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Abnormal Leydig Cell Aggregation in the Fetal Testis of Rats Exposed to Di (*n*-Butyl) Phthalate and Its Possible Role in Testicular Dysgenesis

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Fetal exposure of male rats to di (*n*-butyl) phthalate (DBP) induces testicular changes remarkably similar to testicular dysgenesis syndrome in humans; these include induction of focal areas of dysgenetic tubules in otherwise normal testes. In searching for the fetal origins of the latter, we used image analysis to show that exposure to 500 mg/kg DBP [embryonic day (E)13.5–20.5)] caused abnormal aggregation of Leydig cells centrally in the fetal testis. This aggregation was not due to increase in Leydig cell number, and Leydig cell size was significantly reduced in DBP-exposed animals, as were testosterone levels and immunoexpression of P450 side-chain cleavage enzyme. The Leydig cell aggregates did not exhibit evidence of focal proliferation at E17.5–19.5. Using confocal microscopy and Leydig (3β-hydroxysteroid dehydrogenase) and Sertoli (anti-Mullerian hormone) cell-specific markers, we show that fetal Leydig cell aggregates in DBP-exposed animals trap isolated Sertoli cells within them at E21.5. These areas of intermingled cells are still apparent on postnatal d 4, after cessation of DBP treatment, when they may form misshapen seminiferous cords that trap (intratubular) Leydig cells within them. These centrally located dysgenetic tubules contain germ cells in early puberty, but by adulthood they are Sertoli cell only, implying that presence of intratubular Leydig cells interferes with spermatogenesis. It is concluded that DBP-induced fetal Leydig cell aggregation may be a key event in formation of focal dysgenetic areas in the testis, and identification of the mechanisms underlying these events may give new insights into the fetal origins of testicular dysgenesis syndrome disorders in the human. (*Endocrinology* 146: 613–623, 2005)

TESTICULAR CANCER, CRYPTORCHIDISM, hypospadias, and low sperm counts are all disorders of male reproductive health that are common and/or increasing in incidence in the Western world. For example, cryptorchidism is the most common congenital abnormality in children, affecting 2–4% or more of boys at birth (1–3), and testicular cancer is the most common cancer of young men, and its incidence has risen progressively over the past 50–60 yr in most Western countries (1, 4). Although these disorders manifest at different life stages (cryptorchidism, hypospadias at birth; testicular cancer and low sperm counts in young adulthood), there is strong evidence that these disorders may have a common origin in fetal life. Based on this evidence, these disorders have been hypothesized to constitute a testicular dysgenesis syndrome (TDS), which stems from abnormal development and function of Sertoli and/or Leydig cells during male sexual differentiation (5, 6). As a consequence, there is considerable interest in identifying the events in the fetal testis that might give rise to TDS. We have shown recently that fetal exposure of rats to di (*n*-butyl) phthalate (DBP) offers a potential animal model in which to explore this (7).

In utero exposure to DBP, or to certain other phthalate esters, has been shown to induce cryptorchidism, hypospadias, impaired spermatogenesis, and reduced male fertility in rats (7–12). These postnatal changes are preceded by a decrease in testicular levels of testosterone (7, 13–15) and insulin-like factor 3 (Insl3) mRNA (16) in the fetal testis, induction of apparent fetal Leydig cell hyperplasia, and the widespread occurrence of multinucleated gonocytes. We extended these findings by showing that fetal DBP exposure also results postnatally in focal areas of testicular dysgenesis comprising malformed seminiferous cords/tubules with associated focal Leydig cell hyperplasia, intracordal/intratubular Leydig cells, and immature Sertoli cells (7); these areas occur focally in both cryptorchid testes and otherwise largely normal (scrotal) testes with grossly normal spermatogenesis. Our subsequent goal has been to identify the primary mechanisms in fetal life via which DBP induces testicular dysgenesis because there are very strong similarities between the phenotypic changes observed in adulthood in testes of DBP-exposed male rats and those reported in testes of humans classified as exhibiting TDS (6, 7, 17).

A common finding in studies that have investigated the effect of DBP or other phthalate exposure on male reproductive development is the report of focal areas of Leydig cell hyperplasia in fetal testes (7, 12, 15). Whether this hyperplasia is genuine (*i.e.* there is overproliferation and/or increase

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Abbreviations: AMH, Anti-Mullerian hormone; DBP, di (*n*-butyl) phthalate; E, embryonic day; HRP, horseradish peroxidase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; Insl3, insulin-like factor 3; NRS, normal rabbit serum; PCNA, proliferating cell nuclear antigen; P450scc, P450 side-chain cleavage enzyme; SMA, smooth muscle actin; TBS, Tris-buffered saline; TDS, testicular dysgenesis syndrome.

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in size of Leydig cells) or is merely apparent is unresolved, but it has been largely viewed as being an incidental or secondary change. However, during time-course studies of fetal testes from DBP-exposed males, we noted that abnormal Leydig cell distribution/hyperplasia was the earliest gross morphological change that was evident during treatment. This raised the possibility that the Leydig cell hyperplasia might be an important event in the development of testicular dysgenesis in DBP-exposed males. The present study set out to test this possibility by objectively quantifying Leydig cell numbers and distribution in the fetal testis at various ages after *in utero* DBP exposure and relating this to the postnatal development of certain dysgenetic features (malformed seminiferous cords, intratubular Leydig cells). Our results show that abnormal Leydig cell aggregation, rather than overproliferation, underlies the apparent Leydig cell hyperplasia in the fetal testes of DBP-exposed males. These abnormal aggregates trap isolated Sertoli and other cells within them and in so doing probably give rise postnatally to the formation of dysgenetic areas containing malformed seminiferous cords with intratubular Leydig cells.

Materials and Methods

Animals, treatments, sample collection, and processing

Wistar rats were maintained in our own animal facility according to United Kingdom Home Office guidelines and were fed a soy-free breeding diet (SDS, Dundee, Scotland). Time-mated females were treated from embryonic d (E)13.5 to E21.5 with either 0 (control) or 500 mg/kg DBP (Sigma-Aldrich Co. Ltd., Dorset, UK) in 1 ml/kg corn oil administered daily by oral gavage. This dose was shown previously to result in a high incidence (>60%) of focal dysgenetic areas in postnatal testes of our animals (7). The DBP administered was 99% pure according to the supplier. A total of 64 pregnant rats were used for the present studies, of which 32 were treated with DBP and 32 with corn oil.

Fetal samples. Control and DBP-treated pregnant dams were killed by inhalation of carbon dioxide on E15.5 (n = 2 control, n = 2 DBP), E17.5 (n = 5, 3), E19.5 (n = 4, 5), or E21.5 (n = 5, 5). Fetuses were removed, decapitated, and placed in ice-cold PBS (Sigma-Aldrich). Testes were removed via microdissection, fixed for 1 h in Bouins, and then transferred to 70% ethanol. Testes were weighed before processing into paraffin wax using standard methods. Representative fetuses from the aforementioned litters were subsequently used for the quantitative and immunohistochemical studies detailed below.

