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Treatment of rats with hCG induces inflammation-like changes in the testicular microcirculation

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Summary. Adult rats were injected subcutaneously with 50 i.u. hCG and vascular permeability was compared to that in saline-treated control rats by two independent methods. At 4 h after hCG treatment the rats were injected intra-arterially (i.a.) with FITC-labelled macromolecular dextran (M_r 150 000) and the testicular microcirculation was studied *in vivo* by using a fluorescence microscope. Other rats were injected i.a. with a suspension of colloidal carbon and the location of leaking blood vessels was recorded in sections from the testes by light and electron microscopy.

In hCG-treated animals leucocytes were found adhering to the endothelium in post-capillary venules and in these venular segments dextran was leaking into the interstitium. Carbon particles were deposited in the walls of post-capillary venules and leucocytes migrated through open interendothelial cell gaps in hCG-treated animals. In control animals leucocyte adhesion and migration were not observed, the injected dextran remained in the circulation and the blood vessels were not labelled by carbon.

It is suggested that the hCG-induced increase in testicular interstitial fluid volume, like the tissue oedema in inflammation, is caused by a leucocyte-mediated increase in venular permeability.

Introduction

Leydig cells in the rat testis are situated in large lymphatic sinusoids (Fawcett *et al.*, 1973; Clarke, 1976). Consequently, all communication between Leydig cells, the circulating blood and seminiferous tubules occurs through this interstitial fluid (for review see Sharpe, 1984). It is therefore important to understand how the composition and volume of interstitial fluid are regulated.

Treatment with hCG in high doses results in an increase in volume of interstitial fluid. It was originally suggested that this was due to an increase in capillary permeability (Setchell & Sharpe, 1981; Sharpe & Cooper, 1983; Sharpe, 1979, 1980, 1984), but recent studies have offered other explanations. The capillary blood flow in the rat testis shows large local variations (Damber *et al.*, 1986). Normally, periods of high erythrocyte velocity alternate with periods of no flow (Damber *et al.*, 1986). After hCG treatment the precapillary sphincters dilate and capillary blood flow is continuous. This causes an increase in capillary hydrostatic pressure and the increase in interstitial fluid volume may therefore be caused by increased filtration (Widmark *et al.*, 1986). However, preliminary observations indicate that venular permeability is also increased after hCG treatment (Damber *et al.*, 1986). The increase in interstitial fluid volume is accompanied by a migration of polymorphonuclear leucocytes (PMNs) into the interstitial space and preceded by an intravascular leucocyte accumulation (Bergh *et al.*, 1986). The hCG-induced changes in testicular microcirculation may be similar to those seen in acute inflammation. However, the mechanisms by which hCG increases the volume of interstitial fluid and the physiological role of this remain unknown.

Materials and Methods

Animals

Adult male Sprague–Dawley rats (350–400 g) were injected subcutaneously with 50 i.u. hCG (Pregnyl: Organon, Oss, The Netherlands) dissolved in saline (0·9 g NaCl/l). Control rats were injected with saline only. Before the experiment the rats were anaesthetized with pentobarbitone sodium (40 mg/kg) and the tail artery was canulated as described previously (Damber *et al.*, 1986). Vascular permeability was then investigated by 2 different methods.

In-vivo fluorescence microscopy

At 4 h after hCG or saline treatment (5 rats in each group) the testes were exposed via a scrotal incision. The rat was placed on a microscope stage, the testicular surface was covered by a cover glass and testicular microcirculation was studied using a Leitz fluorescence microscope equipped with epifluorescence optics as previously described (Damber *et al.*, 1986). When the rat was lying under the microscope, 150–200 µl of 5% FITC–dextran 150 (M_r 150 000: Pharmacia, Uppsala, Sweden) were injected intra-arterially (i.a.), making the microvessels clearly visible. The testicular microcirculation was recorded on video tape by a video camera which was connected to the fluorescence microscope. This arrangement allowed both direct observations of testicular microcirculation on a monitor screen and also further analysis by play-back.

