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Biological Effects and Morphological Responses to Estriol, Estriol-3-Sulfate, Estriol-17-Sulfate and Tamoxifen in a Tamoxifen-Resistant Cell Line (R–27) Derived from MCF-7 Human Breast Cancer Cells

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Abstract—*The R-27 cell line is a variant clone derived from the MCF-7 human breast cancer cell line which has lost its inhibitory response to anti-estrogens. In the present study, we have compared the biological responses to estriol* (E_3), *estriol-3-sulfate* (E_3-3-S), *and estriol-17-sulfate* (E_3-17-S) *in these cells and in the parent MCF-7 cells. In the R-27 cell line after* 7 days *of culture, the progesterone receptor (PR) concentrations were greatly increased by* E_3 *and* E_3-3-*sulfate; however, tamoxifen did not block this effect. The effect in PR provoked by* E_3-17-S *was significantly less intense. The concentrations of PR* (pmol/mg *DNA* ± *S.D.*) *in the R-27 cells were as follows: control*: 1.1 ± 0.8; +E_3: 10.5 ± 2.4; +E_3-3-S: 5.4 ± 2.3; +E_3-17-S: 2.6 ± 0.8. E_3 *and* E_3-3-S *also stimulated PR in the MCF-7 cells but to a lesser extent. No stimulation was observed in the* E_3-17-S *treatment. A fraction* (0.5–1%) *of the* E_3-3-S *was found to be hydrolysed in the medium during the incubation in both cell lines, but no hydrolysis occurred after incubation with* E_3-17-S. *Ultrastructural observations showed that in the* E_3 *and* E_3-3-S *treated cells, there was an important development of the ergastoplasm, bundles of filaments and an accumulation of ribosomes. No significant morphlogical alteration was observed in cells exposed to* E_3-17-S. *In conclusion,* E_3 *is biologically very active in both the R-27 and the MCF-7 cell lines and* E_3-3-S *could play a role in the control of the estrogenic activity of* E_3.

INTRODUCTION

ESTROGEN sulfates are one of the most abundant forms of estrogen circulating in the human and some other animal species. Plasma concentrations of estrone sulfate during the menstrual cycle are 3–7 times higher than the combined concentrations of unconjugated estrone and estradiol. Also, considerable plasma levels of estrone sulfate are present even after the menopause [1, 2]. Sulfatase and sulfotransferase activities have been extensively demonstrated in human mammary cancer: formation of estrogen sulfates (sulfokinase activity) has been found by several authors [3–5] and sulfatase activity for estrogen sulfates has also been demonstrated [6–9].

In different mammalian species, estriol is considered to have a short-acting effect and it has been suggested that it could have an antagonistic effect against estradiol [10, 11]. However, when estriol is administered in physiological conditions and continuously, it can act as a full estrogenic compound [12, 13].

The R-27 cell line is a variant clone derived from the MCF-7 human breast cancer cell line, which has lost the inhibitory response to anti-estrogens [14]. Consequently, it was interesting to study the biological responses of estriol, estriol-3-sulfate and estriol-17-sulfate in this cancer cell line and compare these effects in the MCF-7 cell line.

MATERIALS AND METHODS

Radioactive steroids and other chemicals

[17α-methyl-^{3}H]R5020 (17α,21-[17α-methyl-^{3}H]-dimethyl-19-nor-pregna-4,9-diene-3,20-dione) (S.A.: 50 Ci/mmol); non-radioactive R5020; [6,7-^{3}H]-estriol (S.A.: 54 Ci/mmol) were obtained from New England Nuclear Corp. (NEN France, Paris, France). The purity of the different radioisotopes was controlled by thin-layer chromatography in

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the appropriate systems before use. Estradiol, estriol and diethylstilbestrol (3,4-bis(4-hydroxyphenyl)-3-hexene) were purchased from Steraloids (Touzart et Matignon, Vitry-sur-Seine, France); estriol-3-sulfate (1,3,5(10)-estratriene-16α,17β-diol-3-yl-sulfate) and estriol-17-sulfate (1,3,5(10)-estratriene-3,16α-diol-17-yl-sulfate) were obtained from Leo (Helsingborg, Sweden). Tamoxifen [ICI 46,474; trans-1-(*p*-dimethylamino-ethoxyphenyl) 1,2-trans-diphenylbut-1-ene] was a gift from Dr C. Hache (ICI Pharmaceuticals, Le Galien, France); glutaraldehyde (Prolabo, Paris, France); osmium tetraoxide, uranyl acetate and lead citrate (Merck, Darmstadt, German Federal Republic); propylene oxide (FLUKA A.G., Buchs, Switzerland); Epon (Ladd Research Industries Inc., Burlington, U.S.A.).

