

# Selective depletion of testicular macrophages and prevention of Leydig cell repopulation after treatment with ethylene dimethane sulfonate in rats

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Testicular macrophages were selectively eliminated with dichloromethylene diphosphonate-containing liposomes (Cl<sub>2</sub>MDP-lp) to study the role of these cells in the repopulation of Leydig cells after treatment with ethylene dimethane sulfonate (EDS). Right testes were injected with Cl<sub>2</sub>MDP-lp to deplete macrophages and left testes were injected with sodium chloride and served as controls. Injection of Cl<sub>2</sub>MDP-lp produced a 97% reduction in the number of macrophages 10 days after treatment. Twenty-one days after destruction of the existing Leydig cells with EDS, abundant differentiating Leydig cells were present in the left (macrophage-containing) testes. On the contrary, in the right (macrophage-depleted) testes, differentiating Leydig cells were scarce, and was 3% of that found in the control testes. The inhibition of Leydig cell repopulation in macrophage-depleted testes was more evident at 30 days after EDS treatment, when the number of Leydig cells in the right testes was 1% of that found in control testes. The lack of Leydig cell development was also indirectly shown by the lower mass and more atrophic seminiferous epithelium of the right testes, as well as the decreased weight of the ipsilateral epididymis compared with the left testes. These results indicate that testicular macrophages are central to the proliferation and differentiation of new Leydig cells after EDS treatment, and point out the significance of paracrine regulatory mechanisms in rat testes.

## Introduction

Testicular function is under the control of the hypothalamic–pituitary axis. However, it has become evident that actions of gonadotrophin in the testis are modulated by local factors and by cell–cell interactions. Several reviews on the paracrine and autocrine regulation of the testes have been published (Sharpe, 1986, 1990; Saez *et al.*, 1987; Skinner, 1991; Spiteri-Grech and Nieschlag, 1993) and major emphasis has been given to interactions between Leydig cells and Sertoli cells.

Macrophages are a numerically important component of the testicular interstitium (Miller *et al.*, 1983; Dirami *et al.*, 1991) and are a potential source of cytokines in the testes. Several lines of evidence suggest that macrophages play important roles in testicular paracrine regulation. (i) Macrophages are morphologically (Miller *et al.*, 1983) and functionally (Bergh, 1985) coupled to Leydig cells. (ii) Classic secretory products of macrophages, such as interleukin-1 (IL-1) (Le and Vilcek, 1987), tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ) (Xiong and Hales, 1993), and transforming growth factor- $\alpha$  (TGF- $\alpha$ ) (Rappolee *et al.*, 1988) act as local modulators of Leydig cell function (Welsh and Hsueh, 1982; Calkins *et al.*, 1988; Verhoeven *et al.*, 1988; Mauduit *et al.*, 1992; Meikle *et al.*, 1992; Xiong and Hales,

1993). Furthermore, macrophage-conditioned medium increases testosterone secretion *in vitro* (Yee and Hutson, 1985). (iii) Macrophages accumulate in the testis during prepubertal development, in parallel with the proliferation–differentiation of Leydig cells (Hutson, 1990). Treatment of young rats with gonadotrophins increases the concentration of Leydig cells and macrophages (Raburn *et al.*, 1991). (iv) Some macrophage-derived cytokines, such as IL-1 $\beta$  (Xiong *et al.*, 1991) and TGF- $\alpha$ , (Rappolee *et al.*, 1988) stimulate DNA synthesis in immature Leydig cells in culture (Khan *et al.*, 1992a, b).

Treatment with ethylene dimethane sulfonate (EDS) destroys rat Leydig cells (Kerr *et al.*, 1985; Molenaar *et al.*, 1985; Bartlett *et al.*, 1986; Jackson *et al.*, 1986; Morris *et al.*, 1986). A new Leydig cell population develops in the following weeks, and this model has been widely used to assess Leydig cell differentiation in rats (Jackson *et al.*, 1986; Molenaar *et al.*, 1986; Kerr *et al.*, 1987; Teerds *et al.*, 1990; Gaytan *et al.*, 1992). Alternatively, testicular macrophages can be depleted with dichloromethylene diphosphonate-containing liposomes (Cl<sub>2</sub>MDP-lp) (Bergh *et al.*, 1993a), which is a powerful tool for studying the physiological role of these cells.

