was used as an index of relative phagocytic activity.

Thymocyte IL-1 Bioassay

All medium samples were assayed for IL-1 bioactivity using a standard mouse thymocyte proliferation bioassay that shows similar cross-reactivity with IL-1 α and IL-1 β (Mizel et al, 1978), with minor modifications. In order to reduce adherent, nonlymphocytic thymic cell contamination in the dispersed thymocyte preparations, the isolated thymocytes were incubated in 35-mm plastic culture dishes (12×10^6 cells/ml in 3.0 ml) for 30 minutes

(37°C, 5% CO₂/95% air), and the culture supernatants containing the nonadherent thymocytes were collected. The thymocyte-enriched fractions (1.0–1.2 × 10⁶/well) were cultured in DMEM (37°C, 72 hours) in a final volume of 250 μl/well in 96-well culture plates (Costar) with 1 μg/ml phytohemagglutinin (PHA; Sigma) and human recombinant IL-1β standard (Boehringer-Mannheim, Mannheim, Germany) or samples diluted with DMEM and 0.01% BSA. Cultures were supplemented with 2.5% fetal calf serum (FCS; P and D Biologicals, Sydney, Australia) and β-mercaptoethanol (25 μM). At the end of the first incubation period, [³H]thymidine (0.5 μCi/50 μl, 6.7 Ci/mmol) was added to each well, and the cells were incubated a further 16–20 hours at 37°C. The cells were harvested onto glass filters using an automated cell harvester (Packard Micromate 196; Packard Instrument Company, Downers Grove, Illinois) and counted by liquid scintillation in a β-counter (Packard 1900 TR).

In order to obtain relative IL-1 bioactivity estimates, human (h)IL-1β standard (0.06–2 U/ml) and serial 1:2 dilutions of the culture medium samples were assayed in quadruplicate (100-μl standard or sample dilution per assay well), and the log-log transformed dose-response curves were compared using parallel-line bioassay statistics (Finney, 1964). Values are expressed as hIL-1 equivalent bioactivity units, determined against the National Institute for Biological Standards and Control (NIBSC) hIL-1β international standard 86/680 (Poole and Gaines-Das, 1991), because a suitable rat IL-1 standard was not available.

All samples from a single macrophage culture experiment were assayed in a single thymocyte bioassay. The bioassay had an average sensitivity, defined as the minimum detectable concentration in a 100-μl sample, of 0.11 ± 0.07 U/ml hIL-1β, with an average intraassay variation of 30.2 ± 13.5% (mean ± standard deviation [SD]; n = 18 assays). Thymocyte proliferation *in vitro* and the bioassay sensitivity were found to be optimal at a FCS concentration of 1.0–2.5%; in particular, increasing the bioassay FCS concentration from 2.5% to 5% resulted in a corresponding 8- to 16-fold reduction in assay sensitivity.

Dialysis reduced testosterone (and presumably other small molecular weight factors) in the macrophage culture medium below detectable limits (Table 1), but it had no effect on IL-1 bioactivity; recoveries of IL-1 bioactivity after dialysis were 96% (65–139%) and 82% (44–123%) for the peritoneal and testicular macrophage medium samples, respectively (mean and 95% confidence limits). In contrast, charcoal extraction completely removed both testosterone and IL-1 bioactivity (data not shown). Addition of testosterone to the thymocyte cultures at concentrations up to 10 times higher than those found in the testis (10–1,000 ng/ml) had no significant effect on [³H]thymidine incorporation by PHA-stimulated thymocytes in either the presence or absence of IL-1 (data not shown).

Bioassays for IL-1 using primary cell cultures or cell lines are susceptible to cross-reaction with other cytokines with shared biological activities, including interleukin 2 (IL-2), interleukin 6 (IL-6), and TNFα (Dinarello, 1994). In some experiments, human recombinant IL-1ra (a generous gift of Synergen, Boulder, Colorado) was added to the bioassay wells at a concentration (10 μg/ml) that completely inhibited the bioactivity of the IL-1β standard at all assay doses. This protein competes with IL-1α and IL-1β for the IL-1 receptors, but it has no endogenous IL-1-like activity of its own (Arend, 1991).

