Development and validation of a new monoclonal antibody to mammalian aromatase

K J Turner, S Macpherson, M R Millar, A S McNeilly, K Williams, M Cranfield¹, N P Groome¹, R M Sharpe, H M Fraser and P T K Saunders

MRC Human Reproductive Sciences Unit, 37 Chalmers Street, Edinburgh EH3 9ET, UK

¹Department of Biological Sciences, Oxford Brookes University, Oxford OX3 0BP, UK

(Requests for offprints should be addressed to P T K Saunders, Email: p.saunders@ed.ac.uk)

(K J Turner is now at CIIT Centers for Health Research, PO Box 12137, 6 Davis Drive, Research Triangle Park, North Carolina 27709–2137, USA)

Abstract

The biosynthesis of oestrogens from androgens is catalysed by the aromatase complex, an essential component of which is the aromatase cytochrome P450 (P450 arom) protein. Expression of a functional P450 arom is essential for normal fertility in males and females and the sequence of the protein is highly conserved. We have raised a new monoclonal antibody against a conserved peptide and validated it on fixed tissue sections of the rat, common marmoset (*Callithrix jacchus*) and human. The monoclonal antibody was used successfully for Western analysis and specifically reacted with a 55 kDa protein in microsomal extracts. On sections of ovaries in all three species, expression in follicles was specific to the mural granulosa cells of antral follicles and was present in corpora lutea. In the human and marmoset, staining of luteal cells was markedly heterogeneous and did not appear to vary

consistently with the stage of the cycle. The intensity of immunostaining was elevated in corpora lutea from pregnant rats and following human chorionic gonadotropin rescue in the human. In the testis, the highest levels of expression were observed in the Leydig cells within the interstitium. In adult rat and marmoset, and possibly also in the human, some P450 arom was associated with the cytoplasm surrounding elongate spermatids but other germ cells were immunonegative.

In conclusion, a new monoclonal antibody specific for P450 arom recognises the protein in rodent, primate and human. Its ability to work on fixed tissue sections will facilitate identification of individual cells expressing P450 arom within complex tissues.

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Introduction

The biosynthesis of oestrogens from androgens is catalysed by the aromatase complex which consists of aromatase cytochrome P450 (P450 arom) and a flavoprotein, NADPH-cytochrome P450 reductase. P450 arom is highly conserved among mammals (reviewed by Simpson *et al.* 1997, Conley & Hinshelwood 2001). In humans and most other species (the pig being an exception), there appears to be only one gene, CYP19, that encodes P450 arom and in humans this is present on chromosome 15 (Simpson *et al.* 1997, Conley & Hinshelwood 2001). The identification of individuals with defects in CYP19 (reviewed by Grumbach & Auchus 1999) and the generation of aromatase knockout mice (Fisher *et al.* 1998, Honda *et al.* 1998) have added to our understanding of the essential role played by aromatase during fetal life, for normal fertility in adulthood, and in the structure and metabolism of fat and bone.

In rodents, expression of P450 arom is restricted to gonads and brain. In humans and higher primates, expression of P450 arom has been found in a wide range of tissues, including the syncytiotrophoblast of the placenta, stromal cells in adipose tissue, skin fibroblasts, in parts of the vasculature and in several fetal tissues, notably the liver (Toda *et al.* 1994, Harada *et al.* 1999, Simpson 2000). Limited information is available for non-human primates (Hild-Petito & Fazleabas 1997, Sanders & Stouffer 1997). The P450 protein present in each of these sites is identical (Simpson *et al.* 1994); however, analysis of the mRNAs within the individual tissues has shown that a number of different forms of P450 arom mRNA exist due to tissue-specific usage of alternative promoters and alternative splicing of a number of untranslated exons at the 5' end of the mRNAs (reviewed by Simpson *et al.* 1994, Conley & Hinshelwood 2001). Studies have shown that expression of P450 arom in granulosa cells is under the control of pituitary follicle-stimulating hormone (FSH) acting via