We used these techniques to study the role of testicular macrophages in the proliferation–differentiation of Leydig cells *in vivo*.

**Box 1. Scheme of experimental design**

Days after Cl <sub>2</sub> MDP-lp or NaCl treatment	8	10	13	29	31	38	40
30 Rats  Right testes Cl <sub>2</sub> MDP-lp Left testes NaCl	TB (5)	Sc (5)					
	EDS (20)	Veh (5)	Sc (5)				
					TB (5)	Sc (5)	
				Sc (5)			
			TB (5)	Sc (5)			
		TB (5)	Sc (5)				
Days after EDS or vehicle treatment			3	19	21	28	30

TB: trypan blue; 2 ml 3% i.p.; EDS: 75 mg ethylene dimethane sulfonate kg<sup>-1</sup> i.p.; Veh (Vehicle): DMSO:water 1:3; Cl<sub>2</sub>MDP-lp: dichloromethylene diphosphonate-containing (Cl<sub>2</sub>MDP) liposomes 150 µl intratesticular. NaCl: 150 µl 0.9% (w/v) intratesticular. Sc: day on which animals were killed. The number of rats is given in parentheses.

## Materials and Methods

### Animals

Thirty adult male Wistar rats (300–350 g) were used. Animals were maintained under controlled light and temperature and had free access to food and water.

### Chemicals

Dichloromethylene diphosphonate (Cl<sub>2</sub>MDP)-containing liposomes were prepared as described by van Rooijen (1989) and Cl<sub>2</sub>MDP was a gift from Boehringer Mannheim GmbH (Mannheim). Phosphatidylcholine (LIPOID E PC) was a gift from LIPOID KG (Ludwigshafen).

Ethylene dimethane sulfonate (EDS) was synthesized following the method of Jackson and Jackson (1984).

### Experimental design

A scheme of experimental design is shown in Box 1.

Thirty rats were injected intratesticularly with 150 µl Cl<sub>2</sub>MDP-containing liposomes (right testis) and with 150 µl of 0.9% NaCl (left testis) under light ether anaesthesia, using the method described by Bergh *et al.* (1993a). Previous studies have shown that there were no differences between PBS-containing liposomes and NaCl treatment (Bergh *et al.*, 1993a).

After 10 days, five animals were killed by decapitation; these animals had received an i.p. injection of 2 ml 3% trypan blue in saline (Miller *et al.*, 1983) 48 h previously to label testicular macrophages. The other animals were injected i.p. with 75 mg EDS kg<sup>-1</sup> body weight in DMSO:water (1:3) (20 rats) or vehicle (five rats).