Table 1. Testosterone levels in macrophage and Leydig cell culture medium (20 hours) before and after dialysis

Culture medium	Testosterone (ng/ml)	
	Untreated	Dialyzed
Peritoneal macrophages	<0.1	<0.1
Testicular macrophages	0.2	<0.1
Leydig cells	7.7	0.8

Values are result of a single experiment assayed in the same RIA.

Endotoxin Assay

Samples and reagents were assessed for endotoxin (i.e., endogenous LPS) contamination using the Sigma E-toxate *Limulus* amoebocyte lysate (LAL) assay kit (Tomasulo et al, 1977).

Ultrafiltration Studies

Dialyzed macrophage culture medium was fractionated by ultrafiltration using Centricon-10 (molecular mass cutoff 10 kDa) units (Amicon, Danvers, Massachusetts) at 5,000 × g until <10% of the starting volume remained. The supernatant (large molecular weight) fraction was reconstituted to the original volume with DMEM, and both the supernatant and filtrate (small molecular weight) fractions were assayed for IL-1 activity.

Testosterone Radioimmunoassay (RIA)

Medium samples were assayed for testosterone content by ¹²⁵I-labeled testosterone RIA, as previously described (Kerr et al, 1985).

Statistical Analyses

Comparisons between individual values were made by paired *t*-test, sign test, or by Peritz's multiple range test (Harper, 1984).

Results

Macrophage Culture Characteristics

Comparison of isolation conditions indicated that cell attachment was similar for enzymatically or mechanically dispersed testicular interstitial cells when similar numbers of cells were plated (Fig. 1). Culture-to-culture variation in attached cell recoveries was very high (coefficient of variation 80%), but overall, cells attached three times more efficiently to the plastic culture dishes than to the glass substrate. The attached cells in the plastic culture dishes were 93.3 ± 9.9% esterase-positive cells with 2.4 ± 1.0% 3β-HSD-positive Leydig cells (mean ± standard error of the mean [SEM], n = 6 cultures), indicating a macrophage purity (esterase-positive cells – 3βHSD-positive cells) of >90%. The minor Leydig cell contamination was consistent with the presence of low levels of testosterone detected in testicular macrophage medium (Table 1). Peritoneal cells attached approximately four times more efficiently than testicular macrophages to the plastic sub-

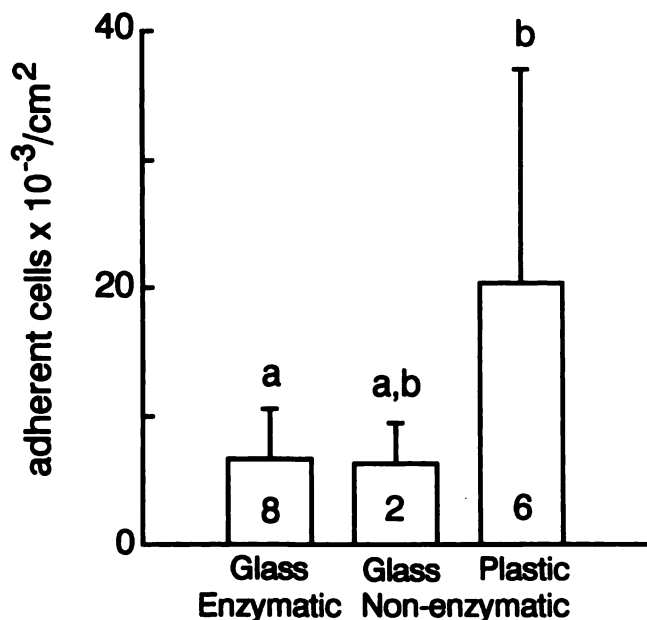


FIG. 1. Attachment of collagenase-dispersed (enzymatic) and mechanically dispersed (nonenzymatic) testicular interstitial macrophages to siliconized glass and plastic substrates after 30 minutes at 32°C. Values are mean \pm SD (n = number of isolations performed under identical conditions). Values with the same letter superscript are not significantly different ($P < 0.05$).

strate ($109,000 \pm 23,700$ attached cells/cm²; mean \pm SEM, $n = 5$ cultures), producing macrophage preparations of comparable purity ($87.3 \pm 14.7\%$ esterase-positive).

