Use of antibodies against LH receptor, 3 β -hydroxysteroid dehydrogenase and vimentin to characterize different types of testicular tumour in dogs

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Testicular tumours in dogs are of Sertoli cell, Leydig cell or germinal origin and mixed tumours are also frequently observed. The cellular components of mixed tumours are usually identified by histological examination but sometimes this is difficult. In this study, a panel of specific antibodies was used to identify the different cell types in testicular tumours by immunohistochemistry. Leydig cells were identified using an antibody against the LH receptor and an antibody against the steroidogenic enzyme 3 β -hydroxysteroid dehydrogenase (3 β -HSD), both of which are characteristic of Leydig cells in testes. Sertoli cells were identified using an antibody against the intermediate filament vimentin. Seminoma cells did not stain with any of these antibodies. Vimentin was used only in histologically complex cases. Eighty-six tumours, diagnosed histologically as 29 Sertoli cell tumours, 25 Leydig cell tumours, 19 seminomas and 13 mixed tumours, were studied. Feminization was observed in 17 dogs. Leydig cell tumours stained positively with the antibodies against the LH receptor and 3 β -HSD, whereas seminomas and Sertoli cell tumours were negative (unstained). The antibody against vimentin stained both

Sertoli and Leydig cells, and tumours arising from these cells, but not seminomas. Immunohistochemistry revealed that three tumours identified histologically as Sertoli cell tumours were actually Leydig cell tumours. In 14 dogs the histological diagnosis appeared to be incomplete, as mixed tumours instead of pure types of tumours were identified in 11 dogs, and in three dogs mixed tumours appeared to be pure types. Hence, the histological diagnosis was insufficient in approximately 20% of dogs. Furthermore, immunohistochemical analysis of testis tumours revealed that feminization occurred in dogs with Sertoli cell tumours or Leydig cell tumours and their combinations, but not in dogs with a seminoma. In conclusion, incubation with antibodies against LH receptor and 3 β -HSD proved to be a consistently reliable method for identification of Leydig cell tumours in dogs. Vimentin can be used to discriminate between Sertoli cell tumours and seminomas. Overall, this panel of antibodies can be very useful for determination of the identity of testicular tumours in which histological characterization is complicated and the pathogenesis of feminization is not clear.

Introduction

Testicular tumours are much more common in dogs than in other mammals. The prevalence varies from 0.068 to 4.6% in male dogs (Machado *et al.*, 1963; Dorn *et al.*, 1968; Bastianello, 1983; Reifinger, 1988) compared with 0.006% in men (Grootenhuys *et al.*, 1990). In studies that included many old dogs, up to 60% of the aged animals appeared to have testicular neoplasms (Mosier, 1989;

Weller *et al.*, 1995). Looijenga *et al.* (1994) reported a peak prevalence of seminomas at 1 year of age.

The three main types of testicular tumour in dogs are Sertoli cell tumours, seminomas and Leydig cell tumours (also referred to as interstitial cell adenomas), and these tumours occur at about equal frequencies (Nielsen and Lein, 1974; Nielsen and Kennedy, 1990). All three tumour types can occur simultaneously in one testis, either as separate tumours or as tumours with intermingled cell types (McEntee, 1990). This feature is rather unusual, as these tumour cells are derived from different cell lineages: Sertoli cells are of epithelial origin, Leydig cells are of mesenchymal origin and seminomas are derived from spermatogenic cells of the seminiferous epithelium (Feldman and Nelson, 1987). In dogs, testicular tumours

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are often bilateral and are often mixed germ cell–somatic cell tumours (Nielsen and Kennedy, 1990), whereas in men testicular tumours are usually unilateral and are rarely of a mixed type. Although seminomas and Sertoli cell tumours in dogs are considered to be potentially malignant, they seldom metastasize (Vitellozzi *et al.*, 1998). Local intratesticular tumour growth is more frequent in dogs, whereas in men, seminomas in particular are usually highly malignant (Swerdlow, 1993).

