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# Local Regulation of Macrophage Subsets in the Adult Rat Testis: Examination of the Roles of the Seminiferous Tubules, Testosterone, and Macrophage-Migration Inhibitory Factor[1]

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## ABSTRACT

**In the adult rat testis, macrophages belong to one of two subsets differentiated by expression or lack of expression of the resident macrophage surface antigen recognized by monoclonal antibody ED2. Local regulation of the testicular macrophage subsets was investigated in normal and 4-wk experimentally cryptorchid adult rats with and without s.c. testosterone implants (T-implants). Macrophage subsets ED2$^+$ (resident-type) and ED2$^-$ (monocyte-like) were identified immunohistochemically and counted in perfusion-fixed frozen testis sections. Depletion of the spermatogenic cells by cryptorchidism had no effect on testicular macrophage numbers. Inhibition of Leydig cell and seminiferous tubule function by low-dose (3 cm) T-implants caused a 40% reduction in ED2$^+$ resident macrophages in both scrotal and abdominal testes. High-dose (24 cm) T-implants, which inhibit Leydig cell function while maintaining normal seminiferous tubule function, also reduced the number of resident macrophages by approximately 40%, although this reduction was at least partially prevented in the abdominal testes. In the scrotal testis only, the ED2$^-$ monocyte/macrophage subset was significantly reduced in number by low-dose, but not high-dose, T-implants. The concentration of the Leydig cell-secreted cytokine macrophage-migration inhibitory factor (MIF) in testicular fluid was reduced by cryptorchidism, but not by the T-implants. When data from all experimental groups were combined, ED2$^+$ resident macrophage numbers showed a significant positive correlation with parameters of Leydig cell function (serum LH and testicular testosterone levels) but a negative correlation with MIF levels. This study indicates that Leydig cells regulate testicular macrophage numbers directly, rather than via an effect upon the seminiferous epithelium, in the adult rat testis. The data also suggest that testosterone and MIF play only a minor role, if any, in this regulation.**

## INTRODUCTION

Mononuclear phagocytes (monocytes and macrophages) represent a substantial cellular population in the normal testicular interstitial tissue of the human and experimental rodent species [1, 2]. In the adult rat testis, these cells belong to one of two distinct subsets, differentiated by the expression of the resident macrophage surface antigen recognized by monoclonal antibody ED2 and a lysosomal marker recognized by monoclonal antibody ED1 [3–5]. The majority of mononuclear phagocytes within the rat testis are ED2$^+$ resident-type macrophages, but there is also a significant population of ED1$^+$ED2$^-$ cells that presumably represents circulating monocytes or recently arrived macrophages (monocyte/macrophages), as well as dendritic cells [5].

There is considerable evidence that the testicular macrophages and the Leydig cells have close functional links [6, 7]. Total rat testicular macrophage numbers gradually decline below normal levels following a brief period of elevation when Leydig cells are ablated by treatment with the specific Leydig cell cytotoxin ethane dimethane sulfonate (EDS) [5]. Similarly, macrophage numbers decline when pituitary gonadotropins are selectively withdrawn [8, 9]. It has been established that these reductions are specifically due to the loss of functional Leydig cells rather than changes in circulating gonadotropin levels [5, 9]. Leydig cells have direct contact with testicular macrophages through intimate structural specializations [10, 11]. Leydig cells also produce locally high levels of androgens, and there is evidence that sex steroids including androgens have the potential to regulate some monocyte and macrophage functions (reviewed in [12]). Recent studies indicate that macrophage-migration inhibitory factor (MIF), a cytokine secreted by lymphocytes and macrophages during inflammation that stimulates monocyte infiltration and proliferation [13], is constitutively produced by adult rat Leydig cells under normal noninflammatory conditions [14]. Finally, Leydig cells regulate the normal spermatogenic function of the seminiferous tubules by producing adequate levels of testosterone, and the seminiferous tubules produce several cytokines with known effects on macrophage function, such as interleukin-1$\alpha$ and transforming growth factor $\beta$ [15]. Consequently, the Leydig cells may regulate testicular macrophage numbers in the adult testis either directly, or indirectly via control of seminiferous tubule secretions.

In the study reported here, the potential roles of the seminiferous tubules and the specific Leydig cell products, testosterone and MIF, in regulating macrophage numbers in the adult rat testis were investigated. This was done by examining the response of the testicular macrophage subsets to manipulation of seminiferous tubule and Leydig cell function by the combined use of surgical cryptorchidism [16] and s.c. testosterone implants [17].

## MATERIALS AND METHODS

### *Animals*

Adult male Sprague-Dawley rats (110–120 days old) were obtained from the Monash University Central Animal

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House and were housed at Monash Medical Centre for the duration of the experimental procedure under conditions of controlled day length (12L:12D) with unlimited access to food and water. This study was conducted in accordance with the Guiding Principles for the Care and Use of Research Animals of the Society for the Study of Reproduction.