The purity of both macrophage populations, as determined by esterase staining, was confirmed by the assay for phagocytic activity. Both macrophage preparations contained similar proportions of phagocytically active cells ($91.0 \pm 1.4\%$ and $90.0 \pm 3.5\%$ for testicular and peritoneal macrophages, respectively [mean \pm SEM, $n = 3$ experiments]), possessing similar phagocytic activities *in vitro*: 8.8 ± 1.1 particles/cell/30 minutes and 8.1 ± 0.8 particles/cell/30 minutes, respectively.

The greater attachment rate of the peritoneal macrophages may be attributable to the smaller number of contaminating cells in the peritoneal lavage compared to that of the testicular interstitial cell preparation. Similar recovery and purity results were obtained with either 15-mm- or 35-mm-diameter culture dishes. In the following studies on the secretion of bioactive IL-1, mechanical separation of testicular interstitial cells and differential attachment to plastic substrate, both of which consistently provided the greatest cell recovery, were employed to isolate testicular macrophages.

Secretion of IL-1 Bioactivity in Response to LPS

LPS-stimulated peritoneal and testicular macrophage culture medium induced a dose-dependent stimulation of

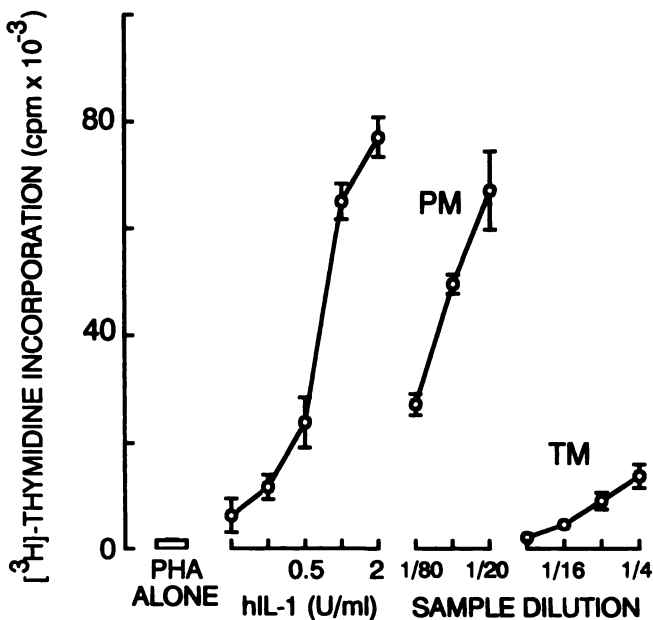


FIG. 2. IL-1 bioactivity in macrophage culture medium. Representative incorporation of [³H]thymidine by mouse thymocytes in response to PHA (PHA alone), in the presence of hIL-1 β standard (0.125–2 U/ml), and serial dilutions of medium from 24-hour cultures of LPS-stimulated peritoneal (PM) or testicular (TM) macrophages. Values are mean \pm SEM, $n = 4$ replicates.

thymocyte proliferation, that was parallel with the hIL-1 β standard following log-log dose-response transformation (Fig. 2). Dose-response studies established that the dose of LPS used ($1 \mu\text{g/ml}$) provided maximal secretion of IL-1 by peritoneal macrophages *in vitro*. However, the level of IL-1 bioactivity equivalents per cell secreted by testicular macrophages in the presence of the maximally stimulating dose of LPS over 24 hours at 32°C was only 2.8% of that secreted by peritoneal macrophages cultured under the same conditions (Table 2). By comparison, collagenase treatment and mechanical disruption in a shaking water bath did not significantly affect the secretion of bioactive IL-1 by LPS-stimulated peritoneal macrophages *in vitro*: 587 (355:1,180) U/10⁶ cells in the untreated group compared with 499 (306:956) U/10⁶ cells in the treatment group (mean \pm 95% confidence intervals in a single experiment).

In the absence of added LPS, bioactive IL-1 was either undetectable in testicular macrophage culture medium ($n = 3$ cultures) or just detectable ($n = 4$ cultures) (Fig. 3; Table 2). In these cultures, LPS significantly stimulated IL-1 bioactivity. However, in three additional testicular macrophage cultures, both basal and LPS-stimulated testicular macrophage culture medium significantly inhibited thymocyte proliferation (data not shown), and these cultures were removed from the final analysis.