In general, Sertoli cell tumours, seminomas and Leydig cell tumours are rare in domestic animals. Leydig cell tumours have been reported occasionally in bulls (Nielsen and Kennedy, 1990; Saez, 1994) horses and mules (Jubb, 1963), and seminomas have been observed in rams (Nielsen and Kennedy, 1990). In men, germ cell tumours account for approximately 95% of testicular neoplasms and occur most frequently in young adults below 40 years of age (Looijenga *et al.*, 1994). Human seminomas are presumed to be derived from gonocytes, the fetal–neonatal spermatogonial stem cells, whereas seminomas are thought to be of spermatocytic origin in dogs (Looijenga *et al.*, 1994).

Testicular tumours may cause feminization in dogs, which is characterized by gynaecomastia, atrophy of the contralateral testis, a pendulous prepuce and attractiveness to other male dogs, as well as a fatal bone marrow depression in severe cases (Bloom, 1954; Cotchin, 1960; Nielsen and Lein, 1974). Feminization has been attributed to excessive secretion of oestrogens by the tumour (Rijnberk, 1996), although there is controversy regarding the capacity of different types of tumour to secrete oestrogens. In dogs, feminization has been reported as a consequence of the presence of Sertoli cell tumours and, to a lesser extent, the presence of Leydig cell tumours, with a prevalence of 19 and 5%, respectively (Peters and van Sluijs, 1996). Seminomas have been associated only rarely with feminization. In men, feminization occurs primarily in conjunction with Leydig cell tumours, which is not surprising given that these cells are the main source of oestrogens in human testes (Bercovici *et al.*, 1985; Haas *et al.*, 1989).

Considering the frequent occurrence of mixed tumours in dogs, the controversy about the type of tumour responsible for the excessive oestrogen secretion resulting in feminization may be caused by a failure to identify specific cellular components of mixed tumours. For example, when using classical histology it can be difficult to distinguish a lipid-rich Sertoli cell tumour from a Leydig cell tumour, in which lipids also accumulate in the cytoplasm of the tumour cells (Cotchin, 1960). In addition, areas that histologically resemble a seminoma can be found in some Sertoli cell tumours (Nielsen and Kennedy, 1990). The use of cell-specific markers could be helpful in such cases to discriminate between different types of tumour cell. Discrimination between cell types would not only be of great importance in dogs, but could also be helpful for the correct diagnosis of testicular tumours in man. For example, it can be difficult to distinguish histologically testicular

adrenal rest tumours from Leydig cell tumours in patients that have congenital adrenal hyperplasia (Solish *et al.*, 1989; Garcia-Mayor *et al.*, 1992; Jódar-Gimeno *et al.*, 1997). Furthermore, different areas of testicular stromal tumours may show an admixture of Leydig and Sertoli cells (Mostofi and Price, 1973) that could be recognized more easily using immunohistochemical markers.

The aim of the present study was to investigate the value of immunohistochemistry compared with classical histological identification of Sertoli cell tumours, seminomas and Leydig cell tumours. Leydig cell tumours were identified using antibodies against LH receptors, which are characteristic of Leydig cells, and an antibody against the steroidogenic enzyme, 3 β -hydroxysteroid dehydrogenase (3 β -HSD). This enzyme has been used as a marker for Leydig cells in other species such as rats and humans (Faustin *et al.*, 1995; Ivell *et al.*, 1997; Mueller *et al.*, 1998). An antibody against vimentin, the major intermediate filament in Sertoli cells (Franke *et al.*, 1979), was used to distinguish Sertoli cell tumours with a complex histology from seminomas.

Materials and Methods

Animals

Testes were collected from dogs with testicular tumours ($n = 86$) and from dogs without testicular disease ($n = 8$). All tumours were detected by physical examination. Dogs with a testicular tumour represented 37 different breeds and 16 dogs were of mixed breed. The age of the dogs varied from 4 to 16 years. The tumour was present in an undescended testis in seven dogs. Signs of feminization were observed in 17 of 86 dogs.