### *Experimental Procedure*

All surgical procedures were performed under ether anesthetic. Rats were made bilaterally cryptorchid by translocation of the testes to the abdominal cavity, which raises the testes to body temperature and depletes most of the developing spermatogenic cells within 7 days with accompanying disruption of normal Sertoli cell function [16]. Briefly, this procedure involved exposure of the testis through an incision in the inguinal canal, transfer of the testis to the abdominal cavity, and closure of the inguinal canal to prevent testicular re-descent. Serum and intratesticular testosterone levels were manipulated by the use of s.c. Silastic implants (medical grade; Dow Corning, Midland, MI) containing testosterone (T-implants), as previously described [17]. Low-dose T-implants (3 cm long) cause a reduction in serum gonadotropin levels, with consequent loss of the developing spermatogenic cells, while high-dose T-implants (24 cm long) similarly cause a reduction in gonadotropins but provide sufficient testosterone to maintain intratesticular testosterone levels adequate to support qualitatively normal seminiferous tubule function [17].

Groups of untreated rats and rats that had been made cryptorchid 7 days previously received low-dose (3 cm) or high-dose (3 × 8-cm implants: 24-cm total) T-implants (s.c.) and were maintained for a further 21 days. Consequently, all cryptorchid rats were maintained for a total of 28 days prior to assessment. Each treatment group consisted of 6 animals, as follows: 1) scrotal testes and no T-implant (untreated controls); 2) scrotal testes and 3-cm T-implants (T3); 3) scrotal testes and 24-cm T-implants (T24); 4) abdominal testes and no T-implant (cryptorchid group); 5) abdominal testes and 3-cm T-implants; 6) abdominal testes and 24-cm T-implants.

At the end of the experimental period, one testis (left or right side at random) was removed under ether anesthesia and weighed. Interstitial fluid (IF) was collected, for measurement of intratesticular testosterone and MIF levels, by drainage from an incision in the testicular capsule (16–20 h, 4°C), as previously described [5]. A serum sample was collected by cardiac puncture, and both IF and serum samples were stored at −20°C prior to assay.

The remaining testis was fixed by lower body perfusion via the descending aorta, with 2% paraformaldehyde, 75 mM lysine, 10 mM periodate, containing 1.0% glutaraldehyde, and then weighed and processed for collection of 6-μm frozen sections, as previously described [5]. Animals were not pretreated with any anti-clotting agents.

After collection of the testes, several androgen-responsive tissues, i.e., the thymus, seminal vesicles (including coagulating glands), and epididymis, were removed and weighed to ensure that the T-implants had been successful, and the vasa deferentia were examined macroscopically to ensure that these had not been damaged during surgical induction of cryptorchidism. This latter observation was considered necessary since vasectomy in certain rat species can cause spontaneous testicular inflammation that could compromise the study [18].

In order to provide additional validation of the intratesticular testosterone levels in cryptorchidism, testes from groups of untreated or 4-wk bilaterally cryptorchid adult rats (n = 6 animals per treatment group) were used for collection of IF as described above (left testis) or immediately snap-frozen in liquid nitrogen (right testis) prior to homogenization in 1.5 ml/g tissue PBS. An additional group of cryptorchid rats was used for collection of testicular IF without drainage (right testis) or after overnight drainage (16 h, 4°C, left testis). The IF and testicular homogenate samples were stored at −20°C prior to assay.

### *Immunohistochemistry and Stereological Analysis*

Sections were stained with the mouse anti-rat monoclonal antibodies ED1, which labels a lysosomal antigen specific to monocytes, dendritic cells, and some macrophages (1:4 dilution), or ED2, which labels resident macrophages specifically (1:800 dilution) [3, 4], using immunoperoxidase detection and subsequent hematoxylin counterstaining as previously described [5]. In order to label all cells of the mononuclear phagocyte and dendritic cell lineages, sections were double-stained with ED1 and ED2 combined. Since dendritic cells represent only a very small cell population compared with the number of mononuclear phagocytes in most tissues, and this also appears to be true of the testis [19–21], this $ED1^+$ and $ED2^+$ double staining provides an estimate of the total testicular monocyte/macrophage population.

Immunostained cell profiles containing a nuclear profile were counted using an Olympus (Tokyo, Japan) BH-2 microscope linked to an Amiga (Bensheim, Germany) 2000 computer with an Impact Vision-24 (G.V.P., King of Prussia, PA) professional video adaptor, as previously described [5]. Briefly, the GRID (Interactivision, Silkeborg, Denmark) software package was used to generate an unbiased counting frame that was superimposed on the microscope image. Fields were sampled with a systematic uniform random scheme using a motorized stage. The numerical density of nuclei associated with immunopositive cytoplasm was calculated and was used to determine the total numbers of cells per testis.

For counting and subsequent analysis, the macrophages were separated into total monocytes and macrophages (labeled by combined ED1 and ED2 immunohistochemistry) and total resident macrophages (all $ED2^+$ cells). In the previous study, the $ED2^+$ resident macrophages represented 80% of the total testicular mononuclear phagocyte population [5]. Intratesticular $ED1^+ED2^-$ monocyte/macrophages and putative ''long-term resident'' macrophages ($ED2^+ED1^-$ cells) were calculated by subtraction, as previously described [5].