Postnatal samples. Male rats aged 4, 25, or 90 d (Adults) were anesthetized via flurothane inhalation and then killed by cervical dislocation. Testes were carefully inspected for normality of the epididymis and vas deferens and then removed, weighed, fixed for 5–6 h in Bouins, and transferred into 70% ethanol. Adult testes were halved after approximately 3 h fixation to aid penetration of the fixative. Testes were embedded in paraffin as described above. The results reported in the present studies derive from control and DBP-exposed males from at least three separate litters per age group and from seven litters for adult animals. At autopsy, testicular position was classified as high abdominal (at level of the kidney), midabdominal, inguinal, or scrotal, which enabled classification of testes in 25- and 90-d males into cryptorchid or scrotal groups. In controls, all testes were scrotal in position. The prevalence of unilateral cryptorchidism in DBP-exposed males was 30 and 57%, respectively, at 25 and 90 d, whereas the corresponding values for bilateral cryptorchidism were 44 and 43%; these values are notably higher than those reported in the literature for similarly treated Sprague Dawley rats (10). In our studies of adult animals exposed *in utero* to 500 mg/kg DBP, we observed that 12% of testes (all of which were cryptorchid) exhibit partial absence of the epididymis. To avoid possible confounding effects of this change on testicular morphology (10, 12), such testes were excluded from present analyses.

Immunohistochemistry

Specific proteins were detected by immunohistochemistry and double immunofluorescence using methods that have been detailed previously (7). Sections of 5 μm were mounted onto coated slides (BDH Chemicals, Poole, UK), dewaxed, and rehydrated. Slides were incubated in 3% (vol/vol) hydrogen peroxide in methanol to block endogenous peroxidase activity and washed in Tris-buffered saline [TBS: 0.05 M Tris, 0.85% NaCl (pH 7.4)]. Immunohistochemistry for phospho-H3 used antigen retrieval by pressure cooking slides for 5 min in 0.01 M citrate buffer (pH 6.0). Nonspecific binding sites were blocked with an appropriate normal serum diluted 1:5 in TBS containing 5% BSA (Sigma) before the addition of the primary antibody and overnight incubation at 4 C. The primary antibodies used in the present studies, their dilutions, and sources are listed in Table 1.

For nonfluorescent immunohistochemistry [proliferating cell nuclear antigen (PCNA), phospho-H3, 3β-hydroxysteroid dehydrogenase (3β-HSD), P450 side-chain cleavage enzyme (P450scc), and smooth muscle actin (SMA)], slides were incubated for 30 min with the appropriate secondary antibody conjugated to biotin at a dilution of 1:500 (rabbit antimouse; swine antirabbit, Dako, Cambridgeshire, UK). The biotinylated antibody was linked to horseradish peroxidase (HRP) by 30 min incubation with avidin-biotin-HRP complex (Dako). Antibody localization was determined by application of diaminobenzidine (liquid DAB$^+$; Dako) until staining in control sections was optimal, and the reaction was stopped by immersing slides in distilled water. Slides were counterstained with hematoxylin, dehydrated, and mounted using Pertex mounting media (Cell Path, Hemel Hempstead, UK).

Fluorescent immunohistochemistry for 3β-HSD and anti-Mullerian hormone (AMH) was performed as described above until before the addition of the primary antibody. 3β-HSD antibody diluted in normal swine serum/TBS/BSA was added and sections incubated overnight at 4 C. After washing in TBS, sections were incubated for 30 min with a secondary antibody, namely swine antirabbit IgG conjugated to HRP (Dako) diluted 1:200 in normal swine serum/TBS/BSA. After washing in PBS, slides were incubated for 10 min with tyramide Cy3 (TSA plus cyanine 3 system; Perkin-Elmer Life Sciences, Boston, MA) diluted 1:50 in the buffer supplied, to amplify 3β-HSD immunostaining and produce red fluorescence. This and all subsequent steps were followed by washing in PBS. Slides were incubated for 30 min with normal rabbit serum (NRS)/PBS/BSA, followed by overnight incubation at 4 C with the AMH antibody diluted in NRS/PBS/BSA. Slides were then incubated for 30 min with biotinylated rabbit antigoat IgG secondary antibody (Vector Laboratories Inc., Peterborough, UK) diluted 1:500 in NRS/PBS/BSA. This was followed by a 1-h incubation with streptavidin-conjugated alexa 488 (Molecular Probes, Poort Gebouw, Holland) diluted 1:200 in PBS, producing green fluorescent AMH immunostaining. Slides were counterstained by incubating for 2 min with a nuclear-specific blue fluorescent label (To-Pro 3; Molecular Probes) diluted

TABLE 1. Antibodies: species, dilution, retrieval, and source

Antibody	Species	Dilution	Retrieval	Source
PCNA	Mouse	1:100	No	DAKO (Cambridgeshire, UK)
Phospho H3	Rabbit	1:6000	Yes	Upstate Cell Signaling Solutions (Milton Keynes, UK)
3β-HSD	Rabbit	1:4000	No	Gift from I. Mason (Edinburgh, UK)
P450scc	Rabbit	1:200	No	Chemicon International Inc. (Temecula, CA)
SMA	Mouse	1:2000	No	Sigma (Poole, UK)
AMH/MIS	Goat	1:80 Fetal 1:40 Neonatal	No	Santa Cruz Biotechnology (Santa Cruz, CA)

1:2000 in PBS. Slides were then washed in PBS and mounted in aqueous mounting medium (Permafluor; Beckman Coulter, High Wycombe, UK).

Image capture

Nonfluorescent images were examined and photographed using a Provis microscope (Olympus Optical, London, UK) fitted with a DCS330 digital camera (Eastman Kodak, Rochester, NY). Fluorescent images were captured using an LSM 510 Axiovert 100M confocal microscope (Carl Zeiss Ltd., Welwyn Garden City, UK). Images were compiled using Photoshop 7.0 (Adobe Systems Inc., Mountain View, CA).

Leydig cell number, size, and distribution

Measurement of Leydig cell cytoplasmic volume and number per testis. Gross inspection of fetal testes from DBP-exposed males in which Leydig cells have been immunostained for 3β-HSD gives the impression of more Leydig cells being present (7, 15). However, such subjective impressions can be very misleading and determination of Leydig cell number, cytoplasmic volume, and distribution are required to establish which, if any, of these parameters are altered in DBP-exposed males.

The volume of 3β-HSD immunopositive cells per testis at E21.5 was determined using stereological methods similar to those described previously (18). The method used Image-Pro Plus 4.5.1 with Stereologer-Pro 5 plug-in software (Media Cybernetics UK, Wokingham, Berkshire, UK) and used an Olympus BH-2 microscope fitted with a Prior automatic stage (Prior Scientific Instruments Ltd., Cambridge, UK). The software was used to select random fields for counting and to place a grid over the tissue. The number of fields counted per animal (~100 per animal) was dependent on obtaining a percentage SE value of less than 5%. Points falling over 3β-HSD-positive cytoplasm or over the nuclei of cells with 3β-HSD-positive cytoplasm were scored separately, and both were then independently expressed as relative volumes per testis. These data were converted to absolute volumes per testis by multiplying by testis weight (equivalent to volume) because shrinkage was minimal. These data were converted to Leydig cell number per testis after determination of Leydig cell nuclear diameter (average of 70–100 nuclei) using standard procedures (18). Average Leydig cell cytoplasmic volume was then computed by dividing total cytoplasmic volume per testis by the number of Leydig cells.