Bioactive IL-1 was detectable in unstimulated peritoneal macrophage cultures, and addition of LPS gave a

Table 2. IL-1 bioactivity in testicular and peritoneal macrophage cultures (values are mean ± SEM)

Culture	Number	IL-1 (U/10 ⁶ cells)
Testicular	no LPS	4* 13.2 ± 4.7
	LPS	7 17.2 ± 3.7†
Peritoneal	no LPS	5 144 ± 20
	LPS	5 609 ± 133†

Comparisons are between unstimulated and LPS-stimulated responses using the sign test: † P < 0.001.

* IL-1 was undetectable in three of the seven cultures analyzed.

further fourfold stimulation of activity (Table 2). These cultures contained no detectable endogenous endotoxin by LAL assay; however, culture medium containing higher concentrations (0.1–1.0%) of “low-endotoxin” BSA from the two independent sources used in this study did give a positive endotoxin result. Significantly, basal peritoneal macrophage IL-1 secretion was substantially reduced by not adding carrier BSA to the culture, resulting in an LPS-stimulation factor of 20.9 (10.4:54.2) (mean ± 95% confidence limits in a single experiment).

Characterization of Activities

Human recombinant IL-1ra (10 µg/ml) inhibited the action of both peritoneal and testicular macrophage culture medium on thymocyte proliferation *in vitro* (Fig. 4). Moreover, IL-1ra significantly inhibited PHA-stimulated

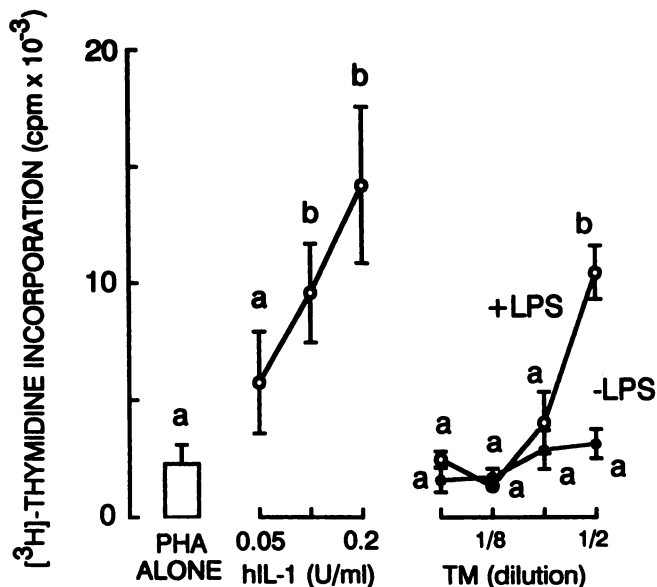


FIG. 3. Incorporation of [³H]thymidine by mouse thymocytes in response to PHA (PHA alone), in the presence of hIL-1β standard, and serial dilutions of medium from 24-hour cultures of testicular (TM) macrophages cultured in the absence (-LPS) or presence (+LPS) of LPS (1 µg/ml). Values are mean ± SEM, n = 4 replicates. Values with the same letter superscript are not significantly different (P < 0.05).