Cell culture

R-27 and MCF-7 human mammary cancer cell lines were a generous gift from Dr. M.E. Lippman (National Institutes of Health, Bethesda, U.S.A.). Cells were grown in a humidified atmosphere containing 5% v/v CO_2 at 37° C and maintained in Minimal Essential Medium (MEM) supplemented with 2mM L-glutamine, 100 U/ml penicillin–streptomycin and 10% v/v dialysed calf serum (Gibco Europe, Paisley, U.K.).

Cells were plated in 75 cm^2 Falcon plastic flasks at a density of approx. 3×10^6 per flask. The medium was changed every 2 days and the culture maintained for 7 days before receptor assays or morphological observations by electron microscopy.

Estrogens, prepared in ethanol, were added at a final concentration of 5×10^{-12}M to 5×10^{-7}M. The final concentration of tamoxifen was 1×10^{-6}M.

Cell fractionation

The cells were washed twice with (Ca^{2+},Mg^{2+})-free Hank's balanced salt solution (HBSS) and then removed by a 10–15 min incubation at 37° C with 1 mM EDTA in HBSS. Cells from two flasks were pooled, cytosol and nuclear fractions were isolated as previously described [15]. Briefly, cytosol fractions were prepared in TED buffer (0.01 M Tris-HCl, 1.5 mM EDTA, 0.5 mM dithiothreitol, pH 7.4) after centrifugation at 200,000 ***g*** for 30 min at 4° C. The nuclear fraction was obtained after extraction of the nuclear-myofibrillar pellet with 0.6 M KCl in TED buffer at pH 8.5 and centrifugation at 200,000 ***g*** for 30 min at 4° C.

Single saturating-dose assays of specific [^{3}H]-R5020 binding sites

[^{3}H]-R5020 was used to determine progesterone receptor binding [16]. Cytosol or nuclear extracts were incubated with 5×10^{-9}M [^{3}H]-R5020 in the absence or presence of a 100-fold molar excess of unlabelled R5020 at 4° C for 16 hr. Unbound steroid was removed by mixing 1 vol. of extract with 1 vol. of dextran-coated charcoal (0.5% w/v charcoal, 0.05% w/v dextran) for 10 min at 4° C. Specific binding was obtained by subtracting the non-saturable binding from the total binding.

Radioimmunoassay of estriol

Unconjugated estriol was determined in culture medium by radioimmunoassay according to a method described by Tulchinsky *et al.* [17] with the modifications indicated previously [18]. After addition of [^{3}H]-estriol (2000 dpm) to calculate analytical losses, 0.5–2 ml of culture medium were extracted with 10 vol. of ethyl acetate : hexane (3 : 2, v/v). The organic phase was evaporated to dryness and redissolved in 0.5 ml 0.1 M phosphate buffer for the radioimmunoassay.

DNA assay

DNA concentrations were determined according to Burton [19].

Electron microscopic examination

Treated and non-treated cells were fixed in cacodylate buffer (pH: 7.2) containing 2.5% v/v glutaraldehyde. After a period of 2 hr, the cells were

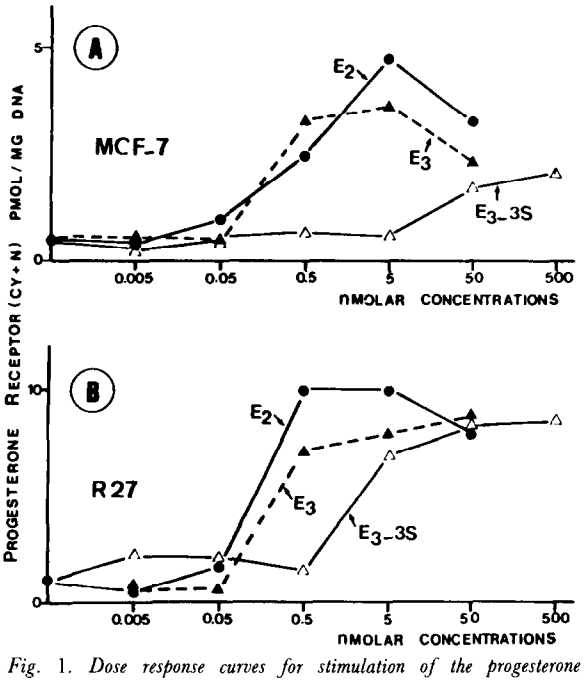

Fig. 1. Dose response curves for stimulation of the progesterone receptor (PR) by estradiol (E_2), estriol (E_3) and estriol-3-S (E_3-3-S). MCF-7 (A) and R-27 (B) cell lines were incubated with various concentrations of E_2 (●——●), E_3 (▲--▲) or E_3-3-S (△——△) *for 7 days. All values represent the mean of two determinations.*