Antibodies

The LH receptor monoclonal antibody (P1B4) was a gift from J. Wimalasena (Department of Obstetrics and Gynecology, University of Tennessee, Knoxville, TN). The antibody was raised against purified rat LH receptors, as described by Indrapichate *et al.* (1992). The antibody was found to bind specifically to LH receptors in gonads of different species and has been shown to bind specifically to the Leydig cells in testes (Bukovsky *et al.*, 1993; Faustin *et al.*, 1995).

The 3 β -HSD polyclonal antibody was a gift from V. Luu-The (Laval University, PQ). This antibody was obtained by immunization of rabbits with 3 β -HSD purified from human placenta and has been used as a Leydig cell marker in adult rats (Teerds *et al.*, 1999). All tumours ($n = 86$) and normal testes ($n = 8$) were incubated with the antibodies against LH receptor and 3 β -HSD.

The vimentin antibody was obtained from Biogenex (San Remon, CA). Vimentin is the major intermediate filament present in the cytoplasm of Sertoli cells and has been used to identify these cells in other studies (Franke *et al.*, 1979; Patnaik and Mostofi, 1993). This antibody has been

used to discriminate between Sertoli cell tumours and seminomas (Patnaik and Mostofi, 1993).

Immunohistochemical staining

After dissection, the entire testis was cut into pieces of approximately 1 cm × 1 cm × 0.5 cm and these were fixed in 4% (v/v) buffered formalin for at least 24 h. One or two blocks that contained macroscopic tumour tissue were selected from each testis and these were embedded in paraffin wax. Several successive sections (5 µm thickness) were cut from each block. One section per testis was stained by eosin and Mayer's haematoxylin for histological examination at the Department of Pathology; the other sections were incubated with the LH receptor, 3β-HSD or vimentin antibodies.

For immunohistochemical purposes the sections were deparaffinized and endogenous peroxidase was blocked by immersion in 1% (v/v) hydrogen peroxide in methanol for 30 min. The slides were washed six times for 5 min in Tris-buffered saline (TBS; 0.01 mol l⁻¹, pH 7.4) and for 30 min in glycine TBS (0.1 mol glycine l⁻¹) to demask epitopes. Subsequently, the slides were washed six times for 5 min in TBS and blocked with 10% normal goat serum in TBS for 30 min. The slides were incubated overnight at 4°C with the LH receptor P1B4 monoclonal antibody at a 1:5000 dilution or with the 3β-HSD polyclonal antibody at a 1:500 dilution in TBS to which 0.05% (w/v) acetylated BSA was added (Aurion, Wageningen). After these incubations, the slides were washed again with TBS and incubated with a biotinylated goat-anti-mouse antibody (Vector Laboratories, Burlingame, CA) for the detection of LH receptor or with a biotinylated goat-anti-rabbit antibody (Vector Laboratories) for the detection of 3β-HSD for 60 min at a 1:200 (LH receptor) or 1:100 (3β-HSD) dilution in TBS-acetylated BSA at room temperature. The slides were washed thoroughly with TBS and incubated with the avidin-biotin (AB) complex of the ABC staining kit (Vector Laboratories) for 60 min, dilution 1:1500 (LH receptor) or 1:750 (3β-HSD) in TBS containing 0.05% (w/v) acetylated BSA. The ABC solution was prepared at least 15 min before use to allow formation of complexes. The slides were washed twice for 5 min in TBS followed by 15 min in a Tris-HCl solution (0.05 mol l⁻¹, pH 7.5). 3,3'-Diaminobenzidine tetrachloride (DAB; Sigma Chemical Co, St Louis, MO) was prepared at a concentration of 0.6 mg ml⁻¹ in Tris-HCl to which 0.03% (v/v) hydrogen peroxide was added. The sections were incubated with this DAB solution for approximately 3 min and counterstained with Mayer's haematoxylin.