Measurement of Leydig cell clusters

To determine whether the distribution of Leydig cells throughout the testis was altered after DBP treatment, testes from five control and five DBP-exposed fetuses at E17.5, E19.5, and E21.5 were serially sectioned and three representative sections from each testis then selected and immunostained for 3β-HSD. The three sections chosen were those corresponding to approximately 25, 50, and 75% intervals through the serially sectioned testis; at E21.5 this corresponded to sections that were 20–30 sections apart from each other, with narrower intervals at younger fetal ages. Sections from fetuses from three to five separate litters for both control and DBP-exposed animals at each age were used for analysis; similar Leydig cell clustering was evident in fetuses from different litters. Quantification of Leydig cell clustering in these sections was undertaken using Image-Pro Plus 4.5.1 software and equipment as described above. Specimens immunostained for 3β-HSD were of sufficient homogeneity, high contrast, and low background to allow computer-assisted thresholding and subsequent computer-assisted counting of Leydig cell (3β-HSD-immunopositive) clusters and determination of Leydig cell cluster area. Digital images of complete testis sections were captured at ×40 magnification. The software was used to trace around each section, creating an area of interest, allowing the area of each section to be calculated. Computer-assisted thresholding was then used to identify and analyze clusters of 3β-HSD-immunopositive cells, generating data on cluster number, area, and the proportion of each section occupied by Leydig cell clusters.

Testicular testosterone analysis

Testicular testosterone levels in control and DBP-exposed pups aged E19.5 (n = 5 control, n = 6 DBP) and E21.5 (n = 4, 7) were measured by RIA, as described previously (7). After dissection, testes were snap frozen on dry ice and stored at −70 C before analysis. Testes were defrosted and homogenized individually in 500 μl PBS, and an aliquot of this was then extracted with 2 ml diethyl ether and shaken for 5 min and placed in a bath of methylated spirits cooled with dry ice. The nonaqueous portion of the extract was then decanted, dried overnight in a fume hood, and reconstituted in phospho-gelatin buffered saline before assay. All samples were assayed together in a single run, and the limit of detection of the assay was 40 pg/testis.

Statistical analysis

Values are expressed as mean ± SEM, and data were analyzed using Student's unpaired *t* test or one-way ANOVA followed by the Bonferroni post test, using GraphPad Prism (version 4, GraphPad Software Inc., San Diego, CA). Data for Leydig cell cluster number per testis were log transformed before statistical analysis using Student's unpaired *t* test due to skewed distribution of the data.

Results

Leydig cell distribution, number, and function in the fetal testis

Abnormal distribution, or clustering, of Leydig cells was apparent as early as E17.5 in animals exposed to 500 mg/kg DBP *in utero* and was most pronounced at E21.5 (Fig. 1). Serial sections of testes from control and DBP-exposed animals at E21.5 indicated that the large aggregations of Leydig cells tended to be centrally located in the testis (Fig. 2). Quantification revealed that there was no significant change in Leydig cell number in DBP-exposed animals at E21.5, when compared with controls, although a marked reduction in average Leydig cell cytoplasmic volume was observed in these animals, indicating that the Leydig cells were smaller (Fig. 3). The abnormal aggregation of Leydig cells in testes of DBP-exposed males could occur through cell migration to these areas or because of higher rates of interstitial/Leydig cell proliferation within these focal areas. The latter possibility was tested by assessing cell proliferation using the mitotic marker phospho-H3. This revealed a similar low level of cell proliferation in interstitial areas of DBP-exposed and control animals at E17.5 (Fig. 3, C and D) and E19.5 (data not shown), ages at which the progressive development of Leydig cell clusters was occurring (see below). Similar findings were obtained when PCNA immunoexpression was assessed (data not shown). As has been reported previously (7, 13–15), testicular testosterone levels in DBP-exposed animals were significantly reduced at E19.5 and E21.5, when compared with controls (Fig. 4). This reduction was associated with a marked reduction in immunoexpression of P450scc in DBP-exposed animals at E17.5–21.5 (Fig. 4), whereas immunoexpression of the 3β-HSD enzyme was unchanged from control levels at these time points (Figs. 1 and 2).

Leydig cell clustering/aggregation

To characterize Leydig cell clustering and assess it objectively, image analysis was used to define the number, size, and distribution of Leydig cell clusters. A decrease in the number of Leydig cell clusters between control and DBP-exposed animals was observed at all ages from E17.5 to E21.5, with a significant reduction evident at E19.5 and E21.5 (Fig. 5A). Because cluster analysis was based on (total) Leydig cell size and this was clearly reduced in

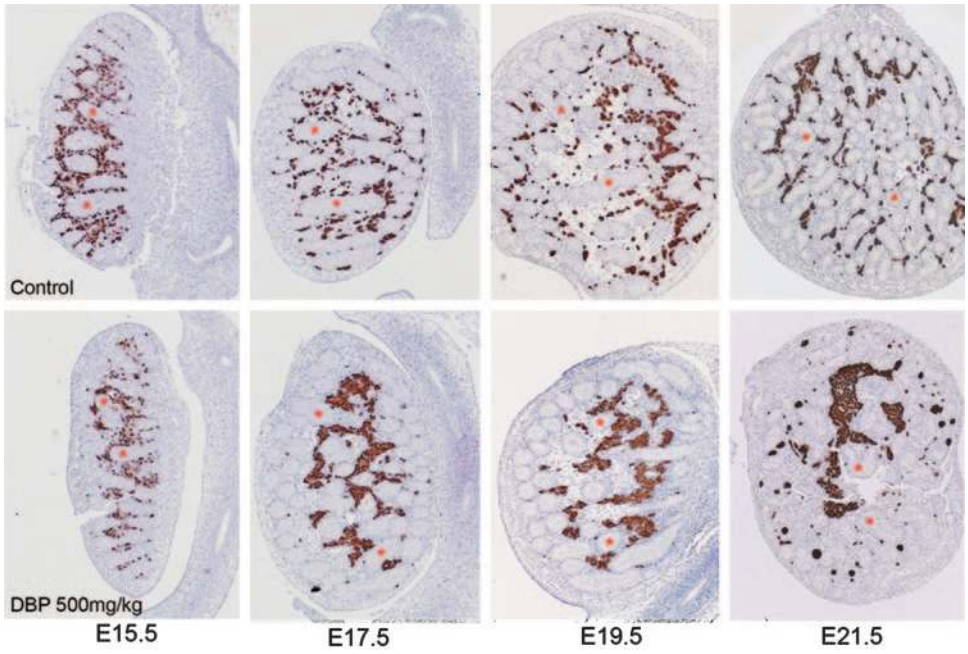

FIG. 1. Representative photomicrographs illustrating the distribution of Leydig (3β-HSD-immunopositive; *brown staining*) cells at E15.5 (n = 2 control, n = 2 DBP), E17.5 (n = 5, 3), E19.5 (n = 4, 5), and E21.5 (n = 5, 5) in the testes of animals exposed *in utero* to 500 mg/kg DBP or corn oil (control). *, Seminiferous cords.