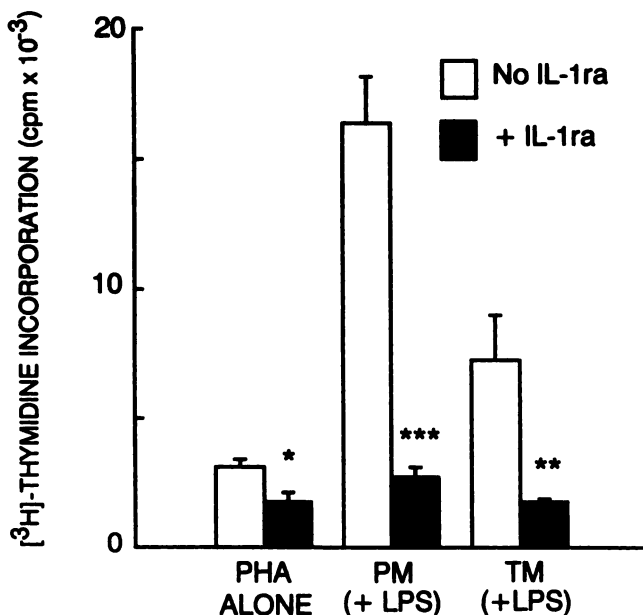


FIG. 4. Effect of interleukin 1 receptor antagonist (IL-1ra; 10 µg/ml) on [³H]thymidine incorporation by isolated thymocytes in the presence of PHA alone or in the presence of medium (100 µl/assay well) from 24-hour cultures of LPS-stimulated peritoneal macrophage medium (PM) diluted 1:10 or testicular (TM) macrophage medium assayed without dilution. Values are mean ± SEM, n = 4 replicates. Comparisons are between responses in the presence or absence of IL-1ra: ***P < 0.001; **P < 0.01; *P < 0.05.

thymocyte proliferation by between 36 and 85% (n = 4 experiments), indicating the presence of exogenous IL-1 in the bioassay. This low level of IL-1 may have been due to production of low levels of IL-1 by the thymus cells, or introduction of IL-1 from the BSA or FCS preparations added to the culture.

Addition of LPS-stimulated testicular macrophage medium had no inhibitory effect on the activity of medium collected from LPS-stimulated peritoneal macrophages in the IL-1 bioassay, and it actually caused a slight increase in activity at higher concentrations (Fig. 5). However, following fractionation by ultrafiltration (10-kDa molecular mass cutoff), an inhibitory activity was demonstrated in the small molecular weight fraction of both testicular and peritoneal macrophage culture medium samples (Fig. 6). Separation of this activity increased the bioactivity of the larger molecular weight fraction of the culture medium in all samples, but it did not equalize activity in testicular and peritoneal cultures. The small molecular weight fraction of the testicular macrophage culture medium did not completely inhibit PHA-stimulated proliferation, and it had no additional effect in the presence of a maximal dose of IL-1ra (Fig. 7). The fact that the inhibitor at higher doses could not completely inhibit proliferative activity indicates that the inhibition was not the result of non-specific cytotoxicity.

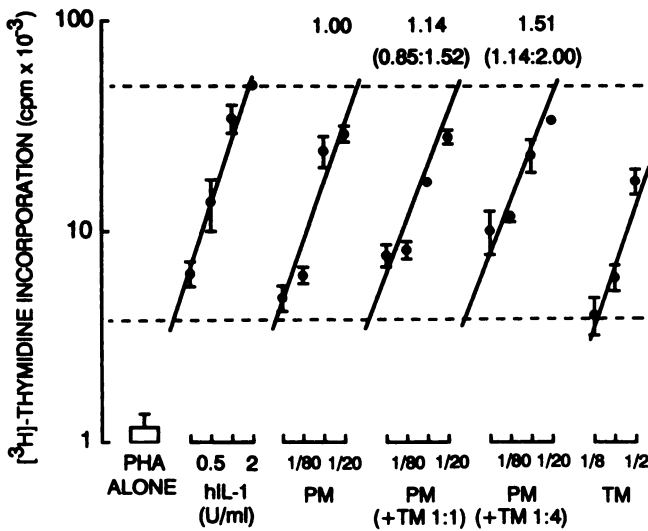


FIG. 5. Bioassay log-log dose-response curves for hIL-1 β , LPS-stimulated peritoneal macrophage conditioned medium alone (PM), LPS-stimulated testicular macrophages alone (TM), an equal volume of peritoneal and testicular macrophage medium (1:1), and peritoneal and testicular macrophage medium volumes representing an equivalent number of peritoneal and testicular macrophages (1:4). The peritoneal macrophage medium dose range is identical in all wells containing peritoneal macrophage medium, and the volume of testicular macrophage medium is proportional to the volume of peritoneal macrophage medium in each well, as indicated (1:1 or 1:4). Values are mean \pm SEM, $n = 4$ replicates. Values above the PM curves are relative activity and 95% confidence limits with respect to the peritoneal macrophage medium. -----, upper and lower limits of the bioassay region for determination of relative activities.