The vimentin monoclonal antibody (Biogenex) was diluted 1:150 in PBS-acetylated BSA. A goat-anti-mouse antibody (Vector Laboratories) diluted 1:200 in PBS-acetylated BSA served as the secondary antibody and the ABC complex was diluted 1:1000 in PBS-acetylated BSA. The other procedures were identical to those described above except that PBS (pH 7.4) was used as washing buffer.

Normal rabbit serum (3β-HSD) or normal mouse serum (LH receptor, vimentin) replaced the primary antibodies in the control experiments. Non-specific staining was negligible in all tumour tissues (representative sections are shown in Fig. 1).

Histology

The sections of each testis stained with eosin and Mayer's haematoxylin were studied at the Department of Pathology by the same pathologist, who did not know the results of the immunohistochemical analysis. The diagnosis was made according to the guidelines of the WHO (Nielsen and Kennedy, 1990). According to these criteria, the cells of a Sertoli cell tumour consist of delicate vacuolated cells, often elongated and slender, with indistinct borders. The cells are frequently lined up like a palisade, perpendicular to the tubular basement membrane. The nuclei are ovoid and vesicular, with small nucleoli, and mitoses are rare. The cytoplasm contains many lipid-filled vacuoles. The tubular basement membranes may appear either as thin, barely visible septa or as thick fibrous strands. Tumour cells may penetrate the basement membrane of the seminiferous tubules and invade the surrounding cells. The more progressive form of Sertoli cell tumour consists of large sheets of uniform cells without recognizable tubular structures. Fibrous septa or vascular stroma separate the tumour masses. Cystic haemorrhages often occur.

Seminoma cells are large, uniform, polyhedral cells resembling spermatogonia. The tumour cells have large vesicular nuclei and prominent nucleoli. The more advanced stage consists of diffusely infiltrating solid sheets of uniform tumour cells that push away tubules, Leydig cells and rete testis. The cells are tightly packed, well delineated, large and round or polyhedral. The nuclei are vesicular and one or two prominent nucleoli are present. Large single nuclei and multinucleated giant cells are frequently present. Vacuolated histiocytes can result in a 'starry sky' appearance. Mitoses often occur and so do lymphocyte infiltrates that are characteristic of seminomas. Sertoli cell tumours as well as seminomas can be intratubular or diffuse.

Leydig cell tumours consist of sheets of uniform, polyhedral and well-delineated cells. Around blood vessels the tumour cells can become elongated and arranged like a radiating palisade. The cytoplasm may contain clear vacuoles of different sizes. The nuclei are small and round, and small single nucleoli are present. Mitoses are generally absent. As it can be difficult to distinguish between hyperplasia of Leydig cells and a Leydig cell tumour, additional criteria have been used to identify this type of tumour. According to McEntee (1990), Leydig cell hyperplasia can be defined as an increase in the number of Leydig cells between tubules without destruction or displacement of surrounding tubules. Therefore, in the present study the process was scored as a local tumour when a discrete nodule or group of interstitial cells locally replaced or displaced the seminiferous tubules.

Fig. 1. Serum control sections of canine testicular tumours, incubated with normal mouse serum (a,e) or normal rabbit serum (c). No brown background staining could be detected. (a) Leydig cell tumour diagnosed histologically as a Sertoli cell tumour. (b) Same tumour as in (a) incubated with the LH receptor antibody. All tumour cells show positive staining. (c) Combined Sertoli cell tumour and Leydig cell tumour diagnosed histologically as a Leydig cell tumour. (d) Same

Combinations of these types of tumours can consist of intermediate differentiation of Sertoli cells and Leydig cells, Sertoli cells and seminomas, or Leydig cells and seminomas. Combinations of three types may also occur within a single testis. When the tumours are not intermingled but remain present as individual nodules they usually retain their histological features. If the different types of tumour grow intermingled it can become difficult to recognize them separately using histology (Nielsen and Lein, 1974; Nielsen and Kennedy, 1990).