cAMP (Steinkampf *et al.* 1987, Whitelaw *et al.* 1992), but stimulation of expression in adipose stromal cells is usually under the control of glucocorticoids and cytokines (Simpson 2000). Synthesis of oestrogens from androgens has been demonstrated using isolated immature Sertoli cells from rat testes and the production of oestrogens was stimulated in the presence of FSH; this effect was shown to be age-dependent (Dorrington & Khan 1993). In adult rats, synthesis and secretion of oestrogens has been demonstrated to occur in Leydig cells under the control of luteinizing hormone (LH), where induction of aromatase activity was shown to be time- and dose-dependent (Valladares & Payne 1979). A more extensive role for oestrogens in male fertility has been proposed following the discovery of aromatase activity in testicular germ cells and epididymal sperm (Nitta *et al.* 1993, Janulis *et al.* 1996b, 1998).

Several antibodies directed against P450 arom have been prepared from purified human placental aromatase (Mendelson *et al.* 1985, Washida *et al.* 1996). One rabbit polyclonal antibody directed against a peptide within rat P450 arom has been described (Sanghera *et al.* 1991), demonstrating that anti-peptide antibodies against P450 arom could be prepared successfully. To enable us to detect P450 arom in multiple species including the common marmoset, a New World monkey, we have prepared a new monoclonal antibody against a conserved peptide highly conserved within the sequence of P450 arom (Conley & Hinshelwood 2001). This reagent has been used successfully to demonstrate the cell-specific expression of P450 arom in fixed sections taken from rat, mouse, marmoset and human. The high specificity and absence of background staining exhibited by this antibody has allowed us to detect very low levels of expression of P450 arom protein in some cells within the ovary and testis.

Materials and Methods

Tissue collection

Cyclical activity of adult female rats was monitored using vaginal smears, and ovaries were recovered at pro-oestrus ($n=7$), oestrus ($n=3$), metoestrus ($n=3$) and dioestrus ($n=4$); one ovary was fixed in Bouin's fluid for 6 h, transferred to 70% ethanol and processed into paraffin wax. The contralateral ovary was frozen in liquid nitrogen and stored at -70°C . Smear results were confirmed using a blood plasma sample collected at the time of death which was used for subsequent measurement of LH concentration. Ovaries were also recovered from three pregnant rats during late gestation (days 18–21).

Human corpora lutea were enucleated at the time of hysterectomy as described previously (Duncan *et al.* 1996). All women were healthy, aged 32–45 years, with regular menstrual cycles and had not received any form of hormonal treatment for at least 3 months before taking part

in the study. The date of the preovulatory LH surge was determined by measuring LH concentrations in serial early morning urine samples collected before operation. On this basis, three corpora lutea were classified as early luteal (LH+2 to LH+4 days), five as mid-luteal (LH+6 to LH+9 days), and four as late luteal (LH+11 to LH+19 days). In addition, five women were given i.m. injections of human chorionic gonadotrophin (hCG; Profasi, Serono Laboratories, Welwyn Garden City, Herts, UK) from LH+7 in daily doubling doses, starting at 125 IU and continuing for 6–8 days until surgery. This regimen has been shown to rescue the corpora lutea and reproduce the hormonal changes of early pregnancy. Tissues were fixed in 4% paraformaldehyde for 24 h (Millar *et al.* 1993). Tissue from term human placentas ($n=3$) was obtained from the Simpson Memorial Maternity Pavilion, Edinburgh, UK and was either snap frozen or fixed in Bouin's fluid for 6 h. Testicular tissues were obtained from men ($n=4$) undergoing surgical investigations for non-obstructive azoospermia or surgical correction of vasectomy. The Lothian Reproductive Medicine Ethics Committee approved the protocols, and volunteers gave informed consent. Human ovarian tissue ($n=3$) was obtained from the Peterborough Hospitals NHS Trust tissue bank. Testicular and ovarian tissues were fixed in Bouin's fluid for 6 h.