DBP-exposed males (Fig. 3B), account was taken of this by expressing each Leydig cell cluster as a percentage of the total Leydig cell cluster area in that animal. Leydig cell clusters were then assigned arbitrarily to one of three groups: small clusters, accounting for 5% or less of the total Leydig cell cluster area per testis; medium clusters, accounting for between 5.1 and 14.9%; and large clusters, which individually accounted for 15% or more of total Leydig cell cluster area per testis. Although numerous analytical paradigms were explored, this was found to be the most appropriate way of presenting the cluster data because it took into account the reduction in cell size and prevented the enormous number of small clusters in the control testes numerically overwhelming the relatively low number of larger clusters in testes of both groups of animals (*e.g.* an average of 156 small clusters, compared

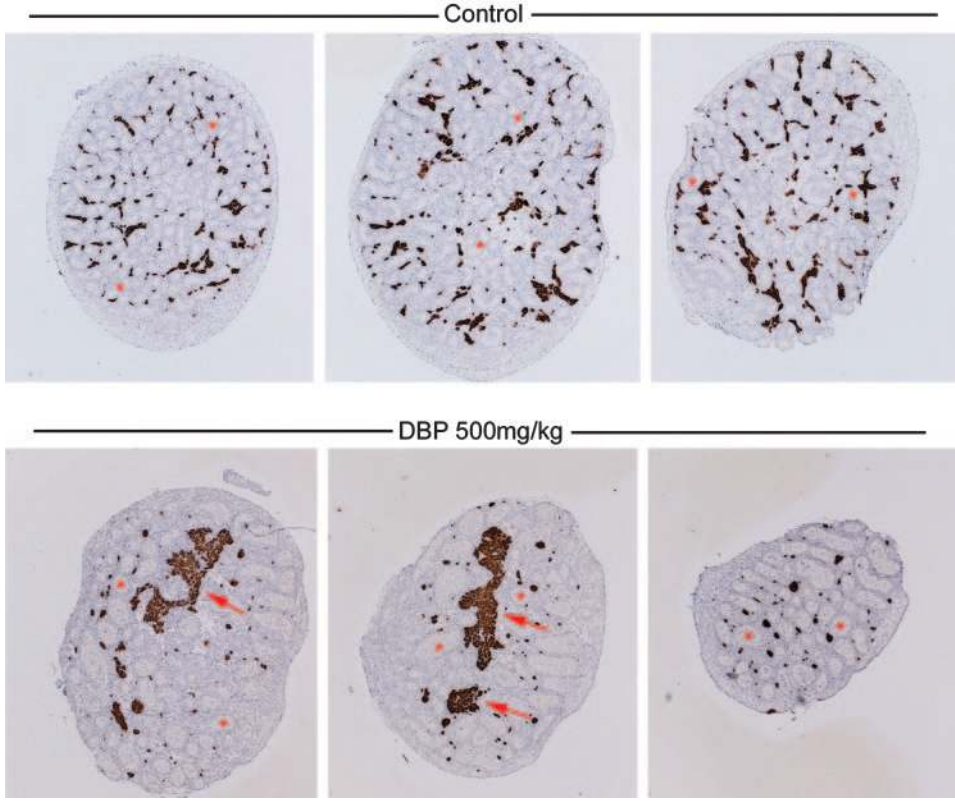

FIG. 2. Changes in Leydig (3β-HSD-immunopositive; *brown staining*) cell distribution throughout the serially sectioned testes of representative control and DBP-exposed animals at E21.5 (n = 5 control, n = 5 DBP). Leydig cell clusters appear to be fewer in number and abnormal aggregates of Leydig cells (*arrows*) are evident centrally in the testes of DBP-exposed animals. *, Seminiferous cords.

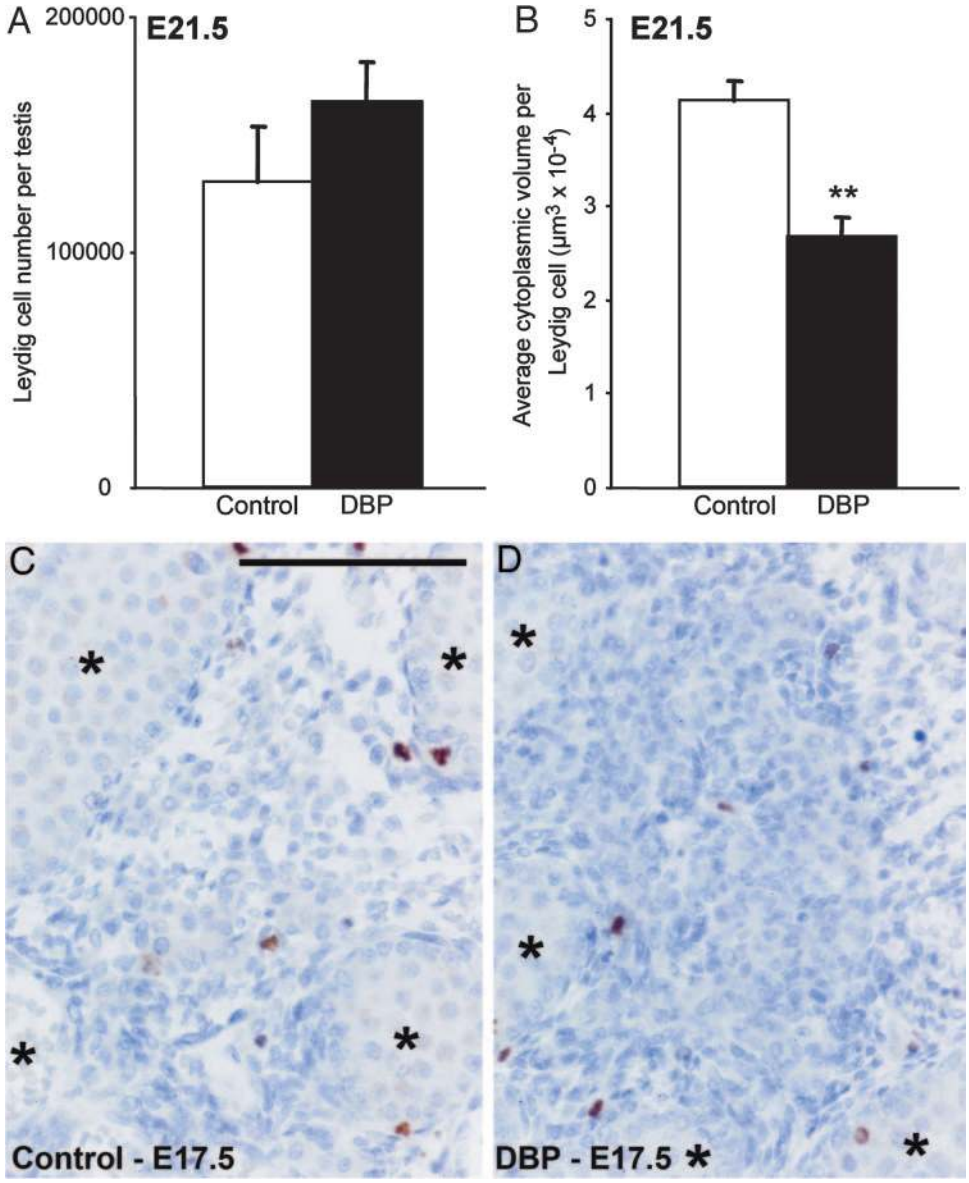

FIG. 3. Comparison of Leydig cell number (A) and average cytoplasmic volume per Leydig cell (B) in controls and rats exposed *in utero* to DBP at E21.5. Values are mean ± SEM for five animals per group (**, $P < 0.005$, compared with controls). Images in the *lower panel* (C and D) illustrate immunolocalization (*brown staining*) of phospho-H3 (mitosis marker) in representative testes from a control and DBP-exposed animal at E17.5 (n = 3 control, n = 3 DBP) in an area with abundant Leydig cells (×40). No difference in cell proliferation was evident between the DBP-treated and control groups. *, Seminiferous cords. *Scale bar*, 100 μm.

with two medium clusters and 0 large in control animals at E21.5). Figure 5B illustrates the contribution that small, medium, and large clusters made to total Leydig cell cluster area in the testes of control and DBP-exposed animals at E21.5. In DBP-exposed animals, there was a significant increase in the percentage of total Leydig cell cluster area accounted for by large clusters, whereas in control animals total Leydig cell cluster area was made up mainly of small and some medium-sized clusters. At all age groups, approximately 30% of the total Leydig cell cluster area in DBP-exposed animals was made up of large clusters, whereas in controls only animals at age E17.5 possessed any clusters of this size (Fig. 5C). It should be noted that comparison of total Leydig cell cluster area per testis in control and DBP-exposed animals at E21.5 (data not shown) revealed a similar reduction in DBP-exposed rats with that determined for Leydig cell cytoplasmic volume by stereological analysis (Fig. 3B), as would be expected.

