CONCERNING THE LOCALIZATION OF STEROIDS IN CENTRIOLES AND BASAL BODIES BY IMMUNOFLUORESCENCE

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ABSTRACT

Specific steroid antibodies, by the immunofluorescence technique, regularly reveal fluorescent centrioles and cilia-bearing basal bodies in target and nontarget cells. Although the precise identity of the immunoreactive steroid substance has not yet been established, it seems noteworthy that exogenous steroids can be vitally concentrated by centrioles, perhaps by exchange with steroids already present at this level. This unexpected localization suggests that steroids may affect cell growth and differentiation in some way different from the two-step receptor mechanism.

KEY WORDS antibody · basal body centriole · immunofluorescence · steroids

In recent years, significant advances have been achieved in the understanding of the biochemistry and biology of microtubule-containing cell organelles (see 23, 25, 26, 31 for recent reviews). The immunofluorescence technique has been instrumental in providing a deeper insight into this knowledge, as monospecific tubulin antibody proved to be a useful tool for characterizing tubulin-containing cell structures (5, 9–11, 20, 28, 29). On the other hand, specific steroid antibodies have been successfully employed for the immunofluorescence tracing of steroids in both source and target tissues (6, 17, 21). In this way, a dynamic monitoring of the steroid kinetics in target cells containing appropriate steroid receptors has been attained (17–19). Quite unexpectedly, in the course of investigations concerned with steroid-receptor interactions, we found that centrioles and cilia-bearing basal bodies were constantly revealed by steroid antibodies in immunofluorescence studies. In the present paper we report this observation, the meaning of which is just tentatively investigated and still open to question, although it could suggest that steroids may be physiologically relevant to the regulation of some microtubule-dependent cell processes.

MATERIALS AND METHODS

Tissue and Cell Preparation

Cryostatic sections were obtained from several tissues of immature Sprague-Dawley rats, male and female, weighing 50–60 g, and then allowed to air-dry. Isolated ciliated cells were scraped from rat tracheal epithelium, washed in phosphate-buffered saline (PBS), and then centrifuged on slides, and air-dried.

Human peripheral blood lymphocytes were separated from fresh heparinized blood from healthy male and female donors, by Ficoll-Hypaque density gradient centrifugation (4). After washing three times with PBS, lymphocytes were resuspended (2×10^6 cells/ml) and incubated at 4°C for 1 h in cold PBS containing bovine serum albumin (BSA) 0.5%, and 17β-estradiol 10^{-8} M. After incubation, cells were thoroughly washed in frequently changed cold PBS-BSA, centrifuged on slides, and air-dried.

The continuous cell line HEp-2, kindly provided by Dr. E. Cassai, Institute of Microbiology, University of Ferrara, was also used for these studies. HEp-2 cells were grown in monolayers in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, penicillin (150 IU/ml), and streptomycin (150 μg/ml). In the experiments involving estradiol, the standard

culture medium was replaced by the same medium supplied with 17β-estradiol 10^{-7} to 10^{-8} M, in which cells were kept for 1 h at 37°C. In the experiments involving vinblastine, HEp-2 cells growing on glass cover slips were treated with vinblastine 5×10^{-5} M for 12–24 h at 37°C; sometimes, 17β-estradiol 5×10^{-5} M was added to the vinblastine-containing medium. Cells were finally washed three times in prewarmed PBS, drained, and air-dried. The presence of tubulin paracrystals was checked by the May-Grunwald-Giemsa stain. At times, the fixation steps recommended for optimal preservation of the microtubule structures (28) were introduced.

Immunofluorescence Procedure

Cryostatic tissue sections and cell preparations were processed for the indirect immunofluorescence technique essentially as described previously (17). Unfixed air-dried and fixed preparations were covered with rabbit anti-steroid antisera (see below) and incubated in a moist chamber for 30 min at room temperature. Slides were then extensively washed in PBS, drained, and covered with fluorescein-labeled goat antiserum against rabbit immunoglobulins, for 30 min at room temperature. After three final washes in PBS, the preparations were mounted in buffered glycerol in PBS (1:1), and immediately inspected with a Leitz Ortholux II microscope equipped with epifluorescent illumination. Pictures were taken on Kodak Rayoscope film.