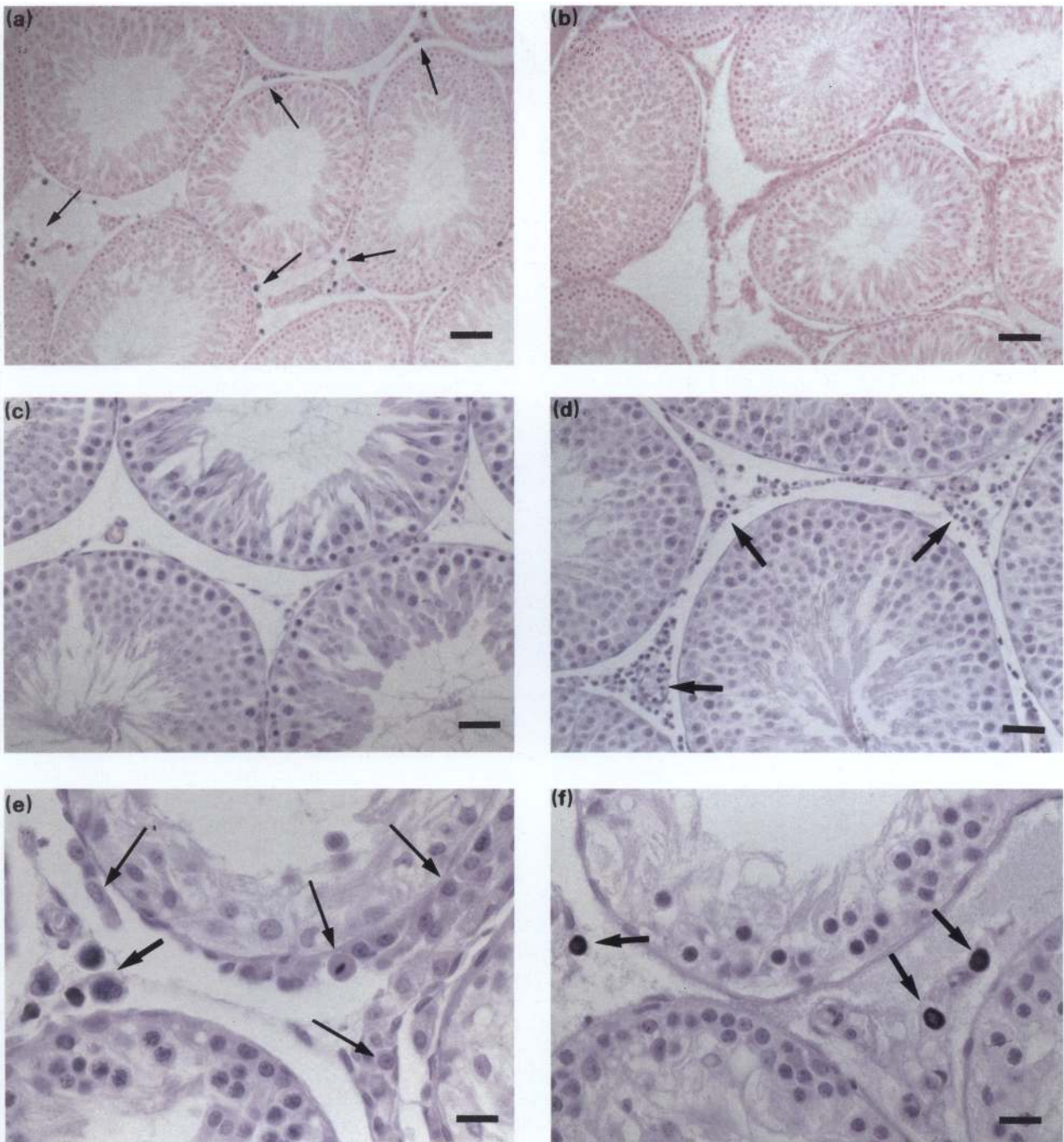
On day 3 after EDS treatment, five animals were killed, and the other animals were killed on day 21 after EDS or vehicle treatment. Five other EDS-treated rats were killed 30 days after treatment. Five animals per group received an i.p. injection of trypan blue 48 h before they were killed.

### Tissue processing

The testes and epididymides were carefully dissected. The testes were weighed and fixed for 48 h in Bouin–Hollande's fluid. After immersion in the fixative, small incisions were made in the tunica albuginea to facilitate fixation. Slabs perpendicular to the longest axis of the organ were then cut and they were dehydrated and embedded in paraffin wax. In all cases, right and left organs were considered separately. Sections, 5 µm, were cut and stained with haematoxylin and eosin or nuclear red, and viewed under a light microscope.