Effects of Testosterone on IL-1 Secretion by Peritoneal Macrophages

Testosterone (10–1,000 ng/ml) had no effect on the production of IL-1 by LPS-stimulated peritoneal cells cultured over 24 hours at 32°C (Fig. 8).

Discussion

This study demonstrates that freshly isolated testicular macrophages secrete a lymphocyte-activating activity that is responsive to LPS stimulation and is blocked by IL-1ra, a naturally occurring antagonist that specifically inhibits both IL-1 α and IL-1 β (Arend, 1991). However, the levels of IL-1 secreted by the testicular macrophages were considerably reduced compared with a representative population of resident macrophages from the peritoneum, cultured under identical conditions. Although both peritoneal and testicular macrophages co-secreted inhibitors of lymphocyte proliferation, this inhibitory activity could not account for the observed differences in bioactive IL-1 secretion. The molecular size characteristics of the inhibitory activity suggest that it was not due to TGF β , IL-1ra, or smaller factors such as steroids that would be removed

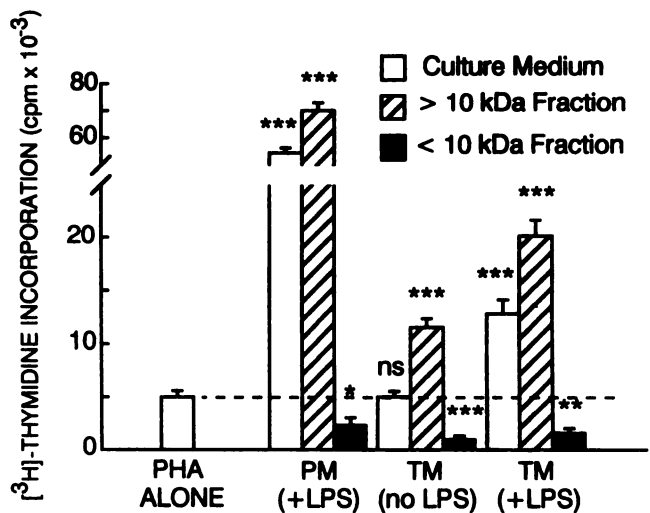


FIG. 6. Ultrafiltration fractionation of macrophage culture medium (10-kDa molecular mass cutoff). Incorporation of [3 H]thymidine by mouse thymocytes in response to PHA (PHA alone), in the presence of untreated medium (\square), and ultrafiltration supernatant fraction (\square) and filtrate fraction (\blacksquare) of peritoneal macrophages cultured for 24 hours in the presence of LPS (PM+LPS), and testicular macrophages cultured in the absence (TM–LPS) or presence (TM+LPS) of LPS stimulation. All PM samples were diluted 1:10 for assay, and TM samples were undiluted (100 μ l/assay well). Comparisons are with the PHA-stimulation control (PHA alone): *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; ns not significantly different ($P > 0.05$).

by dialysis. The presence of this factor(s) may explain why some testicular macrophage preparations were actually inhibitory in the bioassay. These observations are consistent with the phenomenon of prolonged graft survival in the rodent testis (Ferguson and Scothorne, 1977; Head and Billingham, 1985) and *in vivo* data that testicular macrophages do not participate in, and may actually oppose, the intratesticular inflammatory response to hyperstimulation with luteinizing hormone (LH)/human chorionic gonadotropin (hCG) (Bergh et al, 1993).

Earlier studies have reported the presence of IL-1 mRNA in macrophages isolated from the testes of LPS-treated mice, or in mouse testicular macrophages after 24-hour pre-culture in serum (Hales et al, 1992). The significance of the data in the previous study for the resident macrophages would have been obscured by an influx of circulating monocytes in the LPS-treated animals and alterations in endogenous activity during the pre-culture period in the presence of serum. More significantly, mRNA studies do not indicate secretion levels, and several cell types produce IL-1 mRNA but are incapable of its secretion (Schindler et al, 1990).