Labelling of leaking vessels with colloidal carbon

The colloidal carbon technique has been used extensively to label leaking blood vessels in experimental inflammation. Colloidal carbon particles are injected intravascularly. The relatively large particles penetrate open inter-endothelial cell junctions, so called gaps, but are stopped by the underlying basement membrane (Majno *et al.*, 1961; Cotran & Majno, 1964). At 4 h after hCG or saline treatment, 5 rats in each group were injected i.a. with colloidal carbon (1 ml/kg body wt: Pelikan India Drawing ink, Pelikan AG, Hannover, West Germany). The commercial solution was filtered before use. After 1 h the injected carbon has cleared from the blood stream and blackened vasculature caused by sequestration of carbon between endothelial cells and the basement membrane indicated sites of previous leakage (Majno *et al.*, 1961; Cotran & Majno, 1964). The rats were killed at that time and the testes were removed. A small incision was made in one part of the testicular capsule and the testes were fixed by immersion in 4% formaldehyde, 3% glutaraldehyde and 0·05% picric acid in 0·1 M-sodium cacodylate buffer for at least 48 h.

The fixed tissue was examined in 3 ways.

(i) The intact fixed testes were examined using a Zeiss stereomicroscope. Blood vessels under the testicular capsule were studied and the locations of black-stained blood vessels were recorded. Afterwards, the capsule was carefully removed to confirm that the examined vessels were not situated in the capsule.

(ii) Sections, ~1 mm thick, of testicular tissue including a part of the capsule were cut using a razor blade. The sections were cleared in glycerin overnight (Cotran & Majno, 1964). The transparent sections were then examined in transmitted light using a Zeiss stereomicroscope. The presence of black-stained vessels was recorded in relation to the stage of spermatogenesis in the adjacent tubules. This could be done since tubules in different stages of the spermatogenic cycle could be distinguished by their transillumination pattern (Parvinen & Vanha-Perttula, 1972).

(iii) The fixed testes were cut in 1 mm^3 pieces and randomly chosen pieces were post-fixed in 1% OsO_4 in 0·1 M-sodium cacodylate buffer for 2 h, dehydrated and embedded in Epon. Testicular morphology was investigated in 1 µm thick sections stained with toluidine blue using a light microscope and in sections 80 nm thick stained with lead and uranyl acetate by using a Philips 300 electron microscope.

Results

In-vivo fluorescence microscopy

Within 2–3 min after injection of FITC–dextran 150, discrete leakage sites were observed in some, but not all, post-capillary venules in all hCG-treated animals. The leaking venules had diameters ranging from 30 to 40 µm (Figs 1 & 2). No early leakage was observed in arterioles or capillaries. After 20–30 min the whole interstitium was fluorescent and at that time it was no longer possible to exclude minor leakage in capillaries. Using higher magnification we observed leucocytes marginating and adhering to the venular endothelium in hCG-treated animals (Fig. 2). In venular segments with adhering leucocytes, dextran was seen leaking into the interstitium. In control animals some leucocytes were noted in the central blood stream and occasionally leucocytes were seen rolling along the venular endothelium, but without adhesion and the injected dextran

