Immunohistochemical method for detecting lesions in the prostate gland of bulls treated with diethylstilbestrol-dipropionate

J. Weijman, P. Zwart, J. G. Vos, F. C. S. Ramaekers

Hyperplastic and metaplastic lesions in the prostate gland of bulls are used as morphological evidence for the illegal administration of oestrogens or compounds with oestrogenic activity, like stilbenes. Exogenous and endogenous androgens can suppress the effects of these drugs, resulting in suspect or negative results. The detection of epithelial changes immunohistochemically with cytokeratin antibodies appeared to facilitate the diagnosis of hyperplasia and metaplasia in the prostate. A specific and sensitive method for detecting such lesions in formalin-fixed, paraffin-embedded prostate samples has been developed with the commercially available cytokeratin monoclonal antibody 34BE12, and applied to bulls treated with diethylstilbestrol-dipropionate. The highly reproducible staining pattern of 34BE12 can be used for the detection of hyperplasia and metaplasia in the prostate glands of animals treated illegally with oestrogens.

IN the Netherlands the prostate gland ofveal calves is examined histologically in slaughterhouses to control the illegal use of oestrogens as anabolic agents. The test is based on studies showing that exposure to oestrogens, in particular diethylstilbestrol (DES) causes hyperplastic and metaplastic lesions in the prostate gland of several animal species (Ruitenberg and others 1967, 1970, Kroes 1970, Kroes and Teppema 1972, Kroes and others 1976, Deschamps and others 1987, Weijman and others 1987, 1992, Jansen and others 1989, Groot and others 1990, Randles 1990, Lommen and Groot 1993).

In an ultrastructural study of the calf prostate gland Kroes and Teppema (1972) described a basal cell type situated between the basement membrane and the functional glandular cells. It was suggested that the metaplastic changes indicated by DES were the result of the proliferation of these basal cells, and the elevation of the underlying glandular epithelial cells.

In the prostate glandular tissue of veal calves slaughtered at the age of about 18 weeks the lesions induced after the administration of oestrogens are easy to detect (Ruitenberg and others 1967, Kroes 1970, Kroes and others 1976). However, the lesions are less pronounced when combinations of oestrogens and androgens are used, owing to the suppression of the effects of the oestrogens (Kroes and others 1976). Such combinations of hormones lead to less obvious histological changes in the prostatic glandular epithelium. Furthermore, such lesions become less pronounced with increasing age, because of the higher endogenous production of androgenic hormones by older calves (Garbis-Berkvens and others 1985). This is of particular importance because nowadays the fattening time is approximately 30 weeks. For this reason, the category 'suspect lesion' had to be introduced for cases in which only hyperplastic lesions were found after a routine histological examination of the prostate. The authors have shown that immunohistochemistry with cytokeratin antibodies was a promising method for reducing or even eliminating this suspect category (Weijman and others 1987). Comparable changes in the glandular prostatic epithelium could also be detected in goats treated with DES (Weijman and others 1992). On frozen sections of the prostates of these animals the cytokeratin antibodies were
immunoreactive with resting as well as proliferative basal cells in virtually all stages of differentiation. In a previous study (Jansen and others 1989) satisfactory results were obtained with the polyclonal rabbit antisera K40 on formalin-fixed and paraffin-embedded sections of the prostate glands of the bulls used in the present study. However, polyclonal antisera recognise more than one epitope and different lots result in variable immunostaining results.

The aim of the present study was to investigate the well-defined, cytokeratin monoclonal antibody (mAb) 34BE12, which is reactive with basal cells in formalin-fixed, paraffin-embedded prostate tissue, in an attempt to develop a protocol for the detection of oestrogen-induced hyperplastic and metaplastic lesions in samples of bull prostate glands.

Materials and methods

Anabolic preparations

Solutions of diethylstilbestrol-dipropionate (DES-DP) were prepared by Interpet International, either as an emulsion or as an oil composition. The emulsion and oil placebo had the same composition as the corresponding active preparations without DES-DP.