The results of the histological examination were compared with those of the immunohistochemical staining with the antibodies against LH receptor and 3 β -HSD to reach a final conclusion about the type or types of testicular tumour present. In cases of mixed tumours, and in Sertoli cell tumours and seminomas that did not show their typical characteristics, additional staining with the antibody against vimentin was applied.

Results

Incubation with the LH receptor and 3 β -HSD antibodies showed consistent results. In normal testes Leydig cells stained positively with both antibodies, whereas staining was never observed in the seminiferous tubules, in which the Sertoli and germ cells are present. The vimentin antibody stained both Sertoli and Leydig cells but did not stain germ cells. Therefore, this antibody was used to stain a limited number of samples ($n = 49$) to possibly exclude the presence of seminoma in samples diagnosed histologically as mixed seminoma–Sertoli cell tumours.

Twenty-nine tumours were diagnosed histologically as Sertoli cell tumours, 25 as Leydig cell tumours, 19 as seminomas and 13 as mixed tumours. Feminization was present in 11 dogs with a histologically diagnosed Sertoli cell tumour, in one dog with a histologically diagnosed seminoma, in four dogs with a histologically diagnosed Leydig cell tumour and in one dog with a mixed Sertoli cell tumour and seminoma. Tumours identified histologically as Leydig cell tumours stained positively with the LH receptor and 3 β -HSD antibodies (Fig. 2d,e), whereas seminomas (Fig. 2g,h) and Sertoli cell tumours were negative (Fig. 2a,b). The vimentin antibody stained Sertoli cells but also stained Leydig cells and the tumours of both of these cell types (Fig. 2c,f). Vimentin stained Sertoli cell tumours consistently, whereas the staining of Leydig cell tumours was heterogeneous. Sometimes intensive staining was observed in some areas, whereas in other areas staining was negligible. Vimentin staining was never observed in seminomas (Fig. 2i).

All 86 tumours were stained with the antibodies against LH receptor and 3 β -HSD. Staining with the antibody

against vimentin was carried out in the group of 13 histologically diagnosed mixed tumours and in Sertoli cell tumours ($n = 15$) and seminomas ($n = 11$) that contained areas with less characteristic aspects. Five Leydig cell tumours containing areas that did not show homogeneous staining with the Leydig cell specific antibodies were also incubated with the vimentin antibody. In addition, two Leydig cell tumours with classical histology and three normal testes were stained with the antibody against vimentin.

Comparison of histological with immunohistochemical results revealed an incorrect histological diagnosis in three dogs and an incomplete diagnosis in 14 dogs (Table 1). Three histologically defined Sertoli cell tumours showed positive staining with the antibodies against the LH receptor (Fig. 1b) and 3 β -HSD. Therefore, it was concluded that these tumours were Leydig cell tumours and that the histological diagnosis was incorrect in these cases.

Dogs with an incomplete diagnosis had multiple types of testicular tumour instead of a single type of tumour or vice versa. Three histologically diagnosed mixed Sertoli cell and Leydig cell tumours appeared to be pure Sertoli cell tumours after immunohistochemical staining with the LH receptor and 3 β -HSD antibodies. In four dogs with a histologically diagnosed Leydig cell tumour, immunohistochemical staining with the antibodies against LH receptor, 3 β -HSD and vimentin revealed a mixed tumour composed of both Leydig cell tumour and Sertoli cell tumour tissue (Fig. 1d). Two of these dogs had signs of feminization. Two other dogs with a Leydig cell tumour confirmed both by histology and immunohistochemistry also showed signs of feminization. Additional testicular tissue of these dogs was examined immunohistochemically with the three antibodies to ascertain that no other tumour was present. Furthermore, one presumed mixed tumour in which only Leydig cell components were recognized histologically was found to be a combined Sertoli cell tumour and Leydig cell tumour after staining with the panel of antibodies.