### *Hormone RIAs*

Serum samples were assayed for LH and FSH by specific RIAs, as previously described [22]. Assay sensitivity was 0.09 ng/ml for LH and 1.5 ng/ml for FSH. Testicular IF and homogenates were assayed for testosterone without extraction using a direct $^{125}$I-testosterone RIA [23], or after extraction with chloroform-hexane using a [$^3$H]testosterone RIA [24]. Extraction recoveries were 78–82%.

### *MIF ELISA*

Testicular IF was assayed for immunoreactive MIF by a sandwich ELISA employing a monoclonal anti-MIF cap-

ture antibody, a rabbit polyclonal anti-MIF detector antibody, and a purified recombinant mouse MIF standard, as previously described [14]. Note that some samples were not available for MIF assay because of limitations on sample volumes.

### *Western Blot Analysis*

Residual samples of testicular IF from each experimental group were pooled and were subjected to Western blot analysis as previous described [14], with some modifications. The pooled samples were diluted 1:1 with sample buffer (0.125 M Tris, 4% SDS, 20% glycerol, 2.5% β-mercaptoethanol, 0.02% bromophenol blue, pH 6.8). The equivalent of 6–10 μl of IF from each experimental group was run on a 15% SDS-polyacrylamide gel and transferred onto Immobilon P membrane (Millipore, Bedford, MA) in a 20 mM Tris, 150 mM glycine buffer containing 20% methanol at 0.5 mA/cm$^2$ overnight. Excess binding sites on the membrane were blocked with high-salt (350 mM NaCl) Tris-buffered saline, pH 7.4 (TBS), containing 5% (w:v) nonfat dry milk powder, 2% normal goat serum, and 0.2% Tween 20, for 1 h. The membrane was probed (1 h) with rabbit anti-MIF polyclonal antibody diluted 1:1000 in the blocking buffer, followed by a donkey anti-rabbit IgG-peroxidase (Amersham, Arlington Heights, IL) diluted 1:10 000 in the blocking buffer (1 h). The blot was washed five times (5 min each) with TBS, 1% (v:v) Tween 20. After a final rinse with TBS, MIF-reactive bands were revealed using an enhanced chemiluminescence system (Amersham).

### *Statistical Analysis*

Comparisons between experimental groups were made by two-way ANOVA after appropriate transformation to normalize data and equalize variance where necessary, or by Kruskal-Wallis ANOVA on ranks, in conjunction with Student-Newman-Keuls multiple range test. Student's *t*-test was used for comparing data from two groups. Correlations were determined by Pearson's product-moment correlation analysis. All statistical analyses were performed using Sigmastat version 1.0 software (Jandel Corp., San Rafael, CA). All values are mean ± SEM (n = 6 animals per group) unless otherwise indicated.

## RESULTS

### *General Morphological Observations*

Testis weights were reduced by 22% in T3-implanted rats compared with normal controls, but weights were fully maintained in T24-implanted rats (Fig. 1A). In bilaterally cryptorchid rats, testis weights declined by 67% compared with those in controls, but neither the T3- nor T24-implants had any additional effect. These weight changes were attributable to a loss of some late-stage spermatogenic cells in the T3-implanted testes and complete regression of the seminiferous epithelium in abdominal testes (data not shown).

After perfusion-fixation, testis weights were increased only slightly (approximately 5–7%) over the corresponding nonperfused testis weights, with the exception of the nonimplanted abdominal testes group weights, which were increased by 12% (Fig. 1B). These data were consistent with the experimental observation of significant swelling of the flaccid abdominal testes during perfusion.

Total IF volume was not affected by T-implants in scrotal testes (Fig. 1C). However, cryptorchidism caused a 3-fold increase in volume that was at least partially prevented by both the T3- and T24-implants. This result suggested that the greater increase in testis weight following perfusion of the nonimplanted abdominal testes may have been related to an increased capacity of the testis volume to expand under perfusion pressure. Since the expanded interstitial space was lost during subsequent processing and sectioning of the abdominal testis, all following data are based on calculations for the nonperfused testis weights. However, all data analysis was performed using both unperfused and perfused testis weights, and no substantial differences in results were observed.

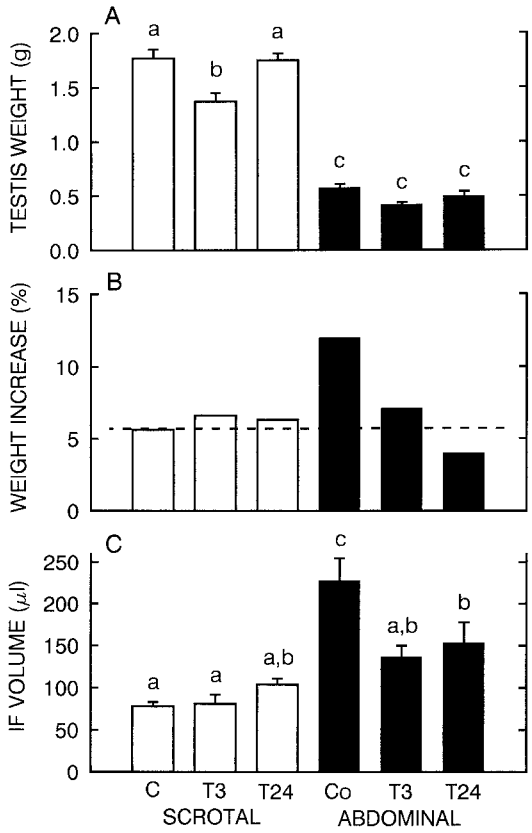


FIG. 1. Testis weight and IF volume data for adult rats with scrotal or abdominal testes without implants (C, Co), with T3-implants, or with T24 implants. **A**) Nonperfused testis weights. **B**) Increase in testis weight (%) following perfusion fixation, as calculated from the difference between the nonperfused and perfused testis collected from each animal. **C**) IF volumes. Values with same letter subscript are not significantly different ($p > 0.05$).