Leydig cell clusters, formation of dysgenetic areas, and intratubular Leydig cells

Focal dysgenetic areas (malformed/misshapen seminiferous cords) are not evident in the fetal testes of DBP-exposed males and only become evident postnatally when, like the fetal Leydig cell clusters, they tend to be centrally located in the testis (7). To explore whether the abnormal formation of large Leydig cell clusters in the fetal testis might cause, or contribute to, the postnatal formation of these dysgenetic areas, double-immunofluorescence staining for 3β-HSD (Leydig cells, *red*) and AMH (Sertoli cells, *green*) at E21.5 was undertaken to establish whether normal segregation of these cells had occurred in the vicinity of Leydig cell clusters; these cell-specific markers were chosen as their immunoexpression level was unaffected by DBP treatment (Fig. 6). This analysis revealed the presence of isolated Sertoli cells trapped within large Leydig cell clusters in DBP-exposed

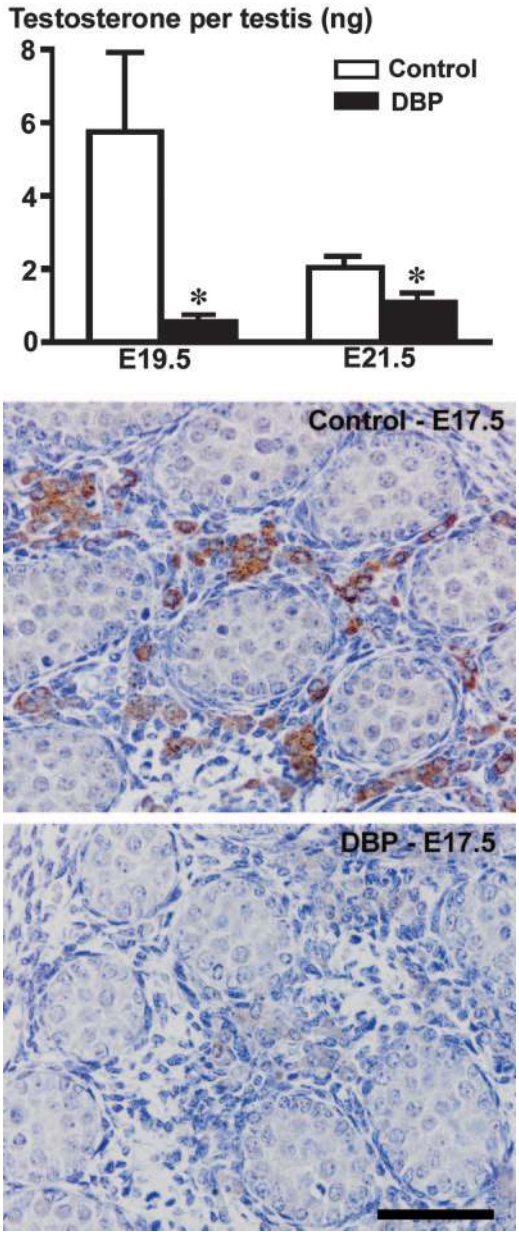

FIG. 4. Comparison of testicular testosterone levels at E19.5 and E21.5 (*top*) and testicular immunoexpression of P450scc at E17.5 in controls (n = 5) and rats exposed *in utero* to DBP (n = 5). Note the marked reduction in intensity of immunoexpression of P450scc in DBP-exposed males. Testosterone values are the mean ± SEM for four to seven animals per group. *, $P < 0.05$, in comparison with respective control value. *Scale bar*, 100 μm.

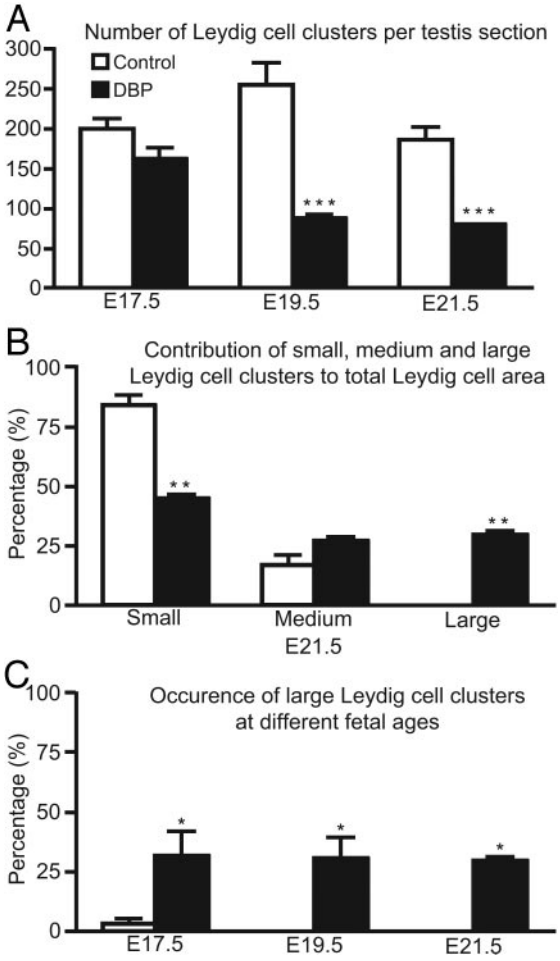

FIG. 5. Leydig cell cluster data from control and DBP-exposed animals at various fetal ages. A, Number of Leydig cell clusters per testis section in control and DBP-exposed animals at E17.5, E19.5, and E21.5. B, Contribution of small, medium, and large clusters to the total Leydig cell cluster area in control and treated animals at E21.5. C, The occurrence of large Leydig cell clusters in the testes of control and DBP-exposed animals at E17.5, E19.5, and E21.5. Values are means ± SEM for five animals per group (*, $P < 0.05$ and **, $P < 0.005$ in comparison with control values). Small Leydig cell clusters were defined as those that accounted for 5% or less of the total Leydig cell cluster area per testis, medium clusters as those that accounted for 5.1–14.9%, and large clusters as those that individually accounted for 15% or more of total Leydig cell cluster area per testis.

animals at a time when seminiferous cords were well established in the remainder of the testis (Fig. 6, A and B). These intermingled groups of Leydig and Sertoli cells were still evident focally on d 4 postnatally in testes of DBP-exposed animals, although it appeared that these areas were now attempting to form seminiferous cords (Fig. 6, C and D).