### Cell counting procedures

The different cell types were counted relative to the number of Sertoli cell nuclei following the methods of Teerds *et al.*



**Fig. 1.** Sections from the left (NaCl-injected) (a,c,e) and right (dichloromethylene diphosphonate-containing ( $\text{Cl}_2\text{MDP}$ )-liposome-injected) (b, d, f) testes. (a, b) Ten days after treatment, macrophages (arrows) were abundant in the left testes and absent in the right testes. Trypan blue was injected 48 h before death. Counterstain nuclear red. Bars represent 50  $\mu\text{m}$ . (c, d) Three days after ethylene dimethane sulfonate (EDS) treatment the testicular interstitium was devoid of Leydig cells, but inflammatory cells (arrows) were present in the right testes. Haematoxylin and eosin. Bars represent 30  $\mu\text{m}$ . (e, f) Twenty-one days after EDS treatment. In the left testes abundant differentiating Leydig cells (long arrows) were found together with macrophages and mast cells (short arrow). In the right testes, mast cells (arrows) were the most prominent cell type. Haematoxylin and eosin. Bars represent 20  $\mu\text{m}$ .

(1988, 1989b) and Gaytan *et al.* (1992). These counts have the advantage of being independent of volumetric changes of the tissue and have been used for the quantitation of interstitial cells after EDS treatment. Macrophages were counted in trypan-blue-

containing vacuoles, either in nuclear red or haematoxylin and eosin stained sections. Leydig cells were recognized by their round to oval nuclei with a characteristic chromatin pattern. Mast cells showed intensely basophilic cytoplasm and central nuclei.

The number of macrophages, Leydig cells and mast cells (counted only when the nuclei were present in the section) and the number of Sertoli cell nuclei (considered only when the nucleolus was present) were counted systematically in five randomly selected sections per testis, up to at least 1000 Sertoli cell nuclei per testis were scored.

### Statistical analyses

Statistical analyses were performed by the Student's *t* test for paired data for comparison between right and left organs, and by ANOVA and Tukey's multiple comparison methods when several groups were compared. Significance was considered at the 0.01 level.

## Results

In a preliminary study, the Leydig cell:macrophage ratio was determined in semithin sections from intact rats and in paraffin wax sections from trypan blue-injected rats. In semithin sections stained with toluidine blue, macrophages can easily be identified by their pale-stained cytoplasm and nuclear characteristics. No differences were found between the methods ( $4.3 \pm 0.2$  Leydig cells:macrophage in semithin sections versus  $4.1 \pm 0.2$  Leydig cells:macrophages in trypan blue-injected rats). This finding indicated that the number of macrophages was not modified by systemically injected trypan blue and that all testicular macrophages have endocytic capacity. Macrophages were therefore counted by their ability to accumulate trypan blue.

### Effects of treatment with $Cl_2$ MDP liposomes

Details on the effects of intratesticular injections of  $Cl_2$ MDP-liposomes ( $Cl_2$ MDP-lp) or NaCl on macrophages have been reported by Bergh *et al.* (1993a). Ten days after treatment, abundant macrophages were present in the NaCl-injected testes (Fig. 1a), whereas macrophages were scarce in  $Cl_2$ MDP-lp-injected contralateral testes (Fig. 1b). Occasionally, a few macrophages were found under the tunica albuginea in some rats. Some areas of tubule disruption at the site of the injection

**Table 1.** Number of macrophages and Leydig cells per 1000 Sertoli cell nuclei 10 days after treatment with NaCl (left testes) or dichloromethylene diphosphonate ( $Cl_2$ MDP) liposomes (right testes)

Treatment	Macrophages	Leydig cells
NaCl	$297.6 \pm 7.2$	$1204.6 \pm 56.6$
$Cl_2$ MDP-liposomes	$9.8 \pm 2.7^*$	$1113.4 \pm 51.8$

Data are means  $\pm$  SEM for five animals.

\*Significantly different ( $P < 0.01$ ) with paired *t* test.

were found either in  $Cl_2$ MDP-lp or NaCl treated testes and these areas were not used for cell counting.

Macrophages in the  $Cl_2$ MDP-lp injected testes represent 3% of the normal population. No significant changes in the number of Leydig cells were found (Table 1).

### Response to EDS treatment

Three days after EDS treatment, the morphology of the testicular interstitium of the left (NaCl-injected) testes was similar to that previously described for intact EDS-treated rats (Morris *et al.*, 1986; Gaytan *et al.*, 1992). Leydig cells were absent and macrophages were the most abundant cell type. No signs of inflammatory reaction were observed (Fig. 1c). An inflammatory reaction was seen in the contralateral ( $Cl_2$ MDP-lp-injected) testes. Leydig cells were also absent and the interstitium was diffusely and moderately infiltrated with polymorphonuclear and mononuclear leucocytes (Fig. 1d).