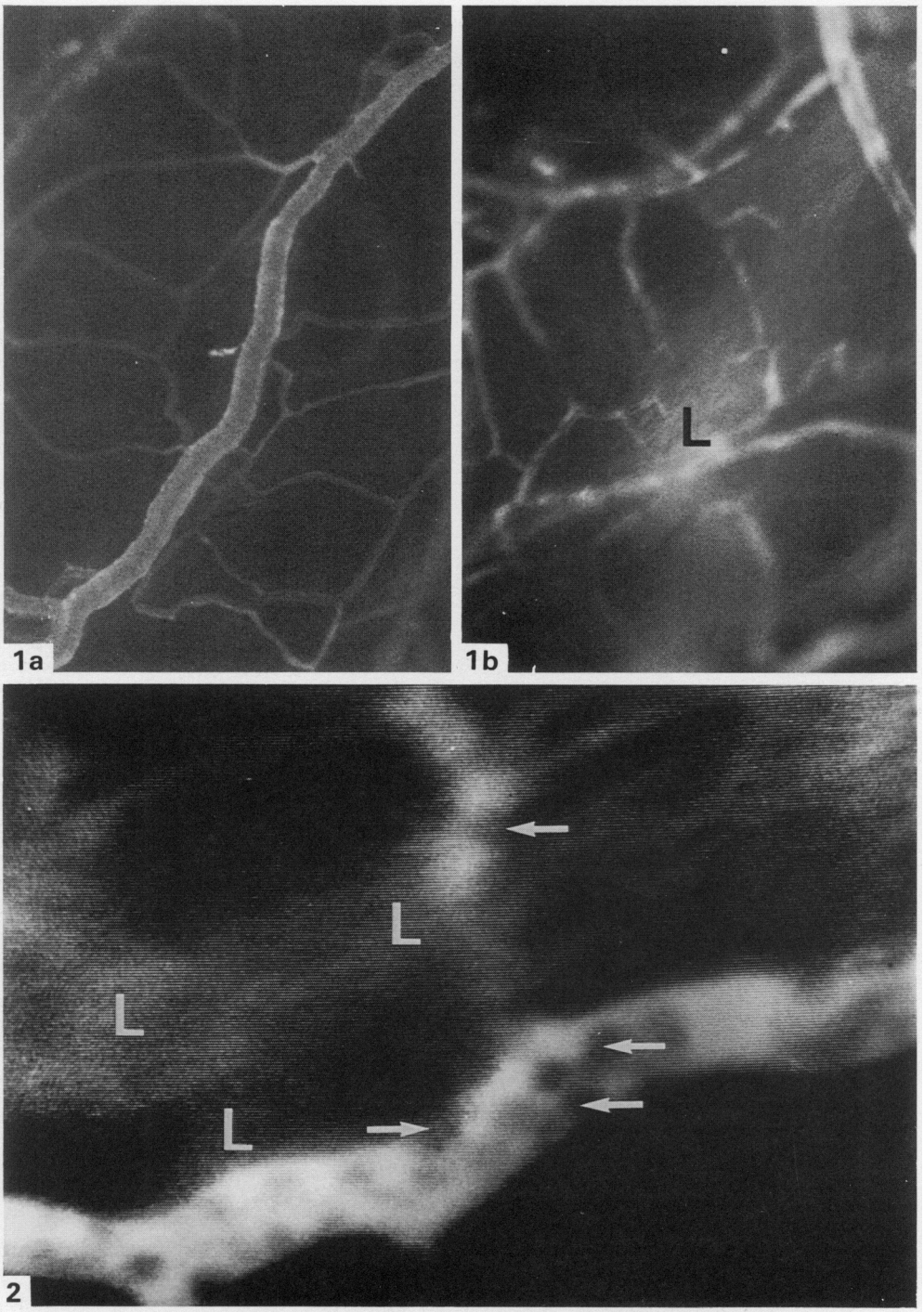
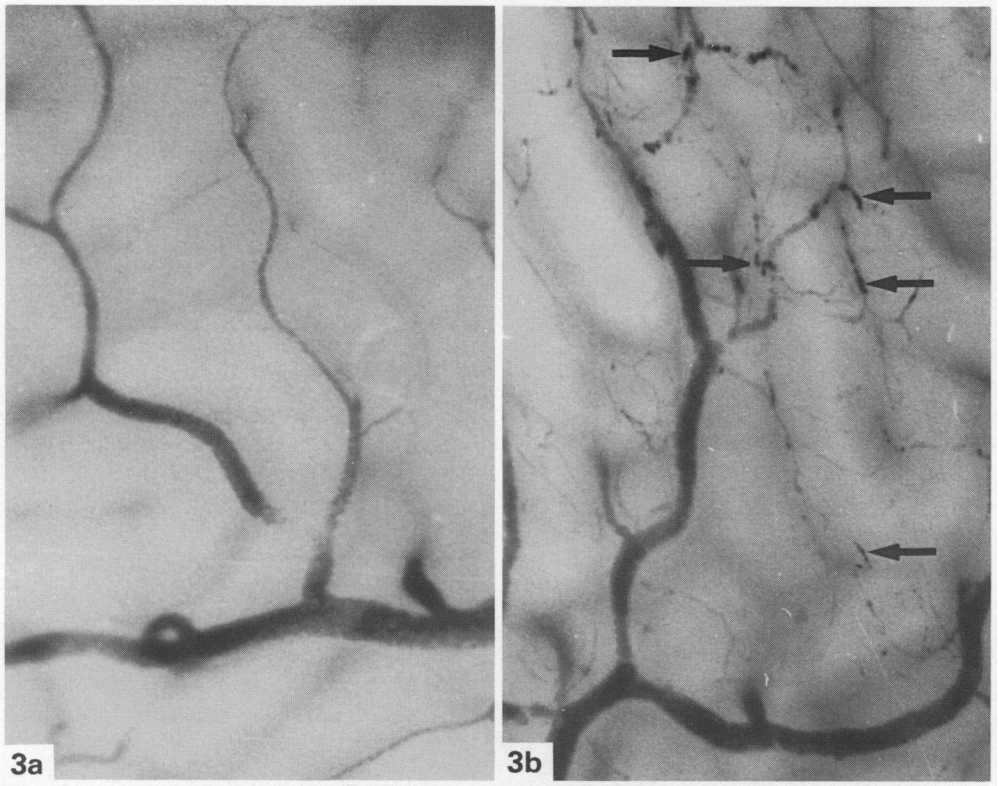