### *Hormonal and Accessory Organ Observations*

Serum LH and FSH levels were increased 2-fold in cryptorchid rats (Fig. 2, A and B). Serum LH was reduced to 30% of normal control levels by either length of T-implant in rats with either scrotal or abdominal testes (Fig. 2A), while serum FSH was reduced to a lesser degree (50–80% of normal) under the same conditions (Fig. 2B).

Testicular IF testosterone levels measured without extraction were significantly higher in the abdominal testis compared with levels in normal controls (Fig. 3A). The T3-implants reduced IF testosterone in both scrotal and abdominal testes to less than 5% of normal, while T24 maintained IF testosterone levels at about 10% of the control value, which was sufficient to maintain testis weights and spermatogenesis. By contrast, seminal vesicle weights were un-

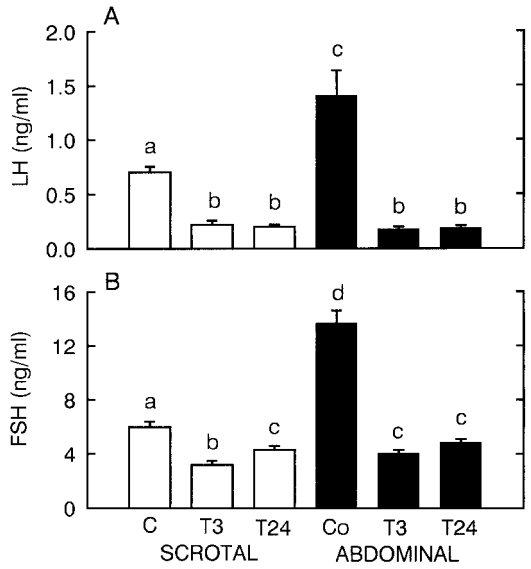


FIG. 2. Serum LH (**A**) and FSH (**B**) levels in adult rats with scrotal or abdominal testes without implants (C, Co), with T3-implants, or with T24-implants. Values with same letter subscript are not significantly different ($p > 0.05$).

affected by cryptorchidism, were slightly stimulated (30–40%) by T3-implants, and were increased 2-fold by T24-implants in rats with either scrotal or abdominal testes (Fig. 3B). Thymus weights were reduced by 50% in the T24-implanted rats but were unaffected by either cryptorchidism

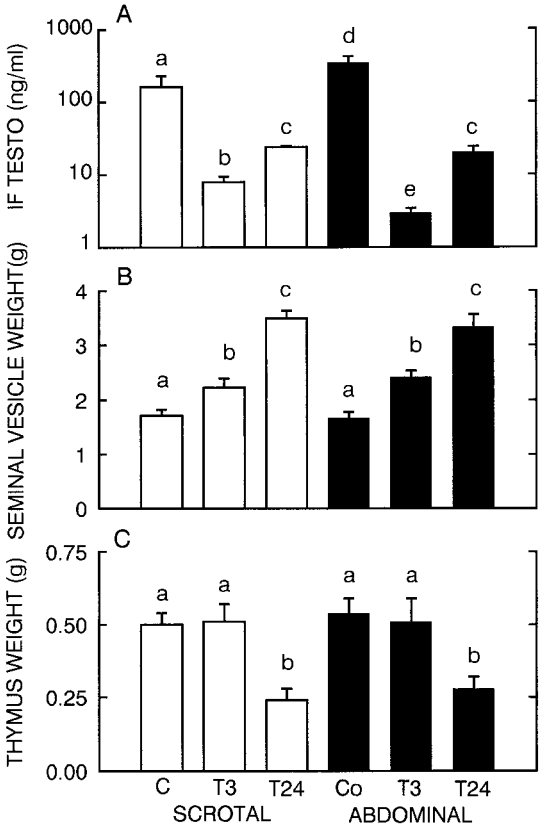


FIG. 3. IF testosterone levels and androgen-dependent organ weights for adult rats with scrotal or abdominal testes without implants (C, Co), with T3-implants, or with T24-implants. **A**) IF testosterone (testo). Note log scale on y-axis. **B**) Seminal vesicle weights. **C**) Thymus weights. Values with same letter subscript are not significantly different ($p > 0.05$).