The reduced ability of testicular macrophages to secrete IL-1 cannot be attributed to a difference in cell viability, because both macrophage populations attached to the culture substrate with comparable efficiency and displayed identical phagocytic activity *in vitro*. Moreover, exami-

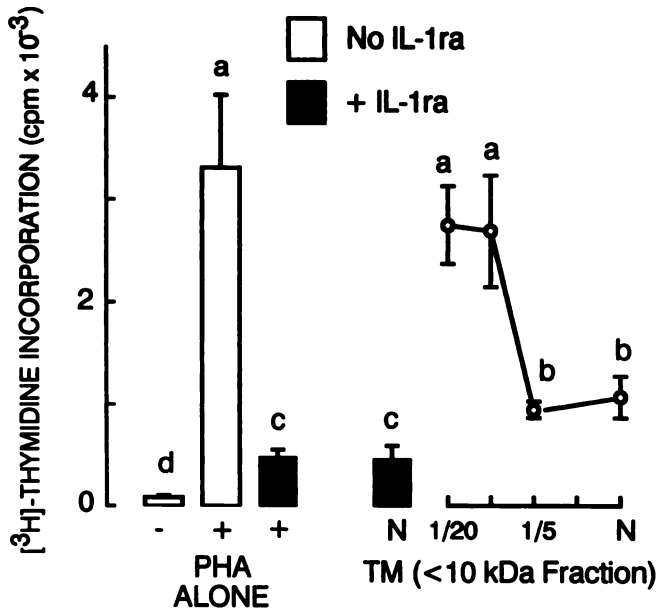


FIG. 7. Incorporation of $[^3\text{H}]$ thymidine by isolated thymocytes in the absence of stimulation (-) and in response to PHA (PHA alone) in the absence (□) or presence of (■) IL-1ra (10 $\mu\text{g}/\text{ml}$); PHA-stimulated $[^3\text{H}]$ thymidine incorporation in the presence of the small molecular mass ultrafiltration fraction (<10 kDa) of LPS-stimulated testicular macrophage culture medium in the absence (○—○) and presence (■) of IL-1ra (10 $\mu\text{g}/\text{ml}$). Values are mean \pm SEM, $n = 4$ replicates. Values with same letter superscript are not significantly different ($P < 0.05$).

nation of the cells before and after culture provided no visual evidence of increased cell death in the testicular macrophage cultures. Finally, mechanical agitation and enzyme treatment had no effect on the secretory activity of isolated peritoneal macrophages collected by lavage.

Resident alveolar and peritoneal macrophages undergo a maturational loss of the ability to secrete IL-1, in comparison with monocytes, due to slower export and conversion of the inactive precursor (Wewers and Herzyk, 1989; Herzyk et al, 1992), and the majority of the testicular macrophages appear to be resident (Miller, 1982; Wang et al, 1994). However, the comparison with resident peritoneal macrophages indicated that there are additional inhibitory influences acting on the testicular macrophages. It is possible that extended exposure of the macrophages to the lower temperature and/or high levels of androgens of the testicular environment may be responsible (Li et al, 1993), although the present studies did not indicate any acute effect of either reduced temperature or physiological doses of testosterone on the ability of the peritoneal macrophages to secrete IL-1 *in vitro*. Alternatively, locally produced cytokines and other regulatory factors may be responsible. Leydig cells appear to regulate macrophage traffic in the testis (Raburn et al, 1993; Wang et al, 1994), and they may also have a direct effect on the inflammatory function of these cells. Although an expla-