On day 21 after EDS treatment, the morphology of both testes was quite different. In the left (NaCl-injected) testes abundant differentiating Leydig cells were found. These cells showed round to oval nuclei and were located in peritubular or perivascular clusters (Fig. 1e). Mitotic figures were frequently seen. Mast cells were also abundant (Fig. 1e). On the contrary, in the right ( $Cl_2$ MDP-lp-injected) testes, differentiating Leydig cells and macrophages were extremely scarce; the most abundant interstitial cell type was the mast cell (Fig. 1f). The seminiferous epithelium of the right testes was more atrophic

**Table 2.** Number of macrophages, Leydig cells and mast cells per 1000 Sertoli cell nuclei 21 days after ethylene dimethane sulfonate (EDS) or vehicle treatment in rats treated with NaCl (left testes) or dichloromethylene diphosphonate ( $Cl_2$ MDP)-containing liposomes ( $Cl_2$ MDP-lp) (right testes)

Treatments	Macrophages*	Leydig cells	Mast cells
NaCl + EDS ( $n = 10$ )	$362.5 \pm 15.9$	$552.3 \pm 79.5$	$252.6 \pm 10.3$
$Cl_2$ MDP-lp + EDS ( $n = 10$ )	$29.6 \pm 12.4^a$	$19.2 \pm 15.9^a$	$279.1 \pm 20.2$
NaCl + vehicle ( $n = 5$ )	$301.2 \pm 13.4$	$1165.4 \pm 63.2^b$	— <sup>†</sup>
$Cl_2$ MDP-lp + vehicle ( $n = 5$ )	$34.9 \pm 12.9^a$	$1079.2 \pm 76.2^b$	— <sup>†</sup>

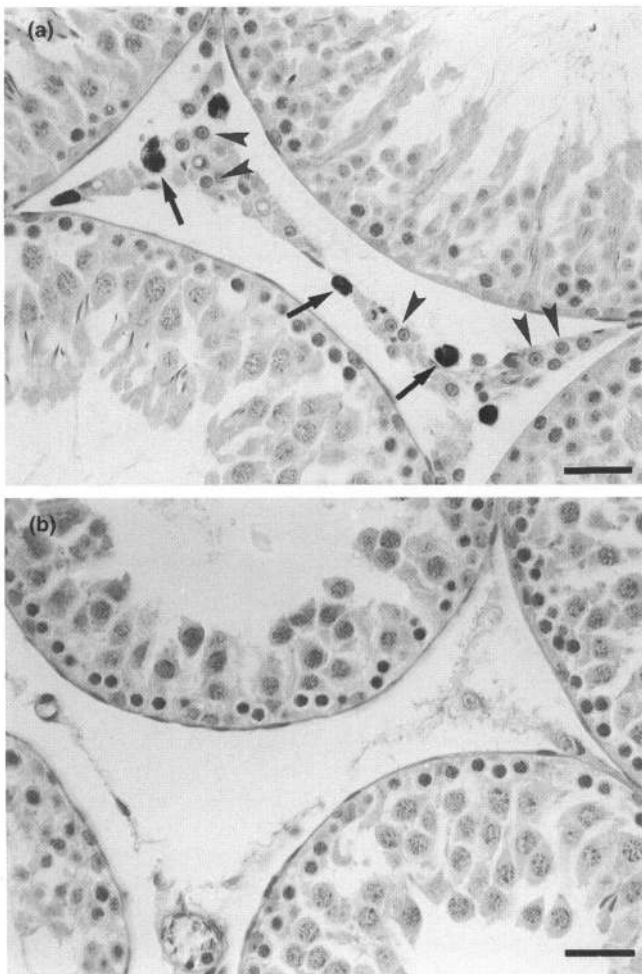
Data are means  $\pm$  SEM.