Fig. 1. Fluorescence micrographs of the testicular microcirculation in (a) saline- and (b) hCG-treated animals. The testes were photographed 15 min after injection of FITC–dextran. In the hCG-treated animal dextran was leaking (L) from post-capillary venules but not from capillaries. Blood vessels were not leaking in (a). × 80.

Fig. 2. A photograph from a video recording showing a leaking venule in an hCG-treated animal. Numerous adhering leucocytes are observed (arrows). × 580.



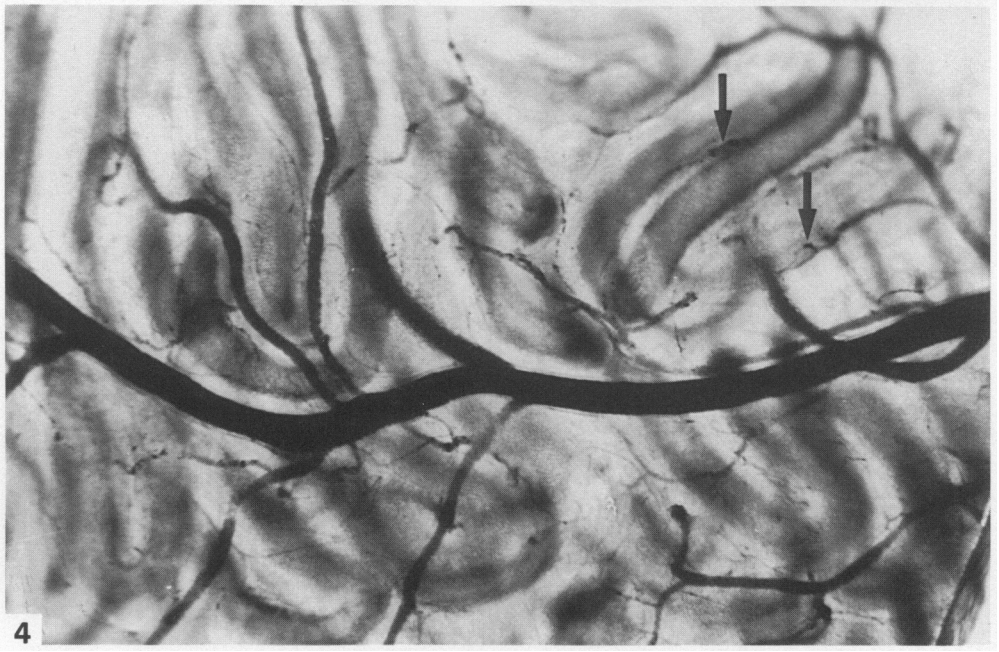


Fig. 3. Stereomicroscopic views of subcapsular blood vessels in the testes of (a) saline- and (b) hCG-treated animals injected with colloidal carbon. In (b) but not in (a) numerous carbon deposits (arrows) are noted in the venular segments. × 42.