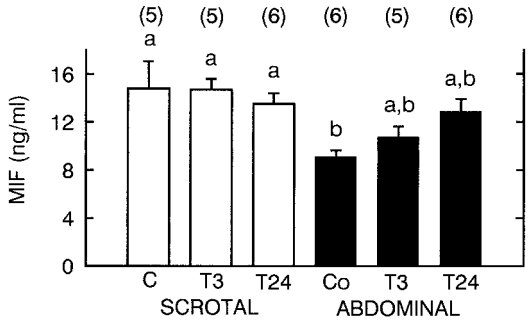


FIG. 4. MIF immunoreactivity measured by ELISA in testicular IF from adult rats with scrotal or abdominal testes without implants (C, Co), with T3-implants, or with T24-implants. Values are mean ± SEM (number of animals in parentheses above histograms). Values with same letter subscript are not significantly different ($p > 0.05$).

or T3-implants (Fig. 3C). These data confirmed that the T3-implants successfully maintained circulating testosterone at close to normal levels, while the T24-implants produced superphysiological levels of testosterone in the serum.

### *Testicular MIF Concentrations*

Immunoreactive MIF levels in testicular IF measured by ELISA (Fig. 4) and by Western blot analysis (Fig. 5) were unaffected in all groups with scrotal testes but were reduced in the abdominal testis. Both T3- and T24-implants at least partially restored immunoreactive MIF levels in the abdominal testes. Accurate quantification of MIF protein levels from the Western data was not considered because of overloading of the gel, which was clearly apparent from the retention of protein between the running lanes at the loading gel/resolving gel interface, and also because the loaded samples were pools of the residual IF available after the testosterone RIA and MIF ELISA.

### *Macrophage Quantitation*

The number per unit testis volume of total monocyte/macrophages, identified by combined immunohistochemical localization of ED1 and ED2, was increased 3-fold following cryptorchidism (Table 1). There was a clear difference between the maximum nuclear diameters of the $ED1^+$ and $ED2^+$ cells (5.7 ± 0.1 and 6.7 ± 0.1 μm, respectively, in scrotal testes), and cryptorchidism had no effect on these

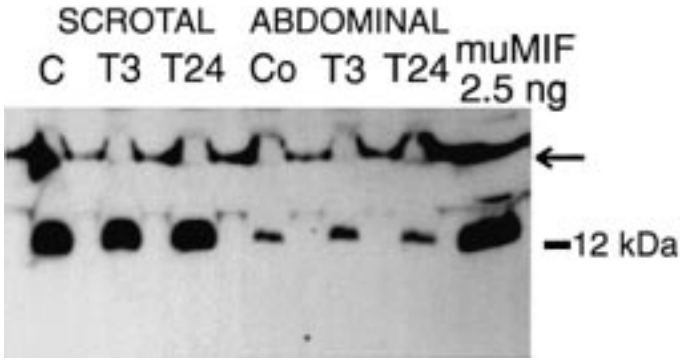


FIG. 5. MIF immunoreactivity detected by Western blot analysis in pooled samples of testicular IF from adult rats with scrotal or abdominal testes without implants (C, Co), with T3-implants, or with T24-implants. Arrow: loading gel/resolving gel interface; muMIF: recombinant murine MIF standard.

TABLE 1. Numerical density estimates for total testicular macrophages in scrotal and abdominal testes.*

| Experimental group | T-implant length | Macrophage density (cells/cm$^3$ × $10^{-6}$) |
|---|---|---|
| Scrotal testes | 0 | 7.50 ± 0.65[a] |
| | 3 cm | 4.96 ± 0.17[b] |
| | 24 cm | 4.88 ± 0.25[b] |
| Abdominal testes | 0 | 21.9 ± 2.2[c] |
| | 3 cm | 16.4 ± 1.5[d] |
| | 24 cm | 18.3 ± 2.3[c,d] |

* Values are mean ± SEM (n = 6 animals); values with same letter superscript are not significantly different ($p > 0.05$).

parameters (5.9 ± 0.1 and 6.8 ± 0.3 μm, respectively, in abdominal testes).

Total testicular macrophage numbers (i.e., all ED1$^+$ cells and ED2$^+$ cells) declined by approximately 40–50% in T3-implanted and in T24-implanted rat testes (Fig. 6A). This decline was primarily the result of a 40% reduction in the number of resident macrophages (ED2$^+$ cells only) (Fig. 6B). There was a more pronounced decline (75%) in the less numerous ED1$^+$ED2$^-$ monocyte/macrophage subset in the T3-implanted animals, while the numbers of this subset were maintained in the T24-implanted group (Fig. 6C). The ED2$^+$ED1$^-$ (long-term resident) macrophages comprised between 25–43% of the ED2$^+$ macrophage population across all treatment groups, and no significant difference in the number of ED2$^+$ED1$^-$ between the treatment groups was detected (data not shown).

In spite of the large increase in macrophage density observed in the abdominal testis (Table 1), there was no change in the total number of intratesticular monocyte/macrophage or resident macrophages (Fig. 6, A and B). This apparent discrepancy between cell density and number was due to the very large decrease in testis volume following cryptorchidism. In contrast to observations in the scrotal testis, the number of ED1$^+$ED2$^-$ monocyte/macrophages was not significantly different from normal control levels in any abdominal testis regardless of the presence or absence of either length of T-implant (Fig. 6C). However, the T24-implants did partially restore ED2$^+$ resident macrophage numbers in the abdominal testis (Fig. 6B) without any apparent effect on the number of ED1$^+$ED2$^-$ monocyte/macrophages (Fig. 6C).