\*Macrophages were counted in five animals per group.

<sup>†</sup>Less than 1 per 1000 Sertoli cell nuclei and located exclusively under the tunica albuginea.

<sup>a</sup>Significant differences ( $P < 0.01$ ) versus NaCl-injected testes. ANOVA and Tukey's test.

<sup>b</sup>Significant differences ( $P < 0.01$ ) versus EDS-treated rats. ANOVA and Tukey's test.



**Fig. 2.** Sections from left (NaCl-injected) (a) and right (dichloromethylene diphosphonate-containing ( $\text{Cl}_2\text{MDP-lp}$ )-injected) (b) testes at 30 days after ethylene dimethane sulfonate (EDS) treatment. Abundant trypan blue-labelled macrophages (arrows) and Leydig cells (arrow-heads) were present in the left testes, whereas they are absent in the right testes. Counterstaining with neutral red. Bars represent 40  $\mu\text{m}$ .

than that in the left testes, and no signs of inflammatory reaction or interstitial fibrosis were observed.

$\text{Cl}_2\text{MDP-lp}$ -injected testes showed significantly fewer macrophages (about 8% of the contralateral testes) and Leydig cells (about 3% of the contralateral testes), whereas no significant differences were found in the number of mast cells (Table 2). On day 30 after EDS treatment, the NaCl-injected testes had abundant differentiated Leydig cells and macrophages (Fig. 2a). On the contrary, both cell types were scarce in the contralateral ( $\text{Cl}_2\text{MDP-lp}$ -injected) testes (Fig. 2b). Testes injected with  $\text{Cl}_2\text{MDP-lp}$  had significantly ( $P < 0.01$ ) fewer macrophages and Leydig cells, whereas the number of mast cells was higher than in control testes (Table 3).

#### Testicular and epididymal mass

No significant differences were found for the testicular mass between NaCl and  $\text{Cl}_2\text{MDP-lp}$ -injected testes 10 or 31 days

after treatment. However, in EDS-treated animals, testicular (Fig. 3) and epididymal (Fig. 4) masses were significantly lower in the  $\text{Cl}_2\text{MDP}$ -injected organs, 21 days after treatment. On day 30 after EDS treatment, macrophage-depleted testes showed a 34% decrease in mass compared with the contralateral, macrophage-containing testes ( $1006 \pm 132$  versus  $1530 \pm 140$  mg,  $P < 0.01$ , Student's *t* test;  $n = 5$ ). The mass of the ipsilateral epididymis was 32% lower compared with the contralateral organs ( $283 \pm 28$  versus  $417 \pm 52$  mg,  $P < 0.01$ , Student's *t* test;  $n = 5$ ).

## Discussion

This study demonstrates that depletion of testicular macrophages prevents the development of new Leydig cells after EDS treatment. Repopulation of Leydig cells depends on LH (Molenaar *et al.*, 1986), and does not occur in hypophysectomized EDS-treated rats (Teerds *et al.*, 1989a; Gaytan *et al.*, 1990) or by blocking pituitary secretion after EDS treatment with exogenous testosterone (Sharpe *et al.*, 1988; Teerds *et al.*, 1989b). The experimental approach used in the study reported here shows that inhibition of Leydig cell development was caused by changes in local regulatory mechanisms, and not by general endocrine alterations. This was indicated by the development of abundant Leydig cells in the contralateral, macrophage-containing, testes. These results point out the significance of paracrine regulatory mechanisms in the testis and the central role of macrophages in this system.