Marmoset (*Callithrix jacchus*) tissues were collected over a period of 3 years, under the Animals Act 1986, from captive-bred animals maintained in a colony that has been self-sustaining since 1973. Progression through the ovarian cycle was determined by monitoring blood progesterone levels and ovaries were obtained during the follicular phase (early, $n=3$; mid, $n=2$; late, $n=3$) and were the same samples as those described by Fraser *et al.* (1999), or during the luteal phase of the non-pregnant cycle and on day 21 of pregnancy. Tissues were fixed by immersion in 4% paraformaldehyde for 24 h and stored in 70% ethanol until processed into paraffin wax as described previously (Millar *et al.* 1993). A term marmoset placenta was also obtained, snap frozen and used for extraction of microsomes.

Preparation of a monoclonal antiserum against aromatase

A peptide (A3, KALEDDVIDGYPVKKC) corresponding to amino acids 376–390 within human aromatase (Corbin *et al.* 1988), plus an extra C-terminal cysteine residue to allow coupling, was synthesised in the Centre for Proteins and Peptides, Oxford Brookes University and conjugated to tuberculin. This peptide maps to a region of the protein that is highly conserved between species (see Conley & Hinshelwood 2001). Peptide A3 was used to immunise mice and monoclonal antibodies were prepared according to standard methods (Groome *et al.* 1990, Groome & Lawrence 1991); positive clones were identified by enzyme-linked immunosorbent assay (ELISA) using purified human aromatase, and those which gave the highest

Figure 1 Detection of aromatase protein in microsomal extracts from rat, marmoset and human tissues. On Western blots, a single protein of ~ 55 kDa was recognised by the monoclonal antibody raised against a peptide within the P450 aromatase protein (A). No signal was seen on blots incubated with antibody that had been pre-absorbed with immunising peptide (B). Extracts were prepared from the following tissues: lane 1, human placenta; lane 2, marmoset placenta; lane 3, marmoset ovary; lane 4, ovary from pregnant rat; lanes 5 and 6, ovaries from two rats killed at pro-oestrus.

reaction were screened by immunohistochemistry on sections of rat ovary (not shown). Immunoglobulins were purified from culture media which had been dialysed against 10 mM phosphate buffer, pH 7.2, using Hi-trap protein G Sepharose columns according to the manufacturer's instructions (Amersham Pharmacia, Amersham, Bucks, UK). IgGs were desalted on PD10 columns (Amersham Pharmacia) which had been pre-equilibrated in phosphate-buffered saline, pH 7.4, then mixed with 20% (v/v) glycerol, 0.02% (w/v) sodium azide (Sigma, Poole, Dorset, UK) and stored as 100 μ l aliquots (0.5 mg/ml) at -20°C .

Western blotting

Microsomes were prepared from ovarian (rat, marmoset) or term placental tissues (human, marmoset) using a modification of the methods outlined elsewhere (Mendelson *et al.* 1985, Hellmold *et al.* 1998). Briefly, frozen tissue was ground using a pestle and mortar containing liquid nitrogen, and allowed to swell in homogenisation buffer (100 mM Tris-HCl, pH 7.4, containing

20% glycerol (v/v), 150 mM KCl (Sigma), 1 mM dithiothreitol (DTT; Sigma), 1 mM EDTA (Sigma), containing two protease inhibitor tablets/50 ml buffer) for 10 min at room temperature (200 mg tissue/4 ml buffer). Samples were homogenised briefly and centrifuged (3500 r.p.m. for 5 min at 4°C) to remove cellular debris, and microsomes were recovered from the resulting supernatant by ultracentrifugation (37 500 r.p.m. for 1 h at 4°C). Microsomal pellets were resuspended in 200 μ l solubilisation buffer (50 mM potassium phosphate buffer, pH 7.4) containing 20% glycerol (v/v), 0.5% (w/v) sodium cholate (Sigma), 0.1 mM EDTA (Sigma), 1 mM DTT (Sigma), 0.2% (w/v) Igepal CO-990 (Aldrich, Gillingham, Dorset, UK), containing two protease inhibitor tablets/50 ml buffer). Aliquots (50 μ g total protein) were added to 5 μ l denaturing/loading buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol, all from Sigma) and boiled for 5 min. Tissue extracts and prestained protein molecular weight markers (BioRad Laboratories Ltd, Hemel Hempstead, Herts, UK) were separated on denaturing minigels containing an acrylamide gradient from 4 to 20% (w/v) polyacrylamide (Invitrogen) run in