Fig. 4. Stereomicroscopic view of blood vessels in a section from an hCG-treated carbon-injected animal. The section is viewed under transmitted light to reveal tubules in different stages of the spermatogenic cycle. By focussing up and down through the section it is possible to demonstrate that leaking blood vessels (arrows) can be observed in relation to tubules in different stages of the spermatogenic cycle. × 30.

remained in blood vessels during the whole observation period of 30 min (Fig. 1a). The intravascular concentration of leucocytes was lower in control than in hCG-treated animals.

Colloidal carbon labelling

When the testicular surface was viewed under the stereomicroscope it was apparent that the subcapsular blood vessels were more prominent and it was easier to detect small-calibre vessels in hCG-treated than in control animals (Figs 3 & 4). In addition to this hyperaemia, numerous black-stained vascular segments were observed in all hCG-treated testes. The leaking vessels were post-capillary venules with diameters of about 15–50 μm. However, carbon deposits were not observed in all post-capillary venules of this size. It was therefore of interest to study whether the location of leakage sites was related to the stage of spermatogenesis in the adjacent tubules. By using a modification of the transillumination technique of Parvinen & Vanha-Perttula (1972), it was possible to recognize tubular segments in various phases of the spermatogenic cycle. No obvious correlation between the location of leakage sites and the spermatogenic cycle was observed (Fig. 4). In control animals black staining of blood vessels was not observed (Fig. 3a).

At the light microscopic level intramural deposits of carbon were observed in venules with diameters ranging from 15 to 50 μm in hCG-treated animals, but staining was never noted in capillaries or arteries. The blood vessels in hCG-treated animals contained more leucocytes than did controls and adhering or migrating leucocytes were often found in close relation to intramural deposits of carbon particles (Fig. 5). Subendothelial deposits of carbon or leucocyte migration were never observed in the control testes.