At d 25 and in adulthood, one to three focal areas per testis of variably shaped, malformed seminiferous tubules (dysgenetic areas) were now evident centrally in the testis (Fig. 6, F and G). In most instances, the malformed tubules in these focal dysgenetic areas also exhibited intratubular Leydig cells (Fig. 7 and Table 2). In adulthood in DBP-exposed animals, all tubules that exhibited intratubular Leydig cells lacked all germ cells (Fig. 7B), whereas at age 25 d, most tubules containing intratubular Leydig cells still contained some germ cells (Fig. 7C). Malformed seminiferous tubules comprising focal dysgenetic areas and intratubular Leydig cells were observed in the majority of testes from adult animals exposed *in utero* to DBP, irrespective of whether the testes were cryptorchid or scrotal in position (Table 2). However, the dysgenetic areas were more common (Table 2), and were usually more extensive and bizarrely shaped, in cryptorchid testes (not shown). In the scrotal testes of DBP-exposed animals, the majority (>90%) of seminiferous tubules were grossly normal, with complete spermatogenesis

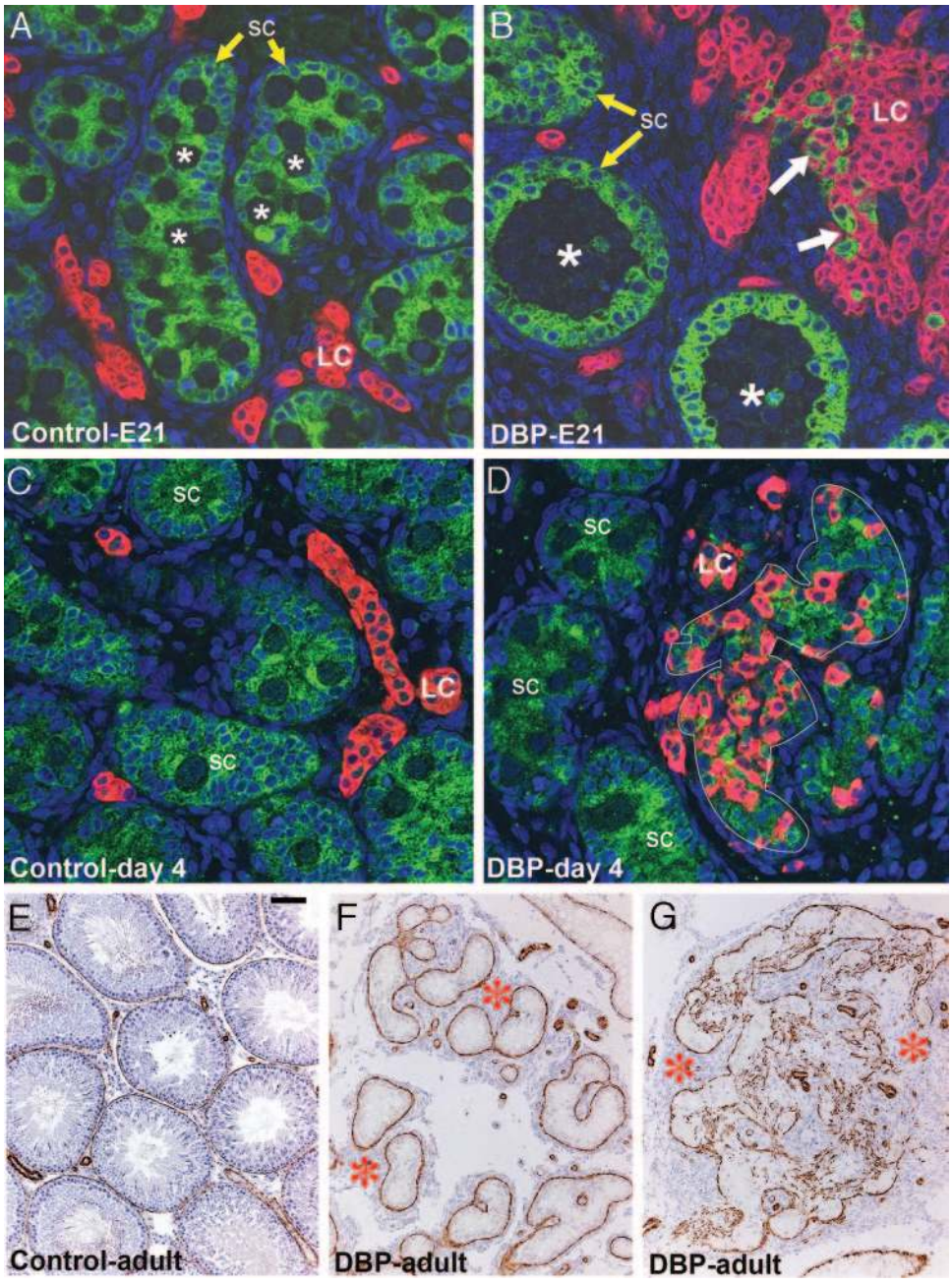

FIG. 6. Distribution of Leydig and Sertoli cells in control (A and C) and DBP-exposed (B and D) animals, illustrated by double immunofluorescence for 3β-HSD (*red*) and AMH (*green*), respectively, at E21.5 and d 4. A large Leydig cell cluster is clearly evident in the DBP-exposed animals both at E21.5 (B) and postnatal d 4 (D). The presence of Sertoli cells within these clusters is also apparent (*white arrows* in B). The *thin white line* (D) has been drawn to illustrate how it is envisaged malformed seminiferous cords may form perinatally that manifest as dysgenetic areas in adulthood (F and G) and in which intratubular Leydig cells are evident (Fig. 7). Note also the abnormal aggregation of gonocytes (*white asterisks*) in DBP-exposed animals (B), compared with controls (A), at E21.5, as described previously (7). Seminiferous tubules in control and treated animals in adulthood are shown in the *lower panel*, visualized by immunostaining for SMA to identify peritubular myoid cells. DBP-exposed animals exhibited one to three dysgenetic areas per testis in which mild (F) to severe (G) malformation of the seminiferous tubules was evident; these areas were never seen in controls (E). Most of the testis in DBP-exposed males appeared comparable with that in controls (E), provided that the testis was in a scrotal position. sc, Normally formed seminiferous cords; *white asterisk*, gonocytes; LC, Leydig cells; *red asterisk*, focal Leydig cell hyperplasia surrounding dysgenetic areas. *Scale bar*, 100 μm.

in adulthood (not shown), even when dysgenetic areas were present centrally in the testis.