Animals and sampling

Twenty-four red and white bulls of the Maas-Rijn-Ussel breed were divided into three groups. The bulls were about one year old with an average weight of 406 kg (range 375 to 440 kg). Twenty animals received DES-DP in a dose of 100 mg DES equivalents and were divided into an ‘oil composition group’ of 10 animals and an ‘emulsion group’ of 10 animals. All the animals were injected intramuscularly in the neck on the same day. The control group consisted of four animals, two of which received oil placebo, and two received emulsion placebo. Two animals from each of the treated groups were slaughtered at the time intervals indicated in Table 1. The last four animals in each group were slaughtered when the concentration of DES in the urine was less than 1 µg litre. The control animals were slaughtered after one, two, six and 10 weeks. Immediately after slaughter, slices of the pars dissecantia of the prostate gland were fixed in 10 per cent neutral phosphate buffered formalin and the tissue was embedded in Paraplast.

A more detailed description of the anabolic preparations, animals and sampling has been given by Jansen and others (1989).

Immunohistochemistry with mAb 34BE12

The mAb 34BE12 (Enzo Biochem; EAB 903) is raised against human stratum corneum and reacts with the high molecular weight cytokeratins 5, 10 and 11 (Gown and Vogel 1982). As a result, it recognises the basal cell compartments of several organs, including the prostate.

The standard procedure for the immunocytochemical detection of hyperplastic and metaplastic lesions in the prostate is as follows: paraffin sections 5 µm thick were placed on glass slides coated with bond fast glue (Permacol; Ede) at a dilution of 1:50. The sections were dewaxed through xylene and hydrated through an alcohol series to water. After three washing steps in 0·01M phosphate buffered saline (PBS) pH 7·4 the sections were exposed to 0·1 per cent Pronase E Type XIX (Sigma Chemical Company) in PBS for 10 minutes at 37°C. The proteolytic reaction was stopped in either 0·05M cold (4°C) tris-buffered saline (pH 7·6) or cold (4°C) PBS (pH 7·4), giving the same final results. The sections were pre-incubated in 20 per cent normal goat serum (NGS)-PBS for 30 minutes at room temperature. After three washing steps in PBS the prostate sections were covered with 0·1 ml of 34BE12, diluted 1:500 in 10 per cent NGS-PBS. To prevent the serum drying, Parafilm was used to cover the slides. The sections were placed in a Miele M 696 microwave oven, of which the bottom plate had been cooled in a freezer (–20°C) (Van de Kant and others 1989). A polystyrene platform was placed between the sections and the bottom plate. The sections, incubated with the primary antibody, were irradiated for five minutes at 80 W. The microwave step was found to enhance the immunoreactivity of 34BE12 without affecting its specificity. This procedure allowed the use of tissue that had been formalin-fixed for a long time. After washing in PBS, the sections were incubated for 30 minutes with rabbit anti-mouse IgG (DAKO A/S) at a dilution of 1:30 in 10 per cent NGS-PBS at room temperature. After another series of washing steps the sections were incubated for 30 minutes with swine anti-rabbit IgG (DAKO A/S) at a dilution of 1:30 in 10 per cent NGS-PBS at room temperature. After washing, the sections were incubated for 30 minutes with the rabbit peroxidase-antiperoxidase complex (Dako A/S) at a dilution of 1:80 in 10 per cent NGS-PBS at room temperature and after another series of washing steps, peroxidase activity was visualised with 3,3′-diaminobenzidine and 0·03 per cent hydrogen peroxide. The sections were counterstained with haematoxylin and mounted permanently with cover slipping resin (Tissue-tek; Miles Diagnostic Division). The coded prostate sections were evaluated by two of the authors (J.W. and J.G.V.), who also read the haematoxylin and eosin stained sections and the sections incubated with the polyclonal antibody K40 used in an earlier study (Jansen and others 1989). The sections were scored negative when only a single layer of basal cells showed immunoreactivity, and positive when immunoreactive clusters of basal cells were visible. Magnifications between 25 x and 400 x were used. Control incubations were carried out with 10 per cent NGS-PBS without 34BE12, or with the non-relevant mAb RK5E 60, which is of the same IgG1 class, but recognises cytokeratin 10 which does not occur in the prostate. No internal peroxidase activity was observed in these sections, because microwave irradiation destroys peroxidase activity.