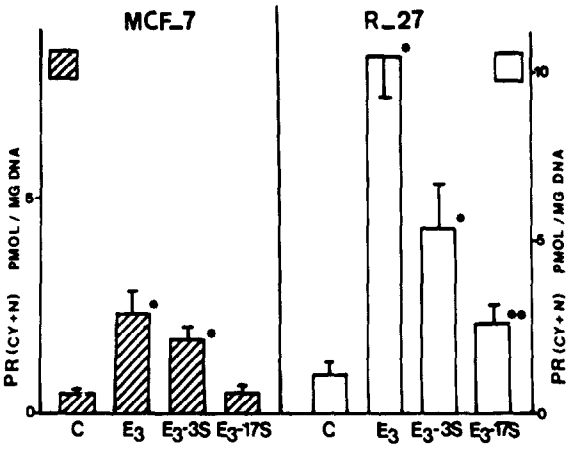

Fig. 2. Effects of estriol (E_3), estriol-3-sulfate (E_3-3-S) and estriol-17-sulfate (E_3-17-S) on the progesterone receptor (PR). MCF-7 (▨) and R-27 (□) cell lines were maintained in culture in MEM containing 5×10^{-8}M E_3, E_3-3-S or E_3-17-S for 7 days. The values represent the mean ±S.E. of 4–20 determinations. Statistical comparisons were made using the Student's t-test.

*$P < 0.01$ (vs. control values);

**$P < 0.05$ (vs. control values).

scraped from this solution and centrifuged. Pellets were rinsed with the cacodylate buffer and post-fixed in osmium tetraoxide (2% w/v), then stained with uranyl acetate (2%, w/v) (20) at 4° C for 1 hr, dehydrated through alcohols and finally in propylene oxide [21]. Fixed pellets were embedded in Epon resin and ultra-thin sections were obtained with an Ultramicrotome LKB (Broma, Sweden) equipped with a diamond knife (Diatome, Bienne, Switzerland), stained with uranyl acetate and lead citrate [22] and examined in a Zeiss EM9 electron microscope (Carl Zeiss, Tübingen, F.R.G.).

RESULTS

1. *Dose response of estradiol* (E_2), *estriol* (E_3) *and estriol-3-sulfate* (E_3-3-S) *on progesterone receptor stimulation*

Figure 1 shows that E_2 or E_3 can be active in both cell lines at a concentration of 5×10^{-10}M but E_3-3-S became active from 5×10^{-9}M. This difference in the dose response curves seemed to indicate that the apparent activity of the estrogen sulfate was due to hydrolysis and not to the sulfate itself.

2. *Progesterone receptors after estriol* (E_3), *estriol-3-sulfate* (E_3-3-S) *and estriol-17-sulfate* (E_3-17-S) *treatments in the* R-27 *and* MCF-7 *cell lines in culture*

It can be observed in Fig. 2 that E_3 and E_3-3-S stimulate the number of specific binding sites for progesterone very intensively in the R-27 cell lines (an average of 10- and 5-fold in relation to the non-treated cells). E_3-3-S also stimulates the PR in the MCF-7 cells but significantly less than that observed in the R-27 cells. In the E_3-17-S treatment of the R-27 cell line, some effect on the progesterone receptor was observed but much less than in the E_3 or E_3-3-S treatments. No effect was observed for E_3-17-S in the MCF-7 cell line.

3. *Measurement of* E_3 *in the culture medium*

In order to explore if E_3-3-S or E_3-17-S could be hydrolyzed to some extent, the radioimmunoassay of estriol was performed in the medium with these two estriol sulfates after incubation in both cell lines. After incubation with E_3-3-S (5×10^{-8}M), significant hydrolysis occurred in both cell lines (130–200 pg/ml of freed E_3), but no hydrolysis was observed in the experiment carried out with E_3-17-S. These data suggest that E_3 liberated from the E_3-3-S is responsible for the biological response because the concentration ($4–7 \times 10^{-10}$M) of E_3 found is sufficient to elicit the biological response (see Fig. 1).

4. *Effect of tamoxifen on the progesterone receptor of R-27 cells*

Because it has been reported that R-27 cells are tamoxifen resistant, it was of interest to explore the action of tamoxifen alone or combined with estriol or estriol-3-sulfate on progesterone receptor levels. Table 1 shows that tamoxifen had no significant effect and it does not block the stimulatory action of E_3 or E_3-3-S when added simultaneously.