Discussion

We (7) and others (8–13, 19) have shown that fetal exposure to DBP, or other phthalates, results in a high incidence of disorders such as cryptorchidism, hypospadias, and infertility that collectively appear similar to those in human TDS (5). Our earlier studies (7) and those of others (20) had also identified the postnatal occurrence of focal dysgenetic areas within the testes of DBP-exposed males in which malformed seminiferous tubules and intratubular Leydig cells were evident. Our search for the mechanisms underlying this focal dysgenesis prompted the present study. In this we have shown that between E15.5 and E21.5, focal, centrally located areas develop in the fetal testes of DBP-exposed males comprising huge aggregations of Leydig cells, which we postulate arise from altered Leydig cell migration. Formation of these Leydig cell aggregates appears to trap isolated Sertoli cells within them and, based on earlier evidence, probably also gonocytes and presumptive peritubular myoid cells (7). These areas are not organized into seminiferous cords in fetal life and are termed dysgenetic. The present findings suggest that early in postnatal life the trapped Sertoli (and peritubular myoid) cells attempt to form seminiferous cords that, because of the abnormal aggregation of cell types, leads to misshapen seminiferous tubules with Leydig cells trapped within them. We believe that this provides a plausible ex-

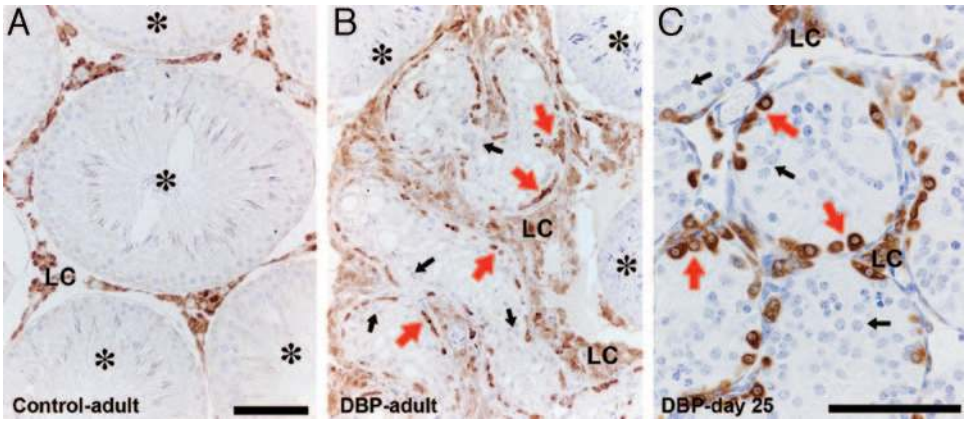

FIG. 7. Postnatal occurrence of Leydig cells (*brown immunostaining* for 3β-HSD) within malformed seminiferous tubules of DBP-exposed animals (*red arrows*) in adulthood (B) and on d 25 (C). No intratubular Leydig cells are found in control animals (A). Note that the intratubular Leydig cells are restricted to dysgenetic areas in which seminiferous tubules are malformed. Note also the presence of some germ cells in dysgenetic tubules at d 25 (C) but their complete absence in adulthood (B). Animal numbers investigated are given in Table 2. *, Tubules exhibiting normal spermatogenesis; LC, Leydig cell clusters in their normal positions within the interstitium; *black arrows*, Sertoli cell nuclei. *Scale bar*, 100 μm.

planation for the origin of the focal occurrence of dysgenetic areas postnatally in central regions of the testes of DBP-exposed animals, and we hypothesize that similar processes may explain the occurrence of similar dysgenetic areas in human TDS (Ref. 17 and our unpublished data). However, the present findings do not provide any clues as to the cause of the abnormal Leydig cell aggregation induced by DBP.

DBP exposure caused changes in Leydig cell distribution in the testis as early as E17.5 and with progressing severity until E21.5, and this was associated with marked suppression of testicular testosterone levels and reduced immunoexpression of P450scc enzyme, consistent with previous reports (7, 13–15). Because Leydig cell clustering and altered steroidogenic function occurred in the same time frame, these changes could have a common cause. Because expression of Insl3 by Leydig cells is also reduced severely in DBP-exposed rats (16), the effect of DBP could be viewed as reflecting a generalized cytotoxic effect. However, this seems unlikely for several reasons. First, DBP has no general or systemic toxicity as evidenced by maternal and fetal body weight (10–12). Second, the effects of DBP are cell selective because there is no change in expression of a range of Sertoli cell protein markers, as exemplified by AMH immunoexpression in our present and previous (7) studies. Third, the effects of DBP on the Leydig cells, although widespread, are also selective as illustrated by the suppression of P450scc immunoexpression and the lack of effect on 3β-HSD immunoexpression, as shown in the present studies.

TABLE 2. Prevalence of dysgenetic areas and intratubular Leydig cells in adult rats exposed *in utero* to corn oil (controls) or to 500 mg/kg DBP

Treatment group	No. of testes	No. of testes with dysgenetic areas[a]	No. of testes with intratubular Leydig cells[b]
Control	24	0	0
DBP-scrotal	8[c]	5	7
DBP-cryptorchid	20[c]	15	18

[a] Based on analysis of testis cross-sections from four to eight blocks per scrotal testis and one to two blocks per cryptorchid testis immunostained for smooth muscle actin.

[b] Based on analysis of testis cross-sections from four to eight blocks per scrotal testis and one to two blocks per cryptorchid testis immunostained for 3β-HSD.

[c] In the DBP-treated group, eight rats were unilaterally cryptorchid and six were bilaterally cryptorchid. All testes analyzed had an intact epididymis.

Using immunohistochemistry for the Leydig cell marker 3β-HSD and image analysis, we have shown a decrease in the number of Leydig cell clusters in the DBP-exposed animals but an increase in the occurrence of large clusters, compared with control animals. These clusters tended to be centrally located, as was illustrated by serial sectioning of E21.5 DBP-exposed testes. We considered two possible explanations for the occurrence of these large Leydig cell aggregates: First, DBP exposure could cause an increase in focal Leydig cell proliferation, or second, DBP exposure could cause Leydig cells to migrate to central regions from other points of origin in the testis. Previous studies, based on nonquantitative observation in DBP-exposed animals, have presumed that such aggregates of Leydig cells are caused by cellular hyperplasia (9, 12, 15, 21), but our study shows that this is not the case. We have shown that not only is Leydig cell number per testis unchanged from control values in DBP-exposed animals at E21.5 but also that Leydig cell size is markedly reduced in DBP-exposed animals due to reduction in cytoplasmic volume. This reduction fits with the reduction in testicular testosterone levels shown at the same fetal ages (this study and Refs. 7, 13–15) because Leydig cell cytoplasmic volume and steroidogenic activity tend to go hand in hand (22). Our present results show no evidence for increased mitosis of interstitial/Leydig cells in fetal testes of DBP-exposed males in the period before E21.5 when Leydig cell aggregation is occurring, and we have found no evidence for altered interstitial cell apoptosis during the same period using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (our unpublished data). These findings conflict to some extent with earlier published studies. For example, one observational study (15) had suggested that there was marginally increased proliferation of interstitial cells in fetal testes of DBP-exposed animals based on

PCNA expression, whereas another study (14) had shown increased expression of factors that might have antiapoptotic effects. However, taken together, the present and earlier (15) findings suggest that if there is a change in Leydig cell proliferation in DBP-exposed testes, it is a minor change and does not result in any significant increase in Leydig cell number on E21.5. By default, this points instead toward altered Leydig cell migration as a more likely explanation for DBP-induced fetal Leydig cell aggregation.

The origin of fetal Leydig cells is still a matter for debate. Two main suggestions have been proposed: first, that Leydig cell precursors migrate into the testis from the mesonephros, as has been described for the peritubular myoid cells (23–25); and second, that Leydig cell precursors arise from the coelomic epithelia of the developing testis and then migrate in between the developing testicular cords (26, 27). Our results lend support to the latter suggestion because we have found no evidence for migration of 3β-HSD-immunopositive cells in from the mesonephros at any of the ages studied, although our analyses do not exclude the migration of 3β-HSD immunonegative Leydig cell precursors into the testis from the mesonephros. Whatever their initial origin, our findings suggest that abnormal aggregation of fetal Leydig cells in DBP-exposed animals most likely occurs via their abnormal migration; however, we cannot exclude other possibilities such as altered sites of differentiation of Leydig (and Sertoli) cells occurring in testes of DBP-exposed animals. We are currently exploring the expression of a number of candidate molecules in DBP-exposed animals, based on reported roles in cell migration/cell attraction in the testis or elsewhere, *e.g.* neural cell adhesion molecule, vanin-1, and the neurotropins (28–31).