When the data for all experimental groups were combined, testicular ED2$^+$ resident macrophage numbers showed a significant positive correlation with LH, seminal vesicle weight (data not shown), and intratesticular testosterone levels (Fig. 7A)—parameters that are indicators of Leydig cell activity. There was an inverse relationship between MIF levels and resident macrophage numbers (Fig. 7B). In the untreated control rats only, both total monocyte/macrophages and total ED2$^+$ resident macrophage numbers were directly related to testis weight ($r = 0.84$ and $r = 0.85$, respectively; n = 6 animals, $p < 0.05$). However, no relationship was detected between resident ED2$^+$ macrophage numbers and testis weight ($r = 0.1$, $p > 0.05$) or IF volume ($r = 0.3$, $p > 0.05$) when all experimental groups were combined.

The number of cells in the ED1$^+$ED2$^-$ monocyte/macrophage subset was not clearly correlated with any of the above parameters but did show a strong correlation with the number of ED2$^+$ED1$^-$ (long-term resident) macrophages (Fig. 7C). There also was a significant relationship between IF volume and the number of these long-term res-

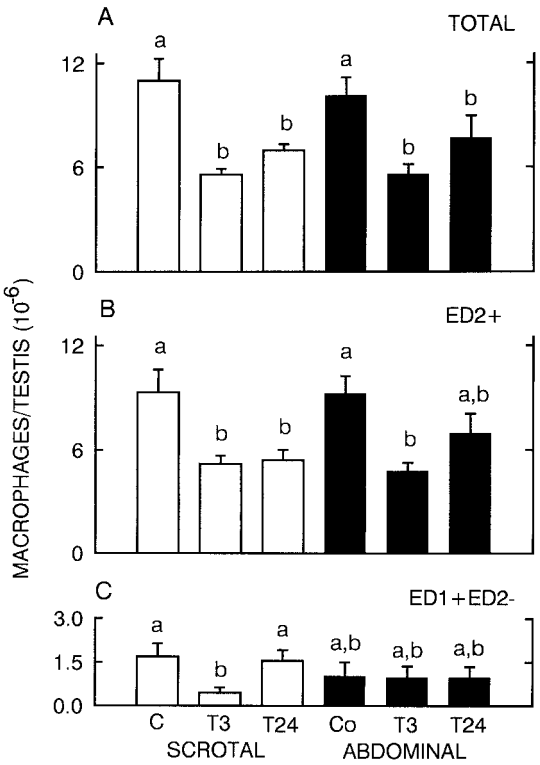


FIG. 6. Total testicular macrophages (**A**), ED2$^+$ resident testicular macrophages (**B**), and ED1$^+$ED2$^-$ testicular monocyte/macrophages (**C**) in adult rats with scrotal or abdominal testes without implants (C, Co), with T3-implants, or with T24-implants. Values with same letter subscript are not significantly different ($p > 0.05$).

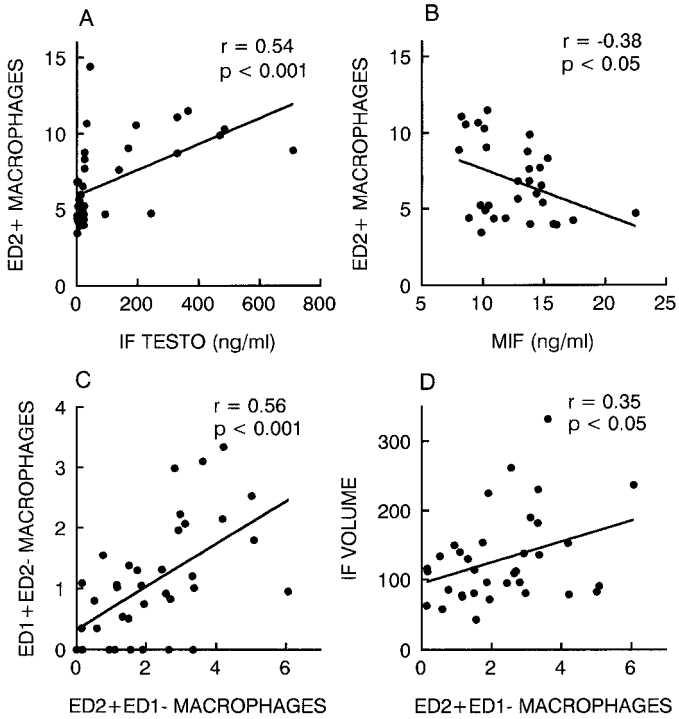


FIG. 7. Correlation analysis of combined data for all rats from each experimental group (n = 36 animals in total): ED2$^+$ resident testicular macrophages with IF testosterone levels (**A**) and MIF immunoreactivity levels (**B**), ED2$^+$ED1$^-$ long-term resident macrophages with ED1$^+$ED2$^-$ monocyte/macrophages (**C**), and IF volume with ED2$^+$ED1$^-$ long-term resident macrophages (**D**).