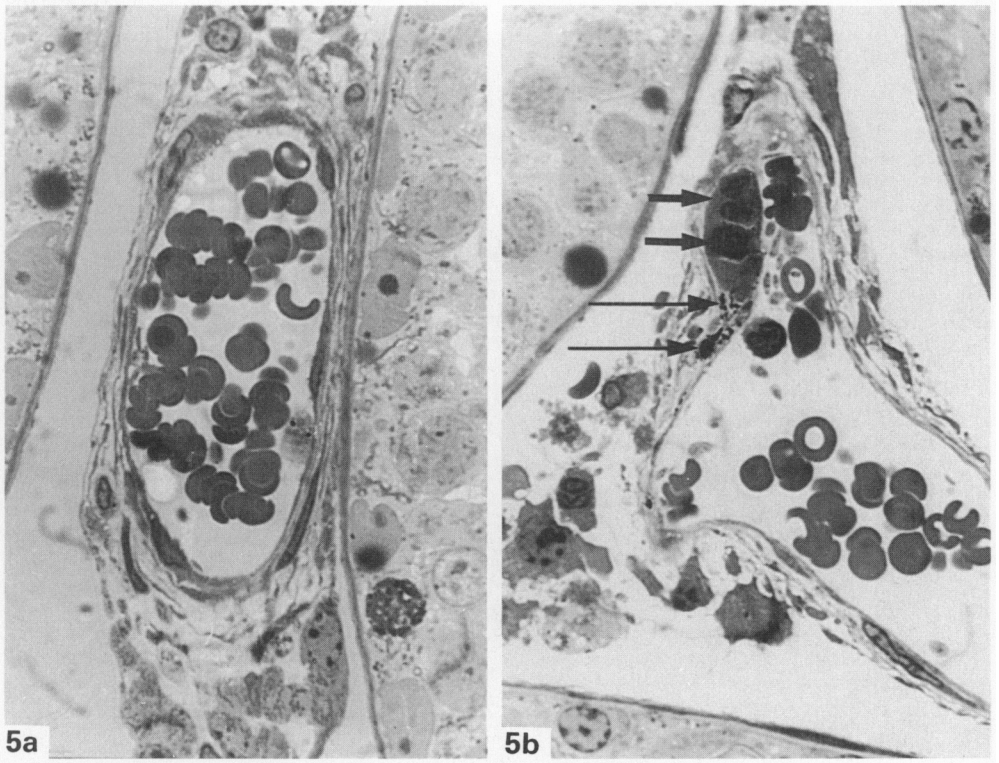


Fig. 5. Light micrographs from the testes of (a) saline- and (b) hCG-treated animals injected with colloidal carbon. In the hCG-treated animals, adhering leucocytes (thick arrows) and intramural deposits of carbon (thin arrows) were observed. In control testes adhering leucocytes or carbon staining was not observed. × 1250.