Five histologically diagnosed Sertoli cell tumours appeared to be mixed tumours after immunohistochemical staining with the panel of antibodies. Four of these were combined Sertoli cell tumours and Leydig cell tumours, as parts of the tumour tissue stained positively with the LH receptor and 3 β -HSD antibodies, and the other tumour was a combination of a Sertoli cell tumour and a seminoma. The latter became clear after additional staining with the vimentin antibody. One dog with a histologically diagnosed seminoma and signs of feminization appeared to have a mixed Sertoli cell tumour and seminoma (Fig. 1f) after immunohistochemical staining with vimentin. On the basis of immunohistochemistry, there were 24 Sertoli cell tumours, 23 Leydig cell tumours, 18 seminomas and 21

tumour as in (c) stained for the presence of 3 β -hydroxysteroid dehydrogenase; only the Leydig cells are stained positively (brown). (e) Mixed Sertoli cell tumour and seminoma diagnosed histologically as a seminoma. (f) Same tumour as in (e) stained with the vimentin antibody. Sertoli cells were positive (brown) and seminoma cells were negative (blue). Sertoli cells are indicated by arrows, Leydig cells by arrowheads and seminoma cells by asterisks. Scale bar represents 15 μ m.

Fig. 2. (a) Sertoli cell tumour with well-defined characteristics (see Materials and Methods) stained with an antibody against 3 β -hydroxysteroid dehydrogenase (3 β -HSD). No positive Sertoli cells with brown staining in the cytoplasm could be observed. (b) A different massive Sertoli cell tumour with many lipid droplets in the cytoplasm stained with the LH receptor antibody. Histologically this tumour resembles the Leydig cell tumour, as shown in (e). No positive staining was observed. (c) Sertoli cell tumour stained with the vimentin antibody. The positive Sertoli cells show brown staining in the cytoplasm. (d) Leydig cell tumour with well-defined characteristics (see

combined tumours. Overall, the histological diagnosis was insufficient in 20% of the cases that were examined. In four of 17 dogs (25%) with signs of feminization the final diagnosis was changed after investigation with the panel of antibodies. Feminization occurred in conjunction with Sertoli cell tumours, in mixed tumours that included a Sertoli cell tumour and in Leydig cell tumours, but not in seminomas (Table 1).

Discussion

The present results indicate that identification of testicular tumours in dogs can be improved greatly using specific antibodies to identify Leydig cells and Sertoli cells. Incubation with an LH receptor antibody and a 3 β -HSD antibody has been found to be a very consistent and reliable method of identifying Leydig cells and Leydig cell tumours in rats and humans (Faustin *et al.*, 1995; Ivell *et al.*, 1997; Mueller *et al.*, 1998). In the present study, it became clear that these two specific markers stained both normal canine Leydig cells and Leydig cell tumours. Thus, either of these markers can be used to detect Leydig cells in dogs. The localization of these two antigens was identical to that observed previously in rat testes (Teerds *et al.*, 1999). The use of Leydig cell-specific antibodies to characterize testicular tumours in dogs considerably increased the accuracy of the pathological diagnosis. In humans, the LH receptor antibody could be useful for distinguishing Leydig cell tumours from testicular adrenal rest tumours in those patients with congenital adrenal hyperplasia. It is known that adrenal rest tumours in the testis as a complication of congenital adrenal hyperplasia are indistinguishable histologically from testicular Leydig cells (Jódar-Gimeno *et al.*, 1997). However, as there are no indications that adrenal rest tumour cells have LH receptors (K. J. Teerds, unpublished), this antibody could potentially considerably improve the characterization of this type of testicular tumour. Other applications in human pathology are also possible. For example, as ovarian theca cells possess LH receptors, the antibody against these receptors could be used to identify these cells in pure or mixed ovarian tumours.