TABLE 2. Testosterone measurements in scrotal and abdominal testes.[a]

| Experimental group | IF (ng/ml) | IF (with extraction) (ng/ml) | Testis homogenates (ng/g tissue) | Testis homogenates (ng/testis) |
|---|---|---|---|---|
| Scrotal testes | 204 ± 45 | 166 ± 38 | 43.9 ± 7.8 | 72 ± 14 |
| Abdominal testes | 255 ± 48[ns] | 179 ± 16[ns] | 141 ± 20** | 86 ± 14[ns] |

[a] Values are mean ± SEM (n = 6 animals); comparisons are between scrotal and abdominal testis values: ** $p < 0.01$, [ns] not significant ($p > 0.05$).

ident macrophages (Fig. 7D), but not between IF volume and the other macrophage parameters.

### *Reevaluation of Testosterone Levels in Abdominal Testes*

To confirm that the relative concentrations of testosterone measured in unextracted IF collected from normal and cryptorchid rat testes (Fig. 3A) were an accurate representation of intratesticular testosterone levels, testosterone in unextracted and chloroform-hexane-extracted IF and testicular homogenates of scrotal and abdominal testes were compared (Table 2). In this experiment, while the level of IF testosterone appeared slightly higher in cryptorchid IF compared with that in normal controls, as was observed in the first experiment (Fig. 3), this difference was not significant. Extraction had no effect on the relative levels of measurable testosterone in testicular IF from scrotal or abdominal testes. In homogenates of the contralateral testes, total testosterone concentrations were about 3-fold higher in cryptorchid testes than in controls on a per gram tissue basis, but total testis content of testosterone was identical (Table 2). Finally, there was no additional accumulation of testosterone in cryptorchid rats' testicular IF collected from the contralateral testis overnight (106 ± 21 ng/ml) as compared with IF collected immediately postmortem (126 ± 22 ng/ml).

## DISCUSSION

In adult rats, reducing Leydig cell function by inhibition of gonadotropin secretion with T-implants led to a decrease in resident testicular macrophages 3 wk later, similar to the decline observed at the same time point after ablation of the Leydig cells with EDS [5], providing further confirmation of the importance of functional Leydig cells for maintaining normal resident testicular macrophage numbers. The decline in macrophage numbers was observed even when seminiferous tubule function was preserved by the use of high-dose T-implants, while disruption of seminiferous tubule function through depletion of the spermatogenic cells by experimental bilateral cryptorchidism had no influence on testicular macrophage numbers or subsets. Altogether, these data indicate that maintenance of macrophage numbers by the Leydig cells is not mediated via the seminiferous epithelium, and most likely involves a direct mechanism. However, neither testosterone nor MIF, a cytokine recently found to be produced by the Leydig cell [14], appeared to be primarily responsible for maintaining resident macrophage numbers. These data also indicate that resident macrophage numbers in the rat testis are not affected by raising the temperature of the testis to normal body temperature or large experimentally induced changes in testicular and/or interstitial tissue volume.

Both nuclear diameter and total macrophage numbers were slightly greater in this study compared with a previous study using identical techniques [5]. This may be attributed at least partially to the fact that the average testis weight also was slightly larger in the present study, because total macrophage number was found to be correlated with organ weight, and hence interstitial tissue volume, in the normal untreated testes. It is most important to note that testicular macrophage numbers and nuclear diameter data both in the present study and in the previous study employing perfusion-fixed frozen testes are approximately the same as testicular macrophage numbers obtained using more ''effective'' glutaraldehyde fixation and plastic-embedded testis tissue [9], which is more resistant to changes in volume caused by processing and sectioning [25]. Comparison of the macrophage density data and number data for normal versus cryptorchid rat testes in the present study reinforces the importance of using absolute cell numbers, rather than density, in studies of changes in cell populations in the testicular interstitial tissue, particularly when one is dealing with treatments that cause large changes in seminiferous tubule volume [5].

The failure of experimental bilateral cryptorchidism to alter macrophage number in the adult rat is entirely consistent with previous studies that have reported no change in total macrophage absolute volume in abdominal rat and mouse testes using a similar experimental model [26, 27], indicating that neither testicular macrophage number nor size is affected by disruption of seminiferous tubule function. Although total macrophage volume has been shown to be decreased in the abdominal testis compared with the scrotal testis in rats that had been made unilaterally cryptorchid at birth, this difference appears to be related to the changes in Leydig cell function in the unilateral cryptorchid model rather than the depletion of spermatogenic cells in the cryptorchid condition per se [6].

Inhibition of LH secretion by s.c. T-implants caused a substantial decline in the resident testicular macrophage population, similarly to previous experimental models that employed Leydig cell ablation by EDS treatment or selective gonadotropin withdrawal by GnRH immunization [5, 9]. This decline was not prevented by the use of high-dose T-implants that provided sufficient intratesticular testosterone levels to maintain seminiferous tubule function. In contrast, the number of monocyte/macrophages ($ED2^-$ cells) was regulated by the changes in testosterone in the scrotal testis. Although there have been reports of direct actions of testosterone on macrophages, and monocytes in particular [28, 29], the observation that the testosterone responsiveness of the $ED2^-$ population was lost in the abdominal testis indicates that this particular action may be mediated via the seminiferous tubules. In the cryptorchid testis there also was a restorative effect of elevated testosterone on resident macrophage numbers, which was not observed in the scrotal testis. The explanation for this restoration is not apparent, but it presumably involves the seminiferous tubules. Altogether, these data are indicative of a minor indirect effect of testosterone on monocytes or macrophage maturation in the adult rat testis, which appears to be mediated via the seminiferous epithelium. Nonetheless, it is unlikely that testosterone is the major factor mediating the direct action of Leydig cells on the number of resident macro-

phages. Consequently, these results are consistent with data provided earlier for the immature testis indicating that Leydig cells regulate testicular resident macrophage development via a non-androgenic mechanism [7].