Results

Morphological changes induced by administration of DES-DP

Jansen and others (1989) described the histopathological changes observed in haematoxylin and eosin stained prostate sections from the bulls used in the present study. Metaplasia in the peripheral prostate glandular tissue is a positive indicator of oestrogen treatment and is therefore used as a criterion in the prostate screening test used in slaughterhouses in the Netherlands.

An example of metaplasia in the peripheral glandular tissue of an animal two weeks after the administration of DES-DP is shown in Fig 1. Prostate sections showing only hyperplasia were considered as suspect. Prostate sections without morphological changes were scored as negative. An examination of the four placebo-treated control animals showed no histological signs of hyperplasia and metaplasia in their prostate glandular epithelium.

Of the 20 DES-DP-treated bulls five gave a suspect result and four gave a false negative result. The results of the histopathological examination are summarised in Table 1.

Hyperplastic and metaplastic changes detected by mAb 34BE12

In the prostate gland of the placebo-treated control animals, the basal cell layer and a part of the suprabasal cells of the urethra and the major collecting ducts, were immunoreactive with mAb 34BE12, and throughout the glandular tissue individual basal cells were also reactive with 34BE12. The immunoreactivity of the basal cells at the periphery of the lobules was stronger than the reaction in the basal cells in the centre of the lobules (Fig 2a, b).

In prostate sections from the DES-DP-treated animals, the numbers of immunoreactive cells in the urethra and the collecting ducts were increased. Clusters of 34BE12-immunoreactive basal cells were observed in the prostate gland of all but two of the DES-DP-treated bulls; 34BE12 immunoreactivity of the hyperplastic or metaplastic lesions occurred at the periphery of the prostate lobules, but was variable. A slight decrease in the number of immunoreactive clusters seemed to occur between 40 and 70 days
TABLE 1: Results of histopathological and immunohistochemical examination of the prostates of bulls treated with a placebo and with diethylstilbestrol-dipropionate in oil and in an emulsion

<table>
<thead>
<tr>
<th>Group</th>
<th>Time of slaughter (weeks after injection)</th>
<th>Histology* (HE sections)</th>
<th>Immunohistochemistry (mAb 34βE12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>1</td>
<td>Negative</td>
<td>Negative†</td>
</tr>
<tr>
<td>Placebo</td>
<td>2</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
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<td>Negative</td>
</tr>
<tr>
<td>Placebo</td>
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<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>DES-oil</td>
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<td>Suspect</td>
<td>Positive‡</td>
</tr>
<tr>
<td>DES-oil</td>
<td>1</td>
<td>Positive</td>
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<tr>
<td>DES-oil</td>
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<td>Positive</td>
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<tr>
<td>DES-oil</td>
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<td>4</td>
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<td>Positive</td>
</tr>
<tr>
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<tr>
<td>DES-oil</td>
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<td>Positive</td>
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<td>Suspect</td>
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</tr>
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<td>Suspect</td>
<td>Positive</td>
</tr>
</tbody>
</table>

* Previous study (Janssen and others 1989), HE Haematoxylin and eosin  
† Only single basal cells positive  ‡ Clusters of immunoreactive basal cells positive

after injection. The immunoreactivity was stronger in the clusters of basal cells than in the individual basal cells.