Figure 2 Immunoexpression of aromatase in ovarian follicles from the rat, marmoset and human. Immunoexpression of aromatase was detectable in the ovarian follicles of the rat (a, dioestrus; b, pro-oestrus), marmoset (c, d) and human (e). The expression of aromatase within the ovarian follicle was confined to granulosa cells and was dependent upon the stage of development of the follicle. In all three species, no aromatase protein could be detected in pre-antral follicles (*). Levels of expression of aromatase were not uniform throughout the antral follicles, being highest in the mural granulosa cells at the periphery of the follicle (arrows) and absent from the granulosa cells surrounding the antrum and within the cumulus (arrowheads). The intensity of immunostaining was increased in the periovulatory follicle (b) compared with less mature antral follicles (a, c, e). Immunopositive staining was present in the human placenta (f, positive control tissue) and no staining was observed after absorption of the primary antibody with the immunising peptide (inset f). Scale bars are 100 μ m (b) or 50 μ m (a, c, d, e, f). O, oocyte.

Figure 3 Immunoeexpression of aromatase in corpora lutea from the rat, marmoset and human. Aromatase protein was detected in cells within the corpora lutea of all three species. In the human and marmoset, expression was confined to luteal cells derived from granulosa cells. Luteal tissue was obtained from the following: (a) rat, dioestrus; (b) rat, pregnant; (c) marmoset, early follicular; (d, e) marmosets, early pregnant (day 22); (f) human, early luteal; (g) human, mid luteal; (h) human, late luteal (inset $\times 40$); (i) human, hCG rescued. Scale bars are 200 μm (h), 100 μm (b) or 50 μm (a, c, d, e, f, g, inset h, i).

an Xcell-II minicell tank using Tris/glycine/SDS running buffer (25 mM Tris, 250 mM glycine, pH 8.3, 0.1% SDS) at 100 V per gel for 2 h and blotted onto polyvinylidene difluoride membrane (Millipore, Watford, Herts, UK). Membranes were blocked for 2–3 h at room temperature in 5% non-fat dried milk powder dissolved in Tris-buffered saline, pH 7.4, containing 0.05% Tween-20 (TBST) and incubated overnight with aromatase antibody diluted 1:5000 in TBST containing 5% normal sheep serum. Bound antibodies were detected using a sheep anti-mouse Ig (Amersham International plc, Amersham, Bucks, UK) diluted 1:5000 in TBST and the enhanced chemiluminescence visualisation system (Amersham International plc) according to the manufacturer's instructions.