Antisera

Highly specific antisteroid antibodies were obtained from Serono Biodata Division, Milan, Italy. These antibodies were raised by immunization of rabbits with BSA conjugated with: (*a*) 17β-estradiol-6-(*O*-carboxymethyl)oxime; antiserum titer: 1/8,000; relative cross-reactivity: <0.001% with testosterone, androstenedione, progesterone, pregnenolone, 11α-hydroxyprogesterone, cortisone, and aldosterone. (*b*) 17β-estradiol-17-hemisuccinate; antiserum titer: 1/11,000; relative cross-reactivity: <0.001% with testosterone, progesterone, corticosterone, androstenedione, androstenediol, and dehydroisoandrosterone. (*c*) Progesterone-11α-hemisuccinate; antiserum titer: 1/32,000; relative cross-reactivity: <0.01% with aldosterone and testosterone; <0.001% with dehydroisoandrosterone, androsterone, cortisone, 5α-dihydrotestosterone, 17β-estradiol, estrone, and estriol. (*d*) Testosterone-3-(*O*-carboxymethyl)oxime; antiserum titer: 1/4,500; relative cross-reactivity: <0.01% with aldosterone, deoxycorticosterone, and progesterone; <0.001% with cortisone and 17β-estradiol. Titer and cross-reactivity of the obtained antisera were tested by radioimmunoassay according to Abraham (1) after extensive treatment with BSA to remove antibodies against the carrier protein.

For immunofluorescence, all steroid antisera were diluted 1:100 into PBS, after several dilutions had been tried.

Fluorescein-labeled goat antirabbit immunoglobulin antiserum (concentration of the fluorescein isothiocyanate (FITC)-conjugated γ-globulin fraction, 11 mg/ml; molar fluorescein/protein ratio, 3:3; total protein concentration after addition of human albumin, 42 mg/ml) purchased from Behring-Werke AG, Marburg-Lahn, West Germany, was used at 1:20 dilution into PBS.

Control Tests

To establish the specificity of the immune tracing of steroids, the standard immunofluoresence technique was varied as follows: (*a*) nonimmune rabbit serum (at 1:10 dilution into PBS) or (*b*) anti-steroid antisera saturated in excess with the respective steroid were substituted for the specific antiserum in the first step of the immunofluorescence reaction; (*c*) unlabeled goat antirabbit antiserum was applied before the fluoresceinated one; and (*d*) only the fluorescein-conjugated antibody was immediately applied.

RESULTS

We have reported previously that steroid hormones can be traced within their target cells by specific steroid antibodies (17). Distinct cytoplasmic and nuclear patterns of fluorescence were displayed in this way, which strictly reflected the well-known dynamics of specific steroid receptors (17–19). The present report deals, in particular, with the intriguing observation of a prominent structure which stood out in both target and nontarget cells, when stained with steroid antibodies by immunofluorescence.

A well-defined, brightly fluorescent cell structure was observed in every examined tissue and cell. This structure usually appeared as a pair of closely arranged small bodies; each member of the pair measured 0.2 μm in diameter. Sometimes, one member of the pair appeared rod-shaped and ~0.3–0.4 μm in length. The intracellular localization of this dual body seemed to depend on the examined cell and to follow a recognizable rule. The structure was usually located at the edge of the nucleus, often associated with a recess of the nuclear outline (Fig. 1). In monociliated cells of the endometrial and renal tubular epithelium, this paired structure was regularly noticed at the luminal pole of the cell. Specialized ciliated cells from several sources (oviductal, tracheal, and ependymal cells) exhibited well-defined sets of small fluorescent granules lined up at the luminal cell surface (Fig. 2); on the contrary, cilia constantly failed to stain.

HEp-2 cells grown under standard conditions (i.e. Eagle's medium added with fetal calf serum)

exhibited the usual fluorescent dual body, close to the nucleus (Fig. 3*a, b*). In metaphase cells, centrioles were recognizable as fluorescent bodies at each side of the equatorial plate (Fig. 3*c*). When HEp-2 cells and human lymphocytes were exposed to a medium containing 17β-estradiol, this cell structure shone more distinctly after cells had been processed as usual with specific estradiol antibodies, but it was no longer demonstrated by anti-progesterone and anti-testosterone antisera. Tubulin paracrystals present in the vinblastine-treated cells never fluoresced, even when formed in the presence of estradiol in the medium. Moreover, these cells often failed to show any fluorescent structure.

No fluorescent labeling was noticed in the preparations treated with nonimmune serum. Saturation of diluted specific antisera with the respective steroid resulted in the total disappearance of positive staining. No fluorescence was seen when only the fluorescein-labeled antibody was applied or the unlabeled goat antirabbit antiserum was used before this one.