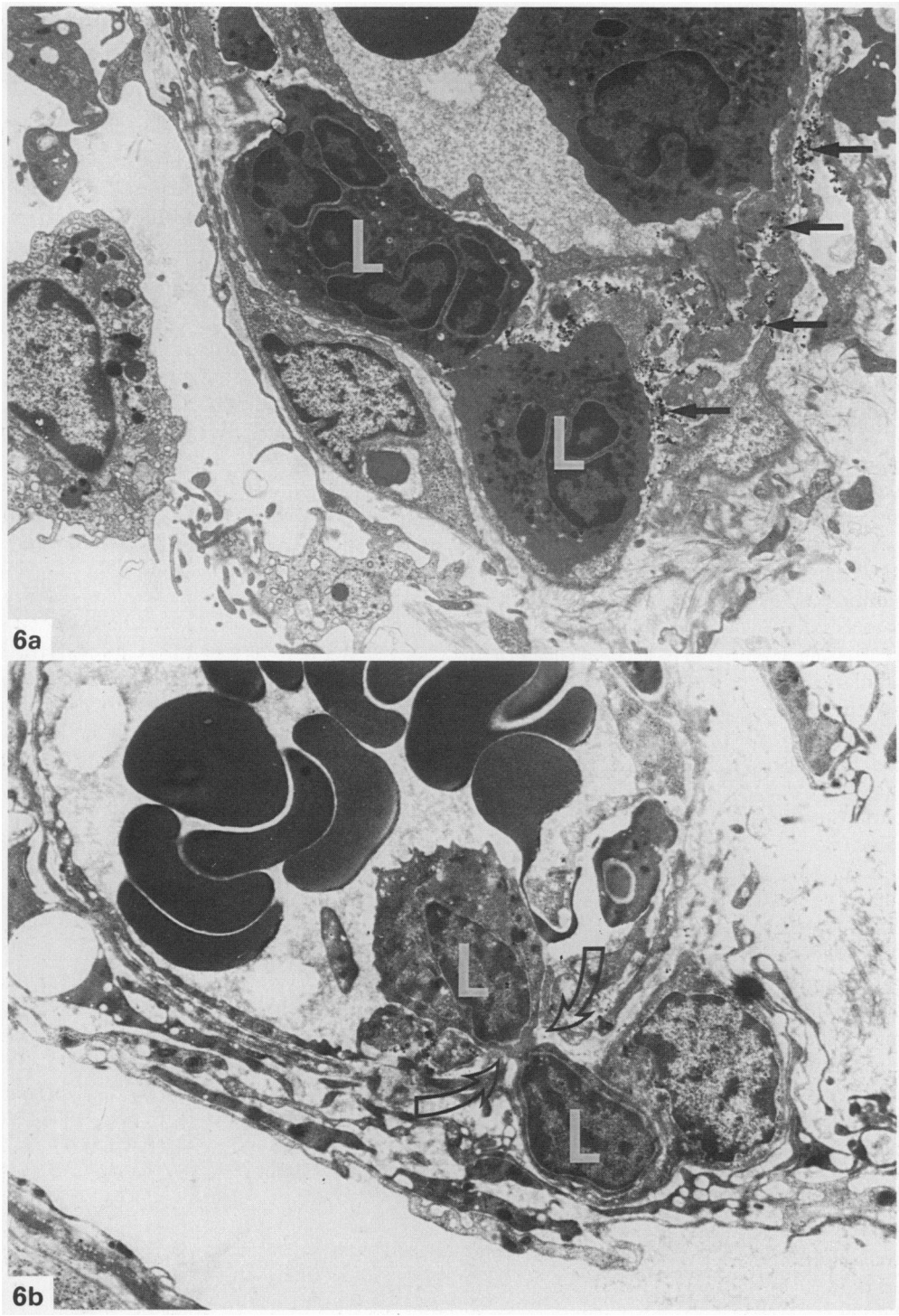


Fig. 6. Electron micrographs from the testis of an hCG-treated animal injected with colloidal carbon. In (a) two intramural leucocytes (L), surrounded by carbon particles (arrows), are observed. In (b) a leucocyte (L) migrating through an open interendothelial cell gap (arrows) is observed. × 6500.

In the electron microscope it was confirmed that the carbon deposits were localized only in venules with diameters of 15–50 μm. Leucocytes migrating through open interendothelial cell gaps and intramural leucocytes surrounded by carbon particles were observed in hCG-treated animals (Fig. 6). Interendothelial cell gaps and carbon staining could not be detected in blood vessels in control animals, even at high resolution, and as observed previously PMNs were not observed in the interstitial space (Bergh *et al.*, 1986). In this study no morphometry of capillary morphology was performed, but no obvious differences in the number of vesicles in the capillary endothelial cytoplasm were observed between hCG- and saline-treated animals and there were no signs of hCG-induced formation of interendothelial cell gaps.

Discussion

The present study shows that the testicular microcirculation can be studied *in vivo* by using a technique originally developed for studying the hamster cheek pouch preparation (Del Maestro *et al.*, 1981a, b; Persson & Svensjö, 1985). Blood vessels lying under the testicular capsule can be observed by using the light microscope, because the transparent capsule is only ~50 μm thick and contains very few blood vessels, although only superficial testicular blood vessels can be studied. The present findings do, however, suggest that these blood vessels are not different from those deeper in the parenchyma. A similar venule leakage as observed *in vivo* was also observed in all parts of the parenchyma by using the colloidal carbon-labelling technique.

This study for the first time demonstrates that hCG treatment results in the formation of interendothelial cell gaps in post-capillary venules and an increased macromolecular permeability in these vascular segments within 4 h. However, no dextran leakage or carbon labelling was observed in capillaries. The subsequent increase in interstitial fluid volume, occurring between 4 and 8 h after treatment (Sharpe, 1984; Widmark *et al.*, 1986), may therefore be caused by an outflow of plasma through leaking venules. An hCG-induced increase in capillary permeability as suggested by Setchell & Sharpe (1981) and Sharpe (1984), especially for molecules smaller than M_r 150 000, cannot be excluded. However, an increased capillary permeability is probably only of marginal importance compared to the large flux of molecules that occurs through the leaking venules.