An example of strong immunoreactivity in an animal one week after the administration of DES-DP is shown in Fig 3a, b. In two of the DES-DP-treated animals no clusters of basal cells reactive with 34βE12 were found. The results of the immunohistochemistry with mAb 34βE12 are summarised in Table 1.

FIG 1: Metaplastic lesions (arrows) in the peripheral glandular epithelium of the prostate of a bull two weeks after the injection of an emulsion of diethylstilbestrol-dipropionate. Haematoxylin and eosin × 75

FIG 2a, b: Immunoperoxidase staining patterns of the prostate of a placebo-treated bull, incubated with mAb 34βE12. The basal cells in the periphery of the lobules (arrowheads) are more immunoreactive than the basal cells in the centre of the lobules (arrows), a) x 150, b) x 300
Discussion

In male calves extensive, squamous metaplastic changes occur in the prostate glandular tissue after the administration of DES. These metaplastic changes are the result of the proliferation and subsequent differentiation of the basal cells. During this process the number of tonofilaments, known to consist of cytokeratins, increases in the basal cells, as seen at the ultrastructural level (Kroes and Teppema 1972).

In adult animals the changes in the glandular prostate tissue are less pronounced, most probably as the result of endogenous testosterone production. Jansen and others (1989) reported that the histological examination of haematoxylin and eosin stained sections of such prostates was of limited value, because five of 20 bulls treated with DES-DP gave suspect results and four animals gave false negative results. Immunohistochemistry, using the rabbit cytokeratin antiserum K40, appeared to be a more sensitive method, because only two of 20 animals gave false negative results. However, these two animals were examined six weeks after injection, when their urinary DES-concentrations were very low (0-2 and 0-4 μg/litre).

In the present study, using mAb 34βE12 with enzyme pretreatment and microwave irradiation of paraffin-embedded sections, similar results were obtained as with the rabbit antiserum. However, immunocytochemistry with 34βE12 was superior to the method using the polyclonal serum, because of the extremely low background and the intense staining reaction observed with this mAb. The polyclonal antiserum also showed a faint staining reaction in the luminal cells of the prostatic gland, as would be expected from such a reagent. The mAb 34βE12 recognises exclusively basal cells in the prostate. Such a reaction pattern was also observed by Gown and Vogel (1984) and by Brawer and others (1985) in human prostate glands. In an earlier study the authors found that mAb RCK 103, which is also specific for the basal cell compartment when examining prostate epithelium, was superior to the polyclonal antiserum, but failed to react in paraffin sections (Weijman and others 1987). Moreover, the cytokeratin epitopes of mAb RCK 103 are poorly defined, because this antibody fails to react in immunoblots of cytokeratins. The advantages of mAb 34βE12 are as follows: first, it reacts against a single epitope on well characterised cytokeratins; secondly, its production is stabilised; and thirdly, its staining pattern is reproducible in formalin-fixed and paraffin-embedded tissues. In the current study the paraffin sections of bull prostates were incubated using microwave irradiation to enhance the immunoreactivity. The staining patterns were reproducible, even on prostate tissues that had been formalin-fixed for a long time, so that the method should be applicable to archival material in retrospective studies.

Most important, however, is the finding that by applying immunohistochemistry with 34βE12 to routinely processed prostate samples, the suspect diagnosis, introduced into the histopathological examination, can be largely eliminated. Because of its reproducibility and strong staining reaction the mAb 34βE12 is recommended for the immunohistochemical detection of hyperplasia and metaplasia in prostate glands from cattle.

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References


FIG 3a, b: Immunoperoxidase staining patterns of the prostate of a bull one week after the injection of diethylstilbestrol-dipropionate in oil: incubated with mAb 34βE12. Strong immunoreactivity is visible in the peripheral lobular tissue. a) x 30, b) x 300
Discospondylitis in an adult horse

M. H. Hillyer, J. F. Innes, M. W. Patteson, A. R. S. Barr

Discospondylitis, of presumed bacterial origin, was diagnosed in an adult thoroughbred racemare. The clinical signs were vague and associated with abnormal mobility of the neck and forelimbs. Clinical pathology showed only a non-specific inflammatory response. A scintigraphic examination revealed the site of the lesion and the diagnosis was confirmed by the identification of radiographic changes affecting two thoracic vertebrae. A prolonged course of antimicrobial agents produced a complete recovery and the horse returned to full athletic use.