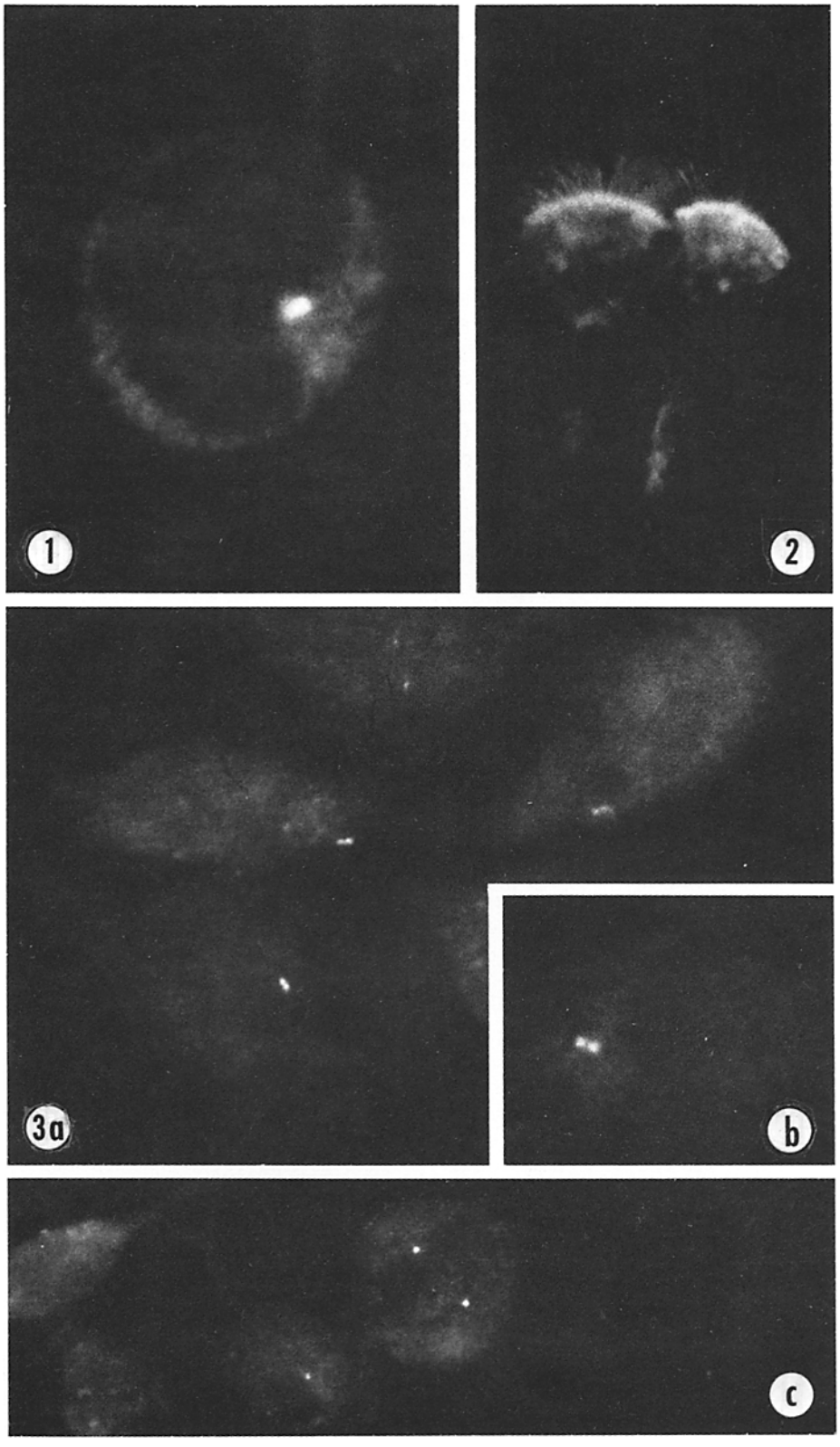
All of the four tested specific steroid antisera proved to be able to detect the concerned structure in each investigated structure; anti-testosterone antibody showed the lower staining power.

DISCUSSION

All the reported features of the cell structures traced by steroid antibodies indicate that we are engaged in the specific demonstration of centrioles and basal bodies. The paired appearance, the orderly localization within the cell (close to the nucleus, or at each pole of the mitotic spindle in metaphase cells, otherwise at the luminal pole in the cells bearing a single primary cilium), and the cortical alignment of ordered sets of small granules in specialized ciliated cells are the characteristics that lend reliability to this statement. The precise identity of the immunoreactive steroid substance has not yet been established, as it can be traced by all the steroid antisera used. This is somewhat disturbing, but it is well known that immunofluorescence is more prone to show cross-reactivity than other immunological methods, especially when antigenic molecules as similar as steroids are bound to cell constituents. Lastly, artifactual relocalization of steroids seems to be ruled out because of the tight binding of stained steroids, which stands extraction by the buffers and solvents used for fixation steps. Moreover, previous studies (17–19) have clearly stated that, when tightly bound, steroids do not undergo relocalization during cell processing.