The results of the present study indicate clearly that the standard morphological criteria given by the WHO (Nielsen and Kennedy, 1990) are not always sufficient to identify testicular tumours in dogs correctly. In some cases it is especially difficult to discriminate between lipid-rich Sertoli cell tumours and Leydig cell tumours in which lipids also tend to accumulate in the cytoplasm (Cotchin,

1960). The histological criteria of the WHO suggest that a canine Sertoli cell tumour differs from both a seminoma and a Leydig cell tumour by the elongated shape of the Sertoli cells, the orientation of the cells, the presence of intracytoplasmic filaments, lipid accumulation, the shape of mitochondrial cristae and the abundance of other organelles (Nielsen and Kennedy, 1990). As Sertoli cell tumours in their malignant form have a lower tendency to palisade and because both Sertoli cells and Leydig cells can both be rich in lipids (Nielsen and Kennedy, 1990), a correct histological diagnosis may be difficult. This observation was also made in the present study, as three tumours characterized immunohistochemically as Leydig cell tumours had been classified incorrectly as Sertoli cell tumours using conventional histological staining methods.

The correct identification of testicular tumour cells is important with regard to the type of tumour that is responsible for feminization in dogs. Feminization is probably the result of excessive production of oestrogens, as has been shown for Sertoli cell tumours in dogs (Rijnberk, 1996). It was generally believed that when a testis tumour caused feminization in a dog, the Sertoli cells were the source of the oestrogen production, although there is some controversy (Peters and van Sluijs, 1996). Feminization in dogs has also been reported to be associated with Leydig cell tumours and seminomas, but it is not clear whether feminization was due to hormone production by the histologically diagnosed tumour or to an inability to detect a coexisting Sertoli cell tumour as the source of oestrogen production (Cotchin, 1960; Lipowitz *et al.*, 1973).

Leydig cell tumours are the main source of oestrogen production in men, which can result in feminization (Abiaad *et al.*, 1994). In the present study, four dogs with histologically diagnosed Leydig cell tumours and one dog with a histologically diagnosed seminoma showed signs of feminization. Immunohistochemical staining with the antibody panel revealed that two of the Leydig cell tumours were actually mixed Leydig cell tumours and Sertoli cell tumours, and the seminoma proved to be a mixed seminoma and Sertoli cell tumour. These findings indicate that the Sertoli cell tumour component of these tumours could be responsible for the signs of feminization. However, the other two histologically diagnosed Leydig cell tumours were still considered to be pure Leydig cell tumours after additional pieces of the tumour were stained with the Leydig cell specific antibodies, implying that Leydig cell tumours can cause feminization. The results of the present study indicate that both mixed Sertoli cell tumours and Leydig cell tumours can cause feminization, and that pure

Materials and Methods) stained with the 3 β -HSD antibody. The tumour cells all stained positively (brown). (e) A different Leydig cell tumour with many lipid droplets in the cytoplasm stained with the LH receptor antibody. All tumour cells stained positively (brown). (f) The same tumour as in (e) stained with the vimentin antibody showing a positive (brown colour) reaction. (g,h,i) Seminoma stained with (g) the 3 β -HSD antibody, (h) the LH receptor antibody and (i) the vimentin antibody. None of the seminomas (g,h,i) derived from different dogs stained positively with any of these antibodies. Sertoli cells are indicated by arrows, Leydig cells by arrowheads and seminoma cells by asterisks. Scale bar represents 15 μ m.

Table 1. Testicular tumours as classified via conventional histological staining and according to immunohistochemistry

Histology	Immunohistochemistry						
	Sertoli cell tumour	Seminoma	Leydig cell tumour	Mixed Sertoli and Leydig cell tumour	Mixed Sertoli cell tumour and seminoma	Mixed Leydig cell tumour and seminoma	Mixed Sertoli cell, Leydig cell and seminoma tumour
Sertoli cell tumour	21 ¹⁰	–	3	4 ¹	1	–	–
Seminoma	–	18	–	–	1 ¹	–	–
Leydig cell tumour	–	–	20 ²	5 ²	–	–	–
Mixed Sertoli cell and Leydig cell tumour	3	–	–	1	–	–	–
Mixed Sertoli cell tumour and seminoma	–	–	–	–	2 ¹	–	–
Mixed Leydig cell tumour and seminoma	–	–	–	–	–	5	–
Mixed Sertoli cell, Leydig cell and seminoma tumour	–	–	–	–	–	–	2
Total (immunohistochemistry)	24 ¹⁰	18	23 ²	10 ³	4 ²	5	2
							86 ¹⁷

Each vertical column represents the number of dogs per type of tumour as defined with immunohistochemistry and each horizontal row represents the number of dogs per type of tumour as defined by histology.