Immunohistochemistry

Tissue sections (5 µm) were cleared in histoclear (BS & S Scotland Ltd, Edinburgh, UK) for approximately 10 min, hydrated in decreasing concentrations of ethanol, washed briefly in tap water and incubated in hydrogen peroxide (3%, v/v) in methanol for 30 min to block endogenous peroxidase. After washing in water, slides were rinsed in Tris-buffered saline (TBS; 0.05 M Tris, pH 7.4, 0.85% NaCl) for 5 min and blocked in normal rabbit serum (NRS; Diagnostics Scotland, Carlisle, Lanarkshire, UK) diluted 1:4 in TBS containing 5% bovine serum albumin (BSA; Roche Diagnostics Ltd, Lewes, E Sussex, UK) for 30 min at room temperature. On sections of human and marmoset tissues, an avidin–biotin block was performed using reagents from Vector (Peterborough, Cambs, UK) as follows: eight drops of avidin were added to each 1 ml TBS used, this was incubated on sections for 15 min, which were then rinsed in TBS and washed in TBS for 5 min. Thereafter, eight drops of biotin were added per 1 ml TBS, incubated on sections for 15 min, rinsed in TBS and then washed in TBS for a further 15 min. Sections were incubated with anti-aromatase IgG diluted 1:50 in NRS/TBS/BSA overnight at 4 °C; antibody preincubated with immunising peptide (tenfold excess concentration) was used as a negative control. A rabbit anti-mouse biotinylated secondary antibody (DAKO, Cambridge, Cambs, UK) was diluted 1:500 in NRS/TBS/BSA and incubated with the tissue sections for 1 h at room temperature. Bound antibodies were visualised by incubating the sections with ABC–HRP complex (DAKO) followed by incubation with 3,3'-diaminobenzidine tetrahydrochloride (Sigma). Sections were counterstained with haematoxylin. Images were captured using an Olympus Provis microscope (Olympus Optical Co., London, UK) equipped with a Kodak DCS330 camera (Eastman Kodak Co., Rochester, NY, USA). Captured images were stored on a Macintosh G4 computer and assembled using Photoshop 5.0 (Adobe, Mountain View, CA, USA).

Results

Specific detection of P450 arom in tissue extracts from the human, marmoset and rat

A single protein band with an apparent molecular weight of 55 kDa was detected in microsomal extracts from human placenta (positive control tissue), marmoset placenta and ovary, and ovaries from pregnant rats or those in pro-oestrus (Fig. 1A). In other experiments, a protein with identical mobility was present in testicular extracts from day 6 and adult rats and an extract of human placenta recovered at 18 weeks of gestation (not shown). Incubation of the placental samples with antibody that had been pre-absorbed with the immunising peptide did not result in detection of any protein (Fig. 1B), demonstrating that the antibody was specific and that no proteins were detected by the second antibodies.

Immunolocalisation of P450 arom in ovarian tissues from the rat, marmoset and human

In the rat, marmoset and human, no P450 arom was detected in pre-antral ovarian follicles (Fig. 2). Within the granulosa cell layers of the developing antral follicles, expression of P450 arom within the cytoplasm was highest in the mural granulosa cells at the outer edge of the follicle (arrows) and immunoreactive protein was undetectable in granulosa cells around the antral cavity and within the cumulus (arrowheads). The marked compartmentalisation of P450 arom expression within granulosa cells was particularly striking in the mature follicles from rats at pro-oestrus (Fig. 2b). Expression of P450 arom protein was not detected in either the oocyte (Fig. 2) or theca. A similar pattern of expression has been found in the follicles of mice (McNeilly *et al.* 2000) (not shown). Consistent with previous reports (Naganuma *et al.* 1990), intense immunoreactivity was localised to the syncytiotrophoblast of the human placenta (Fig. 2f, positive control tissue) and this staining was absent when the antibody was pre-absorbed with the immunising peptide (Fig. 2f, inset).

Immunoexpression of P450 arom was detected in corpora lutea in the rat, marmoset, human (Fig. 3) and mouse (not shown). Staining was specific to the cytoplasm of large cells and was not seen in endothelial cells lining the blood vessels (Fig. 3, arrowheads). In the marmoset and human, staining of the large luteal cells was heterogeneous and in some samples appeared particularly striking adjacent to the cell membrane (see Fig. 3c). In the human, staining was not observed in theca-derived luteal cells and we did not see a consistent change in intensity of immunostaining in larger granulosa-derived cells associated with duration of the luteal phase. In the rat, intense immunostaining for P450 arom was detected in corpora lutea from females killed during the second half of pregnancy (Fig. 3b) and, in humans, expression was highest in tissue recovered

following stimulation by hCG (Fig. 3i). In marmosets, tissue recovered from pregnant animals (22 days post ovulation) showed marked heterogeneity in the level of staining (compare Fig. 3d and e).