Since the classical study of Hartman *et al.* (1950) it has been known that hCG treatment results in testicular hyperaemia within 6 h. We have previously shown that hCG treatment alters the capillary blood flow pattern in the testis by dilating precapillary sphincters (Damber *et al.*, 1986). In addition, the concentration of intravascular PMNs is increased within 4 h after hCG treatment and by 8 h PMNs are observed in the interstitial space (Bergh *et al.*, 1986). Treatment with hCG therefore appears to induce changes in testicular microcirculation with striking similarities to those in acute inflammation. Inflammation is characterized by hyperaemia, dilatation of precapillary sphincters, leucocyte adhesion, formation of endothelial cell gaps in post-capillary venules, leucocyte migration, increased permeability for macromolecules and oedema (Hurley, 1978; Williams, 1985). The oedema is caused both by dilatation of precapillary sphincters resulting in increased hydrostatic pressure in the venules, and by increased venular permeability (Williams, 1985). The increase in venular permeability can be induced by two different mechanisms. The inflamed tissue may secrete substances like histamine with direct effects on the venular endothelium resulting in the formation of interendothelial cell gaps (Majno *et al.*, 1961; Williams, 1985; Persson & Svensjö, 1985). Alternatively the inflamed tissue may secret chemotactic factors attracting neutrophil leucocytes and adhering leucocytes open interendothelial cell junctions in post-capillary venules (Wedmore & Williams, 1981; Williams, 1985; Persson & Svensjö, 1985). The effects of histamine and other substances with direct effect on the endothelium are of short duration and influence only venules with diameters of 9–15 μm (Björk *et al.*, 1982; Svensjö & Roempke, 1984; Persson & Svensjö, 1985). In contrast, the leucocyte-mediated effect is more sustained and in addition influences larger venules (Björk *et al.*, 1982; Svensjö & Roempke, 1984). Moreover, histamine

treatment does not, as in most other organs, result in vascular leakage in the testis (Gabbiani *et al.*, 1970). It therefore appears that leucocytes may be involved in mediating the hCG-induced increase in venular permeability.

To our knowledge leucocyte adhesion, formation of large endothelial cell gaps in venules, and leucocyte migration have previously been described only in pathological conditions such as inflammation (with the possible exception of the ovary, see below). The present observation that such phenomena occur in the testis during maximal hormonal stimulation is therefore surprising. Two explanations are possible. In inflammation, leucocytes play a central role in regulating the supply of macromolecules to the tissue (Wedmore & Williams, 1981; Williams, 1985). The testis may be using an inflammation-like mechanism to regulate its supply of macromolecules during maximal hormonal stimulation. This hypothesis requires that the testis secretes chemotactic factors for leucocytes in response to hCG treatment. Whether this occurs is presently not known but indirect evidence suggests that this may be the case. Leydig cells secrete leukotriene B4 *in vitro* (Cooke *et al.*, 1984), one of the most potent leucotactic factors known (Björk *et al.*, 1982; Persson & Svensjö, 1985; Williams, 1985). The testicular interstitium contains macrophages, these macrophages may be activated by hCG treatment (Bergh, 1985a, b) and macrophages in general are an accepted source of chemotactic factors (Takemura & Werb, 1984). Observations of the ovary also suggest that leucocytes could play a physiological role in the gonads: the LH peak that occurs after mating results in perifollicular leucocyte accumulation, interstitial oedema, and anti-inflammatory drugs inhibit ovulation (Zachariae *et al.*, 1958; Espey, 1980).

Alternatively, the leucocyte-induced venular leakage could be a sign of testicular pathology caused by stimulation with a non-physiological hormone in high doses. If so, this information is of great importance since hCG in doses of this magnitude has generally been used in in-vivo studies of testicular endocrine function. We have previously observed that hCG in doses of $\geqslant 50$ i.u. is needed to induce leucocyte accumulation and to increase interstitial fluid volume. In our hands, 50 i.u. was roughly the lowest dose giving a maximal testosterone response. A half-maximal response, obtained with 12·5 i.u., did not result in an increase in interstitial fluid volume or in leucocyte migration (Bergh *et al.*, 1986; Widmark *et al.*, 1986). Apparently the hCG-induced inflammation-like change in testicular microcirculation only takes place during maximal stimulation. It remains to be studied whether similar changes can be induced by an endogenous LH pulse.

In conclusion the hCG-induced increase in testicular interstitial fluid volume is probably related to a leucocyte-mediated formation of interendothelial cell gaps in post-capillary venules. The physiological significance of this observation remains unknown.

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