Macrophages play important roles in the regeneration of many cell types after tissue injury. For instance, macrophages are central to the wound-healing response which requires the proliferation of several regenerating cell types, through the secretion of several growth factors (Rappolee *et al.*, 1988). Similarly, Leydig cell destruction with EDS led to the disposal of dead Leydig cells by macrophages (Jackson *et al.*, 1986; Morris *et al.*, 1986; Kerr *et al.*, 1987) and the consequent regeneration of the Leydig cell population (Molenaar *et al.*, 1986; Jackson *et al.*, 1986). Previous studies have shown that macrophage-derived cytokines, such as IL-1 $\beta$  and TGF- $\alpha$ , stimulate DNA synthesis in immature Leydig cells *in vitro* (Khan *et al.*, 1992a, b). However, macrophages are not the only source of these cytokines in the testes. Other cell types, such as Sertoli cells (Syed *et al.*, 1988) and peritubular-myoid cells (Skinner *et al.*, 1989), also have the capacity to secrete these growth factors. The contribution of each cell type to the total amounts of IL-1 and TGF- $\alpha$  in the testis, as well as their effects on the proliferative activity of Leydig cells *in vivo*, are not known.

Interstitial cell proliferation after EDS treatment follows a biphasic pattern. A first proliferative wave (involving many different cell types) occurs on days 2–3 after treatment (Teerds *et al.*, 1990; Gaytan *et al.*, 1992). It is thought that some of these proliferating mesenchymal cells correspond to undifferentiated Leydig cell precursors. From day 3 to day 18 after treatment, abundant morphologically recognizable Leydig cells are present (Jackson *et al.*, 1986; Morris *et al.*, 1986; Gaytan *et al.*, 1992). In addition, testosterone concentrations reach normal values by day 21 (Bartlett *et al.*, 1986). A second proliferative wave, involving mainly differentiating Leydig



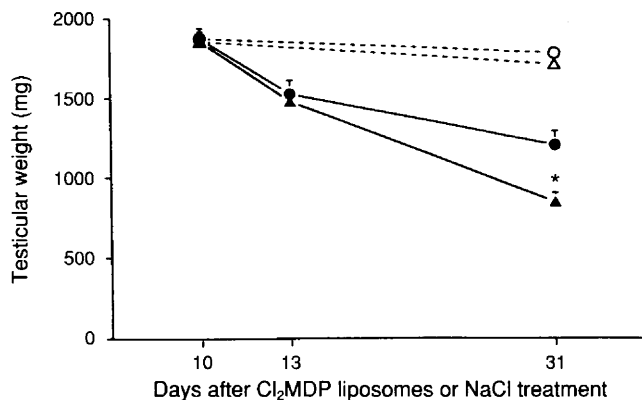
**Table 3.** Number of macrophages, Leydig cells and mast cells per 1000 Sertoli cell nuclei 30 days after ethylene dimethane sulfonate (EDS) treatment in rats treated with NaCl (left testes) or dichloromethylene diphosphonate (Cl<sub>2</sub>MDP)-containing liposomes (Cl<sub>2</sub>MDP-lp) (right testes)

Treatment	Macrophages*	Leydig cells	Mast cells
NaCl	327.3 ± 12.4	994.6 ± 40.5	210.7 ± 20.1
Cl <sub>2</sub> MDP-lp	24.3 ± 17.6 <sup>a</sup>	10.3 ± 6.5 <sup>a</sup>	308.6 ± 24.4 <sup>a</sup>

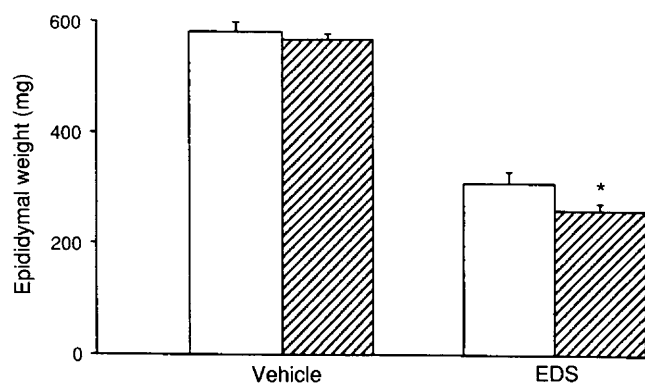
Data are means ± SEM (n = 5).

\*Macrophages were counted in five animals per group.

<sup>a</sup>Significant differences (P < 0.01) versus NaCl-injected testes. Student's *t* test for paired data.