Immunolocalisation of P450 arom to testicular tissue from the rat, marmoset and human

Within the testis of the rat, marmoset and human, the most intense immunoreactivity for P450 arom was detected in Leydig cells within the interstitial compartment (Fig. 4). In the rat (Fig. 4a) and marmoset (Fig. 4c), specific immunostaining was also clearly present in the cytoplasm surrounding the heads of elongate spermatids; this was much less distinct in the human (Fig. 4e). A faint immunopositive reaction, not present in the negative controls (insets Fig. 4b, d and f), was also apparent in the cytoplasm of Sertoli cells (arrows). Immunoexpression was always less intense in the testicular samples than in mature ovarian follicles, corpora lutea or the placenta and there was some variation in the intensity of the staining in the cytoplasm of elongate spermatids between different animals even within the same species (e.g. compare Fig. 4a with b and c with d).

Discussion

By choosing a peptide within P450 arom conserved between species we have produced a monoclonal antibody which recognises the protein in all species so far examined. The antibody was used successfully for both Western analysis and to localise the P450 protein in fixed tissue sections. The detection of a single protein band with an apparent molecular size of 55 kDa in microsomal extracts of placenta and ovary is consistent with the reported size of the protein (Mendelson *et al.* 1985, Steinkampf *et al.* 1987).

Aromatase activity is high in the human placenta and this has made it the tissue of choice for the purification of the P450 arom protein (Mendelson *et al.* 1985, Yoshida & Osawa 1991). Naganuma *et al.* (1990) reported that the expression of P450 arom in the human placenta was restricted to the syncytiotrophoblast. We have detected P450 arom protein in protein extracts of marmoset placenta and on tissue sections (not shown) consistent with a syncytiotrophoblast cell type in the placenta of this species.

On ovarian sections, follicular expression of P450 arom was confined to mural granulosa cells in mature follicles. Our results contrast somewhat with results claiming that P450 arom is present in thecal cells in the rat and human (El-Maasarany *et al.* 1991, Inkster & Brodie 1991). However, they are in agreement with results showing that expression in granulosa cells is developmentally regulated and confined to mature follicles (Sasano *et al.* 1989, Inkster

& Brodie 1991, Tamura *et al.* 1992, Suzuki *et al.* 1993, Tetsuka & Hillier 1997). In rats, consistent with the developmental regulation of expression of P450 arom, its mRNA was increased by treatment with FSH, whereas expression of androgen receptor mRNA was decreased (Tetsuka *et al.* 1995). In human granulosa cells retrieved from mature follicles, P450 arom has been shown to be increased by FSH *in vitro* (Steinkampf *et al.* 1987). Granulosa cells isolated from marmoset ovaries also synthesise aromatase in response to FSH treatment (Hillier & Miro 1993). Yoshinaga-Hirabayashi *et al.* (1990) found, in rats, that granulosa cells of antral follicles became strongly immunopositive 12 h after stimulation with hCG, and administration of pure recombinant FSH to rats induces specific expression of P450 arom mRNA (Whitelaw *et al.* 1992). In both cases, the highest expression is in the mural granulosa cells, in agreement with our own findings in the ovaries examined.