In the present study, we show for the first time that the abnormal Leydig cell aggregates in fetal testes of DBP-exposed animals contain isolated Sertoli cells. We are unaware of any other reports that show the presence of Sertoli cells outside seminiferous cords at such a late stage of gestation and intermingled with Leydig cells. The present and earlier (7) findings show that the abnormal Leydig cell aggregates in DBP-exposed animals are still evident postnatally at d 4 when Sertoli cells, gonocytes, and presumptive peritubular myoid cells are also evident in these areas. Such areas are restricted to one to three central locations in the testes of DBP-exposed animals, whereas in most of the same testes the seminiferous cords are normally formed. At postnatal d 4 these abnormal cell aggregates appear to be trying to form seminiferous cords, and it seems plausible that the malformed cords that result correspond to the focal dysgenetic areas that are evident in later postnatal life, in particular in adult testes, because these are also restricted to one to three central areas of the testis (7). The present findings suggest that abnormal Leydig cell aggregation in fetal life may give rise to these unsegregated collections of testicular cells, which in turn give rise to dysgenetic areas in early postnatal life. The occurrence of Sertoli and other cells in the fetal Leydig cell aggregates may be the result simply of physical trapping, and/or it may result from interference with normal cell-cell signaling.

The preceding reasoning also provides a logical explanation for the abnormal occurrence of Leydig cells within seminiferous cords/tubules in postnatal life after DBP-exposure (present study and Ref. 7). These intratubular Leydig cells are mainly found within, or bordering, dysgenetic areas, and most tubules that contain Leydig cells are misshapen. There are two possible mechanisms via which these intratubular Leydig cells could occur. The most obvious explanation, supported by the present findings, is that differentiated fetal Leydig cells are simply trapped when seminiferous cords form in early postnatal life in the aforementioned dysgenetic areas in which fetal Leydig cells have aggregated. Another possibility is that precursors of either fetal or adult Leydig cells are trapped at the same time during dysgenetic cord formation and that these cells subsequently differentiate *in situ* into Leydig cells. We have some (unpublished) evidence to support the latter possibility, based on the appearance of intratubular Leydig cells coincident with differentiation of the normal adult Leydig cell population at approximately 15 d of age plus evidence of these intratubular Leydig cells undergoing mitosis. However, it also seems highly likely that simple trapping of fetal Leydig cells within late-forming seminiferous cords accounts for at least some of the intratubular Leydig cells evident in postnatal testes of DBP-exposed animals.

Intratubular Leydig cells have only rarely been reported in the literature. A recent example is in mice deficient in dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome, gene 1 (Dax1) in regions adjacent to peritubular myoid cell disruption and impaired seminiferous cord formation (32). Intratubular Leydig cells have also been reported in patients with infertility and cryptorchidism (both features of TDS) (5) and have been implicated in the impaired spermatogenesis exhibited by these patients (33–35). We also analyzed testicular tissue from patients with testicular cancer (the most severe manifestation of TDS) (5) and have found intratubular Leydig cells to be present in approximately 30% of patients, in each instance in grossly abnormal tubules devoid of germ cells (our unpublished data). Because intratubular Leydig cells can be identified easily only if Leydig cells are immunostained (*e.g.* for 3β-HSD), we consider that their occurrence in some patients (and perhaps in animal models) may have gone unrecognized. In our rodent studies with DBP, we found that Sertoli cells within tubules containing aberrant Leydig cells were always immature, and no spermatogenesis occurred within their immediate vicinity, at least in the adult testis (7). We hypothesize that intratubular Leydig cells interfere with Sertoli cell development and cell-cell interactions. However, the present findings also indicate that at earlier postnatal ages (d 25), germ cells are found within dysgenetic tubules that harbor intratubular Leydig cells. This contrast with what is found in adulthood in DBP-exposed males implies that the physical presence of the intratubular Leydig cells may disrupt the function of the Sertoli cells, such that long-term survival of germ cells and progression of normal spermatogenesis is not possible. This might in turn explain the variable occurrence of Sertoli cell-only tubules in the adult testes of animals exposed *in utero* to DBP (7) and merits investigation in TDS patients in whom a high prevalence of Sertoli cell-only tubules has been reported (17). Whether this physical obstruction causes the failure of the Sertoli cells to mature or whether the Sertoli cells in the dysgenetic areas of DBP-

exposed testes are inherently incapable of maturing is unclear and certainly warrants further investigation.

Exposure of fetal male rats to 500 mg/kg DBP, as in the present study, results in a high incidence of cryptorchidism (7, 10); a rate of 80–100% unilateral or bilateral cryptorchidism was found in the present and a previous study (7) in our Wistar rats. This cryptorchidism is presumed to result from DBP-induced suppression of Insl3 (16), and perhaps testosterone (7, 13, 36), production by fetal Leydig cells. In the present studies of fetal testes, it was not possible to classify testes as destined to descend or become cryptorchid because testis descent was still in progress. However, two pieces of evidence indicate that DBP-induced fetal Leydig cell aggregation and its sequelae are not restricted to testes that are destined to become cryptorchid. First, the dysgenetic areas, which we suggest form in areas of prior fetal Leydig cell aggregation, are evident in adulthood in scrotal and cryptorchid testes, although these areas tend to be more extensive in cryptorchid testes. Second, significant clustering of fetal Leydig cells is still demonstrable in all rats exposed to a 5-fold lower dose of DBP that results only rarely in cryptorchidism (our unpublished data). Partial agenesis of the epididymis can also occur in rats exposed to 500 mg/kg DBP (12, 20), although we have found only a 12% incidence of this phenomenon in our adult rats (all associated with cryptorchid testes). To avoid confounding, we chose to exclude such testes from our present analyses.

In conclusion, the present study suggests that the abnormal aggregation/migration of fetal Leydig cells as a consequence of fetal DBP-exposure is probably a key event in precipitating dysgenetic changes in the testes of such animals that then persist for life. The resulting focal occurrence of malformed seminiferous tubules and intratubular Leydig cells in adulthood may stem from this initial effect. This supports the view that the fetal Leydig cell may be a primary site of action of DBP (37), especially as abnormal Leydig cell aggregation is the earliest effect of DBP that we have found in the fetal testis. Identification of the molecular mechanisms that underlie fetal Leydig cell aggregation is therefore an important future research goal, with implications for understanding the origins of TDS disorders in man. However, it is difficult to see how DBP-induced focal aggregation of fetal Leydig cells can account for all of the changes observed in the fetal testis, in particular the occurrence of multinucleated gonocytes (7, 13, 15). The latter occur throughout the fetal testes of animals exposed to 500 mg/kg DBP, including in normally formed seminiferous cords. It is therefore more likely that multinucleated gonocytes result from effects of DBP on the gonocytes or Sertoli cells. It is also possible that even the observed changes in Leydig cell aggregation and function occur secondary to a primary effect of DBP on Sertoli cells, but such mechanisms remain to be demonstrated.

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