**Fig. 3.** Evolution of testicular weight in rats injected intratesticularly with NaCl in left testes (○, ●) or dichloromethylene diphosphonate containing (Cl<sub>2</sub>MDP)-liposomes in right testes (△, ▲) and with ethylene dimethane sulfonate (EDS) (●, ▲) or vehicle (○, △) 10 days later. \*Significant difference (P < 0.01) by Student's *t* test for paired data versus contralateral testes.



**Fig. 4.** Epididymal weight in rats injected intratesticularly with NaCl in left testes (□) or dichloromethylene diphosphonate containing (Cl<sub>2</sub>MDP)-liposomes in right testes (▨), 21 days after treatment with ethylene dimethane sulfonate (EDS) or vehicle. \*Significant differences (P < 0.01) by Student's *t* test for paired data.

cells, occurs from day 18 to day 23 after treatment (Teerds *et al.*, 1990; Gaytan *et al.*, 1992).

The scarcity of recognizable Leydig cells in macrophage-depleted testes at least up to 30 days after EDS treatment could be due to inhibition of different steps: (i) the first proliferative wave and therefore the expansion or commitment of undiffer-

entiated precursors; (ii) the differentiation of precursor cells to morphologically recognizable, LH-responsive, immature Leydig cells; (iii) the proliferative activity of differentiating Leydig cells. The stage of Leydig cell development that was inhibited was not determined in the study reported here and different experimental approaches are needed to test these possibilities. In normal conditions, macrophages are present throughout Leydig cell differentiation. It is noteworthy that the number of macrophages relative to Leydig cells was higher during Leydig cell repopulation (65.6 macrophages per 100 Leydig cells in NaCl injected testes, 21 days after EDS treatment) than in control testes (24.4 macrophages per 100 Leydig cells before EDS treatment), because fewer Leydig cells were present in these animals. In addition to Leydig cells, mast cells also proliferate and accumulate in the testicular interstitium after EDS treatment (Gaytan *et al.*, 1990, 1992). In the study reported here, mast cell accumulation was not modified in macrophage-depleted testes at 21 days after treatment. The proliferation of different cell types may depend on different, sometimes overlapping, combinations of growth factors. This contention is in agreement with previous studies indicating that mast cell proliferation-differentiation in rat testes is related to the absence of fully differentiated Leydig cells (Gaytan *et al.*, 1990, 1992). It is also in accordance with the existence of a large number of mast cells in macrophage-depleted testes, in which Leydig cells were absent at 30 days after EDS treatment.

An inflammatory response was present in macrophage-depleted testes 3 days after EDS treatment, whereas no signs of inflammatory reaction were found in the contralateral testes. This finding seems to indicate that testicular macrophages inhibit acute inflammatory reactions after massive cell death. It has been reported that hCG-induced inflammation-like response in the rat testis is enhanced in macrophage-depleted testes (Bergh *et al.*, 1993b). Macrophages release several factors, such as TGF-β and proopiomelanocortin-derived peptides (Wahl *et al.*, 1989; Li *et al.*, 1991), which inhibit polymorphonuclear leucocyte accumulation (Mason and van Epps, 1989). Whether this inhibitory action is limited to the testis or is also present in other tissues is unknown.

Surprisingly, infiltrating monocytes did not repopulate the testicular interstitium in macrophage-depleted testes, as the number of macrophages was equivalent in EDS or vehicle-treated animals. Recovery of the macrophage population was slow: only 8% of the normal population was present 31 days after depletion, and this was not dependent on the presence of Leydig cells.

The early inflammatory response found after EDS treatment seems to be time-limited since the stimulus (dead Leydig cells) had disappeared by day 3 after EDS treatment and no signs of inflammation or tissue damage (fibrosis) were found after 21 days. However, the possible disturbance of interstitial cell regeneration by acute inflammation cannot be disregarded from the data of the study reported here.

However, it is clear that macrophages play a crucial role in the repopulation of Leydig cells after EDS treatment. Additional studies are needed to determine the role of macrophages in the proliferation–differentiation of Leydig cells either during recovery from EDS treatment or during prepubertal development.

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