Consistent with our previous data that testicular MIF mRNA and protein levels are maintained following hypophysectomy [14], MIF was only slightly affected by the changes in LH, FSH, and testosterone induced by T-implants in the present study. The results did indicate that MIF levels decline in the abdominal testis, but there was no apparent positive relationship between the levels of this cytokine in testicular IF and monocyte or macrophage numbers in the testis. In fact, there was evidence of a slight negative relationship between MIF and resident macrophage numbers. While this study does not exclude a role for MIF in regulating other functions of monocytes or macrophages in the testis, for example by modulating their inflammatory abilities [13], these data suggest that MIF has no major stimulatory effect on testicular resident macrophage proliferation or development. However, approximately 30–50% of the testicular resident macrophage population is not acutely sensitive to the withdrawal of gonadotropins or Leydig cell function ([5, 8, 9]; present study), and it remains a possibility that persistence of some resident macrophages in the rat testis is MIF-dependent.

In spite of the considerable experimental evidence that Leydig cells maintain the elevated number of macrophages in the testis, the mechanism involved remains to be established. It may involve direct contact via Leydig cell-macrophage specializations [10, 11], although the relatively slow decline in macrophage numbers following Leydig cell destruction by EDS treatment suggests that simple physical attachment is not the mechanism responsible [5, 30]. Other cytokines may be involved; for example, both colony-stimulating factor-1 (CSF-1) and interleukin-1α (IL-1) have been implicated in regulating testicular macrophage function [31, 32]. Normal resident macrophage development in many tissues including the testis is dependent upon CSF-1 [33], while IL-1 is a key mediator of the inflammatory response including monocyte recruitment [34]. However, CSF-1 expression in the adult testis appears to be extremely low, and the role of this cytokine in stimulating testicular macrophage development appears to be exerted systemically, possibly at the level of bloodborne tissue macrophage precursors [31, 35]. Moreover, the principal site of IL-1 production in the testis in the absence of an inflammatory stimulus is the Sertoli cells and spermatogenic cells, rather than the Leydig cells [32, 36]. These data point to the involvement of another, as yet unidentified, Leydig cell cytokine or product.

In the present study, s.c. T-implants partially reversed the increase in IF volume that follows the loss of normal vascular regulatory influences from the seminiferous tubules in the cryptorchid testis [37, 38]. These data suggest that the accumulation of IF following seminiferous tubule damage is partially dependent upon gonadotropin action. This effect is most likely mediated by LH, which stimulates IF volume in other physiological and pathophysiological conditions [39]. Interestingly, there also was a relationship between the number of long-term resident macrophage numbers and the volume of the IF, which may indicate an as yet unidentified role for these cells in controlling vascular responses in the testis.

Consistent with previous observations, peripheral testosterone levels were effectively normal in the bilaterally cryptorchid rats, as indicated by the seminal vesicle and thymus weights [40, 41]. Testicular IF testosterone levels also appeared to be either normal or slightly elevated in the cryptorchid rat. Although similar results have been reported elsewhere [40, 42], this latter observation was analyzed further because it was not consistent with previous results obtained in our laboratory and in some other laboratories using the same experimental model [41, 43, 44]. Continued synthesis of testosterone after testis collection, leading to artificially elevated testosterone levels in testicular IF, has been raised previously as a potential technical problem [45, 46]. However, there was no evidence of continued synthesis in the cryptorchid testis, and measurement of total testosterone in testicular homogenates collected immediately postmortem also confirmed that intratesticular testosterone levels in the cryptorchid rat were as high as, if not higher than, those in normal testes. Comparison of the IF testosterone levels and the total testicular testosterone values also indicated that testosterone is concentrated within the testicular interstitial tissue, as reported in previous studies [47, 48]. Finally, extraction of testicular IF did not indicate the existence of any hidden differences in bound reserves of testosterone not detected by the direct testosterone RIA.

The reason for the discrepancy in testosterone levels within the cryptorchid testis in different studies is not known, but it may involve differences in blood flow and testosterone clearance. Induction of experimental cryptorchidism requires considerable manipulation of the testis and its associated vasculature. This manipulation may have variable effects on blood supply and drainage, including the countercurrent exchange mechanisms that regulate testosterone release from the testis via the blood [49]. Slight differences in surgical technique could account for differences in the vascular mechanisms that regulate testosterone levels within the interstitial tissue, with potentially different outcomes for the eventual intratesticular testosterone levels. In any case, these data emphasize the importance of confirming intratesticular testosterone levels in all experiments where testicular function is altered.

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