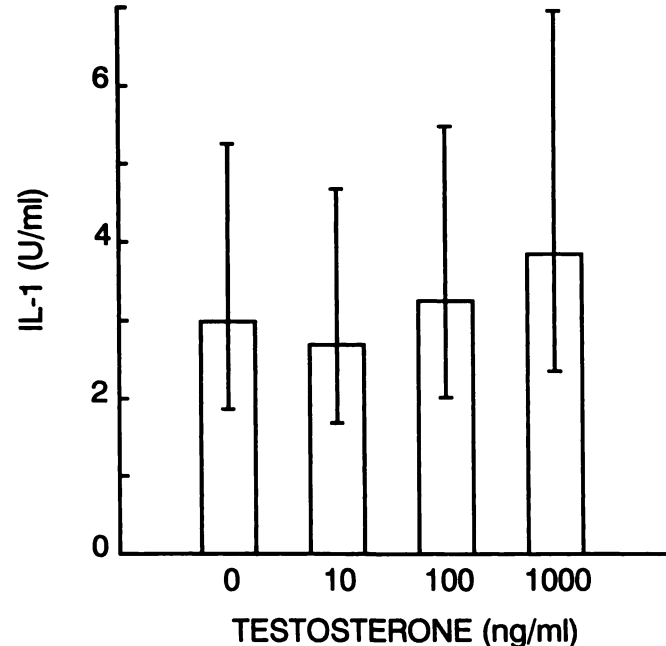


FIG. 8. Effect of testosterone (10–1,000 ng/ml) on IL-1 secretion by peritoneal cells *in vitro* (24 hours at 32°C). Values are mean and 95% confidence intervals for four pooled replicate culture medium samples, assayed by thymocyte *in vitro* bioassay.

nation for the deficiency in the ability of the testicular macrophages to secrete bioactive IL-1 remains to be established, there is evidence in the case of $\text{TNF}\alpha$ that this may not be a general phenomenon affecting all inflammatory cytokines (Hutson, 1993; Moore and Hutson, 1994).

Resting monocytes and macrophages normally do not express IL-1 (Dinarello, 1994). A recent study has suggested that treatment of macrophages with collagenase, the established method for isolation of testicular interstitial cells, including macrophages, may act as a nonspecific stimulus of cytokine release *in vitro* (Moore and Hutson, 1994). In the present study, physical dissection of the interstitial tissue without enzymes by modification of a previously published method (Hedger and Eddy, 1986) provided a suitable alternative approach. However, detectable IL-1 still was present in most samples of medium from peritoneal and testicular macrophages cultured in the absence of added LPS. This observation was consistent with reports that attachment of cells to glass or plastic substrate induces IL-1 mRNA and IL-1 secretion by monocytes and peritoneal macrophages (Fuhlbrigge et al, 1987; Burchett et al, 1988; Schindler et al, 1990). An additional possibility may be the presence of endogenous LPS in the reagents used. So-called "low-endotoxin" BSA from different sources, including those used in this study, contains detectable endotoxin by LAL assay, and there is evidence that macrophages actually may be even more

sensitive to endotoxin than the LAL assay itself (Schindler and Dinarello, 1990). Taken together, these data suggest that it is likely to be impossible to establish resting levels of secretion of IL-1 by the testicular macrophages using any *in vitro* approach.

Several technical modifications to the thymocyte bioassay described in this study were considered essential to the measurement of the very low levels of IL-1 secreted by testicular macrophages. The sensitivity of the bioassay was increased by reducing the FCS concentration, consistent with previous reports that FCS contains a potent inhibitor of lymphocyte proliferation *in vitro* (McKenzie et al, 1990). Second, the adherent thymic epithelial cells, which are the primary androgen-sensitive cells in the thymus (Sasson and Mayer, 1981), were depleted prior to plating of the thymocyte cultures. Finally, all medium samples were dialyzed, effectively removing steroids as well as other small molecular weight factors, including polyamines, that could inhibit lymphocyte growth (Allen and Roberts, 1986).

In conclusion, the reduced responsiveness of the testicular macrophage to stimulation of this key inflammatory cytokine indicates that, although the bactericidal/phagocytic activities of testicular macrophages are retained, the specific leukocyte-enhancing activities of these cells are down-regulated. This inhibition may be important from a physiological or homeostatic point of view, because IL-1 has additional actions within this tissue on spermatogenesis and Leydig cell function that may compromise testicular function during inflammation (Calkins et al, 1990; Söder et al, 1991; Hales, 1992). In addition, IL-1 secreted at very low levels by the resident macrophages may be involved in more discrete local actions within the interstitium, consistent with an intratesticular autocrine, rather than inflammatory, role for this cytokine. Nevertheless, it is important to note that monocyte-derived or macrophage-derived IL-1 β production is induced within the testis under appropriate conditions, such as following systemic administration of LPS (Hales et al, 1992), and this remains a potential mechanism for the down-regulation of fertility and androgen secretion that accompanies testicular inflammation and chronic disease (Adamopoulos et al, 1978).

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