Superscript numbers represent the number of dogs with signs of feminization.

Leydig cell tumours can also cause feminization. Pure seminomas were not found in dogs with feminization. Additional immunohistochemical studies should be performed with an antibody against aromatase (the enzyme responsible for conversion of testosterone into oestradiol) to determine whether Leydig cells in dog testes are capable of oestrogen production, as is the case in humans and rats (Saez, 1994). Concomitantly, Leydig cells or Leydig tumour cells could be isolated and their oestradiol production *in vitro* could be measured.

A mixed testicular tumour was found in 21 of 86 dogs. Immunohistochemistry revealed more mixed tumours than did histology. The histological diagnosis was incomplete in 13 of 21 cases of mixed testicular tumours (62%), indicating that it is easy to misinterpret histological features. An incomplete characterization of mixed tumours occurred more frequently when Leydig and Sertoli cell tumours were involved than in cases of seminomas. Most seminomas differ morphologically from Sertoli cell tumours and Leydig cell tumours because of their characteristic histological appearance with lymphocyte infiltrates, although some anaplastic Sertoli cell tumours have areas resembling the morphology of seminomas. It is possible that Sertoli cell tumours and seminomas occur simultaneously without clearly demarcated borders. In such cases, the gradual transition from Sertoli cell tumour tissue to the smaller areas with seminoma cells can easily be overlooked. Previously it was not clear whether such areas in a tumour represented atypical differentiation of Sertoli cells or were a true mixture of Sertoli cell tumour and seminoma (Nielsen and Kennedy, 1990). Studies using the antibody against vimentin, which stains Sertoli cells but not seminoma, showed that two tumours diagnosed histologically as a pure Sertoli cell tumour and a pure seminoma, were actually both combined Sertoli cell tumour and seminoma.

Vimentin is the major intermediate filament in Sertoli cells and the antibody against it can aid in the histological classification of normal and tumour tissue (Patnaik and Mostofi, 1993). As seminomas in dogs do not stain positively with vimentin or any of the other antibodies used, and Sertoli cells do not stain with the LH receptor or 3 β -HSD antibodies, incubation of successive sections with this panel of antibodies results in a reliable immunohistochemical characterization and diagnosis of tumour type.

Peters *et al.* (2000) observed that in addition to palpable macroscopic tumours, small tumours that could not be detected by palpation were common in the testes of ageing dogs. It is now possible to characterize these neoplasms more easily using the methods developed in the present study.

In conclusion, traditional histological classification of canine testicular tumours is not sufficient. The results of the present study indicate that the use of cell-type-specific antibodies considerably improved the diagnosis of complex forms of testicular tumours in dogs. The antibody against the LH receptor could also be useful in the differ-

ential diagnosis of human testicular tumours or ovarian tumours. Therefore, it is recommended that testicular tumours, especially complex mixed tumours, should be investigated immunohistochemically. These advanced staining methods, together with measurements of hormone concentrations, may help to resolve the controversy about the association between feminization and specific tumour types.

The authors are very grateful to all the veterinarians, especially P. H. Gijsman, S. Ratker, J. Weeber and R. Burger, who collected testis material for this study. The authors would also like to thank M. de Boer-Brouwer for skilful technical assistance and K. Junker for supplying some complicated cases. The authors are grateful to H. Halsema, W. Voorhout and F. Breeman (Culemborg, The Netherlands) for their help in preparing the plates of photomicrographs. The critical review of this manuscript by B. E. Belshaw is greatly appreciated.

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Revised manuscript received 9 August 2000.

Accepted 29 August 2000.