It is worth noting that the preliminary in vitro studies suggest that steroids can be vitally concentrated by centrioles from the steroid-containing medium. The possibility should be taken into account that exogenous steroids may be exchanged with steroids already present at this site. These observations could suggest that some component of the centriolar complexes is able to bind steroids. A preliminary investigation has been made to establish whether stained steroids are bound to structural elements of centrioles (12) and basal bodies (33), such as tubulin, or to some associated pericentriolar materials (22). Tubulin, the heterodimer subunit of microtubules, is well known to be endowed with specific binding sites for natural ligands and various drugs (25, 30–32). However, the failure of tubulin-containing cell structures (such as the cytoplasmic network, the mitotic spindle, and tubulin paracrystals) to stain, even when exposed vitally to steroid hormones, seems to suggest that some centriolar component other than tubulin is accountable for steroid binding. Incidentally, the unsuccessful visualization of centrioles in many vinblastine-treated cells is in agreement with the fact that tubulin paracrystalline inclusions assemble preferentially around centrioles (3); it is conceivable that these inclusions may interfere with the access of the antibody to centrioles. A more precise definition of the centriolar component responsible for steroid binding could be achieved by means of the immunoperoxidase technique with electron microscopy (work in progress).

Some puzzling findings arise from the comparison of the immunofluorescence results obtained by the use of tubulin and steroid antibodies, respectively. Monospecific tubulin antibody, indeed, did not seem able to visualize the centriole itself in interphase cells. Even when the cytoplasmic microtubule network has been disrupted by disassembling drugs, the centrospheric region was reported to appear as a fluorescent ring with a dark center, while centrioles failed to stain (5, 10). Also, the immunofluorescence studies concerned with mitotic figures and microtubule-organizing centers, though showing some centriole-associated structures, never revealed centrioles themselves (10, 11, 20, 28). On the other hand, a unique specific decoration of centrioles and basal bodies was produced by steroid antisera, as reported here, whereas no other microtubule


1
2
3a
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structure was visualized. At this moment, we have no satisfactory explanation of this intriguing contrast.

In conclusion, evidence is provided for the presence of immunoreactive steroids in centrioles and basal bodies. Whatever role steroids may play at this level is not immediately obvious. A wide variety of mechanisms have been suggested to explain steroid hormone action. Current models assume that, on entering target cells, steroid is tightly bound to a specific cytoplasmic receptor, and that this hormone-receptor complex is then transferred to the nucleus where it binds to the chromatin, altering the pattern of gene expression (see 2, 7, 8, 13, 16, 34 for recent reviews). Present biochemical evidence favors this common primary mechanism for steroid hormone action; however, it is noteworthy that certain steroid-sensitive processes can proceed without the mandatory involvement of receptors (2, 13, 14, 16). To these latter processes could likely be related the specific demonstration of steroids in centrioles and basal bodies. Circumstantial evidence is now available that steroid-induced changes of the diplosome may play an ancillary role in mediating steroid hormone action. Indeed, steroids have proved to affect the structure, the duplication, and the normal array of the paired centrioles, and to regulate directly the process of ciliogenesis, the relation of which to centrioles and basal bodies is well known (15, 24, 27). Therefore, the presence of steroids in centriolar complexes might suggest that an adjunctive pathway should be envisaged, by which steroid hormones affect cell replication, growth, and differentiation.

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FIGURE 1 Human lymphocyte from a male donor, stained with antiestradiol antibody. A brightly fluorescent structure fills the centrospheric region invaginated in the nuclear outline. × 1,800.

FIGURE 2 Ciliated epithelial cells scraped from tracheal mucosa of a 10-day-old male rat, stained with antiestradiol antibody. Ordered rows of fluorescent granules lie in the luminal region below the ciliated border. × 1,200.

FIGURE 3 HEp-2 cells exposed to estradiol (see text). In (*a*), antiestradiol antibody reveals brightly fluorescent paired centrioles near the nucleus. × 800. In (*b*), note the diplosomic configuration of the stained structure. × 1,800. In (*c*), fluorescent centrioles are located at each side of the equatorial plate of a metaphase cell. × 800.

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