DISCOSPONDYLITIS is a destructive, inflammatory and proliferative process involving intervertebral discs, their associated endplates and vertebral bodies. It has been well recognised in dogs (Hurov and others 1978, Kernegay and Barber 1980, Moore 1992) but appears to be less common in cats (Malik and others 1990) and horses (Adams and others 1985, Chaffin and others 1995). In all species it is more common in the neonate than in the adult animal (Stashak and Mayhew 1984, Markel and others 1986). Vertebral body osteomyelitis has been reported in adult horses (Collins and others 1971, Kelly and others 1972, Markel and others 1986) and may be more common in foals (Giguere and Lavoie 1994, Olchowy 1994, Chaffin and others 1995). The clinical and pathological features of discospondylitis and vertebral body osteomyelitis in the horse are very similar and may overlap (Markel and others 1986).

The only previous specific reports of discospondylitis in horses (Adams and others 1985, Chaffin and others 1995) describe the condition in four adults and two foals. Of these only two survived, a thoroughbred mare after medical therapy, and a quarterhorse foal after extensive medical and surgical treatment.

This report describes the clinical signs, diagnosis, treatment and outcome of a case of discospondylitis in an adult thoroughbred. A scintigraphic examination located the lesion accurately and after prolonged antimicrobial therapy the horse recovered and was able to return to full athletic function.

History

A five-year-old thoroughbred gelding was examined with a two month history of generalised stiffness, unwillingness to lower its head and neck and expiratory grunting when moving. At the onset of the condition the horse was reported to have had a slight pyrexia. Previously it had been in full work, having been in the same training yard since it was a yearling and had raced successfully during the previous two seasons. Previous treatments had included anthelminths and antibiotics, the latter resulting in a transient improvement in the clinical signs.

Clinical examination and further investigations

The gelding appeared bright and alert and was in moderate body condition. Its rectal temperature, pulse and respiratory rates were normal. It moved stiffly in the stable and was unable/unwilling to lower its head and neck fully without adopting an abnormal stance, with one foreleg markedly protracted and the other retracted (Fig 1). Voluntary lateral flexion of the neck was normal to the left but restricted to the right. When at rest the gelding tended to adopt a hunched stance with all four legs drawn together beneath the abdomen. At the walk and trot the horse had a bilateral shortened forelimb stride. Circling and walking backwards appeared normal but it was reluctant to walk up or down an incline. Firm manual palpation over the withers was resisted and continued pressure caused the horse to knuckle on the forelegs. Auscultation of the heart and lung fields was normal, as was an endoscopic examination of the upper respiratory tract and trachea. Cytological and bacteriological examinations of a sample of airway lavage fluid revealed nothing abnormal. Standing, lateral thoracic radiographs obtained for evaluation of the lung fields were considered normal, as were lateral radiographs of the cervical spine. The results of an echocardiographic examination were normal.

Blood samples were collected for routine haematological and biochemical examination. The results were normal except for a hyperproteinaemia of 98.3 g/litre (normal range 50 to 70 g/litre) caused by a hyperglobulinaemia of 69.4 g/litre (normal range 25 to 45 g/litre) and a hyperfibrinogenaemia of 6.9 g/litre (normal range 1 to 4 g/litre). Serological tests for brucella and boella antibodies were negative.

Rectal examination revealed no palpable abnormalities in the caudal abdomen and repeated attempts at abdominal paracentesis did not yield any peritoneal fluid. Percutaneous abdominal ultrasonography revealed nothing abnormal.