In the present study we were able to examine luteal specimens from both the human and the marmoset. In both species, the intensity of the immunopositive signal in cells of similar size was markedly heterogeneous and endothelial cells lining the blood vessels and other small luteal cells were immunonegative. In the marmoset, more luteal cells appeared to be immunopositive than in the human and this would be consistent with the different proportions of steroidogenically active cells reported in the two species (Webley *et al.* 1990). The reason for the variation in expression within individual cells is not known but similar results have been reported for rhesus macaque corpora lutea (Sanders & Stouffer 1997). In human corpora lutea, the intensity of the immunopositive signal was greater in corpora lutea from women following treatment with hCG than from those in the normal menstrual cycle. Suzuki *et al.* (1994) have reported a correlation between immunointensity and aromatase enzyme activity per luteinised granulosa cell in human corpora lutea during the menstrual cycle. In the baboon, an increase in expression of aromatase mRNA during pregnancy following the rise in chorionic gonadotrophin has also been reported (Hild-Petito & Fazleabas 1997). We did not find a consistent up-regulation in expression of P450 arom in marmoset corpora lutea during early pregnancy although, at this time, marmoset corpora lutea are active in the synthesis of oestradiol, and expression of 17 β -hydroxysteroid dehydrogenase type 7 is high (Schwabe *et al.* 2001). Expression in rat, marmoset and human corpora lutea is consistent with a recent study in which a specific 278 bp region identified upstream of human CYP19 was used to direct ovary-specific expression of an hGH reporter gene (Hinshelwood *et al.* 2000), resulting in high levels of expression of the transgene in murine corpora lutea.

In the present study, the highest levels of expression of P450 arom protein on sections of testes from adult rat, marmoset and human were detected in interstitial Leydig

Figure 4 Immunorexpression of aromatase in the testes of human, marmoset and rat. Note that aromatase was detected in the cytoplasm of Leydig cells (L) in all three species. In the rat (a, b) and marmoset (c) some immunostaining was also found specifically within the cytoplasm surrounding elongate spermatids (E), although this was less convincingly specific to elongate spermatids in the human (e). A faint immunopositive reaction, not present in the negative controls (insets b, d, f), was also apparent in the cytoplasm of Sertoli cells (arrows). Testes were obtained as follows: (a, b) rat; (c, d) marmoset; (e, f) human. Scale bar (50 μ m) in (a) refers to all sections. Insets in b, d, and f are sections of testes from the same species stained with antibody that had been absorbed with the immunising peptide.

cells in all three species. Our findings are consistent with results previously reported using testicular tissue from the human (Inkster *et al.* 1995), rat (Kurosumi *et al.* 1985, Levallet *et al.* 1998) and bank vole (Bilinska *et al.* 2000); testes from non-human primates have not been examined previously. These findings are supported by those of Valladares & Payne (1979) who used cell-free homogenates of the different testicular compartments obtained from adult rats to demonstrate that aromatase activity was detectable in the interstitial compartment and was increased following LH treatment, consistent with metabolism occurring in Leydig cells. Using fixed tissue sections, we found expression of P450 arom in germ cells and Sertoli cells difficult to detect, with the most convincing data being obtained in the rat, where significant immunorexpression was present in the cytoplasm of the most mature elongate spermatids and a faint immunopositive signal was present in Sertoli cells. Similar results were obtained in the marmoset although, in this species, the volume of spermatid cytoplasm stained was less abundant due to the presence of multiple stages of spermatogenesis in a single cross section (Millar *et al.* 2000). The ability of the rat Sertoli cell to synthesise oestrogens and to be stimulated to do so by FSH is reported to be markedly age dependent with highest levels in cells from prepubertal animals (Dorrington & Khan 1993) and, because we only used material from adults, this may explain the low level of staining observed. Although some studies have reported aromatase activity or mRNA in germ-cell populations other than elongate spermatids (Janulis *et al.* 1996a, 1998), we were unable to detect consistent immunopositive staining in these cells. We were also unable to reach an unequivocal conclusion regarding the presence/absence of P450 arom immunorexpression in the cytoplasm of elongate spermatids in the human for two reasons. First, elongate spermatid cytoplasm was sparse. Secondly, there was considerable variation between tissues from individual testes. The latter variability was also evident for rats and marmosets. This variability may indicate that the level of expression of P450 arom protein in germ-cell cytoplasm is close to the limit of detection by immunohistochemistry.

In conclusion, we have developed a new monoclonal antibody, specific for P450 arom, which gives clear cytoplasmic staining with low background on fixed tissue sections from a wide range of species including the human and the rodent. This reagent provides a valuable addition to the panel of reagents directed at increasing our understanding of the role(s) of oestrogens in human health and disease.

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