5. *Transmission electron microscopy (TEM) of R-27 cells after different treatments*

Non-treated R-27 cells (Fig. 3A) have a dense

Table 1. Effect of tamoxifen, tamoxifen + estriol or tamoxifen + estriol-3-sulfate on the progesterone receptor of R-27 mammary cancer cell lines

	Progesterone receptor (cytosol + nuclei, in pmol/mg DNA)
Control	1.13 ± 0.33
+ Tamoxifen	2.12 ± 0.55
+ [Tamoxifen + Estriol]	7.24 ± 1.80*
+ [Tamoxifen + Estriol-3-sulfate]	4.19 ± 0.56*

R-27 cells were maintained in culture in MEM containing 1×10^{-6}M tamoxifen or tamoxifen combined with 5×10^{-8}M estriol or estriol-3-sulfate for 7 days.

The values represent the mean ±S.E. of four to seven determinations.

Statistical comparisons were made using the Mann–Whitney distribution-free test.

*$P < 0.01$ (vs. control values).

cytoplasm, and the Golgi apparatus or rough endoplasmic reticulum (RER) cannot be clearly differentiated. Large nuclei with condensed chromatin and small nucleoli are also observed. Mitochondria are small, dense and well distinct. Details at higher magnification are indicated in Fig. 4A. In estriol-treated cells some microvilli are evident, mitochondria are numerous and larger than those in non-treated cells. RER with cisternae is associated with numerous peri-cytoplasmic ribosomes. Some bundles of filaments lie in the clear cytoplasm. Small nuclei are observed with condensed chromatin and fibrillar nucleoli. The dense accumulation of ribosomes near the cytoplasmic membrane is indicated in Fig. 3B (for details see 4B). After the E_3-3-S treatment, the cells show an important development of a fibrillar system close to the well distinct nuclear membrane. Many small vacuoles, surrounded by numerous ribosomes, are localized near the cytoplasmic membrane and large nuclei present a dispersed chromatin (Figs. 3C and 4C). On the other hand, in the E_3-17-S treated cells (Fig. 3D), no significant morphological changes are observed.

DISCUSSION

Mammary cancer tumors are considered hormone-dependent when they contain significant quantities of estrogen receptors [23]. Because the progesterone receptor is a function of estrogen action, this parameter was chosen to establish the biological activity of estrogens in these tumors, as well as in isolated mammary cancer cell lines. Patients who have both receptors respond to a high degree to antiestrogen treatment (70–80%)[24–26].

The present data indicate that E_3 and E_3-3-S significantly increase the specific binding sites for progesterone in the R-27 cells, 10- and 5-fold, respectively, compared to the non-treated cells and 3–4 times in the MCF-7 cells. The amount of E_3 measured in the medium ($4–7 \times 10^{-10}$M) for both cell lines indicates that a fraction of E_3-3-S was hydrolysed. This concentration of E_3 ($4–7 \times 10^{-10}$M) is sufficient to elicit a biological response (Fig. 1). Different authors have also found that E_2 at 10^{-10}M induces biological effects in MCF-7 cells [27, 28]. Consequently, we propose that the biological effects of E_3-3-S are carried out through the freed E_3. It is to be remarked that E_3-17-S provokes a limited stimulation of PR in the R-27 but not in the MCF-7 cell line. The factors which are involved in this difference (cell penetration, hydrolysis) need to be explored.

The present data may have physiological relevance because: (1) estrogen-3-sulfates are the natural form of estrogen sulfoconjugation, and (2) estriol-3-sulfate is accumulated in great proportion (100–6000 pg/ml) in human breast cyst fluid [29].

It is also to be remarked that tamoxifen does not stimulate the PR and it does not block the effect provoked by E_3 or E_3-3-S. The lack of effect of TAM on PR is in contradiction with the data of Vignon *et al.* [30] in which these authors found a significant increase in PR in the incubation of the same cell line with TAM.

The ultrastructural observations from transmission electron microscopy indicate that some morphological characteristics of the non-treated R-27 cells are similar to those which have been observed in MCF-7 cells [31, 32]. However, in E_3 and E_3-3-S treated cells a greater pericytoplasmic accumulation of ribosomes is observed, as well as an important development of the ergastoplasm and bundles of filaments, considered to be a cellular transport system. All of these modifications may be related to the increase observed in progesterone receptor concentrations since no significant morphological alterations (in relation to the non-treated cell) were observed in the E_3-17-S treated cells.

In conclusion, E_3 is biologically very active in MCF-7 and R-27 mammary cancer cell lines and E_3-3-S, an abundant estrogen in breast tissue, could play an important role in the regulation of E_3 effects, which suggests the potential importance of estrogen-3-sulfates in the action of estrogens in the mammary gland.

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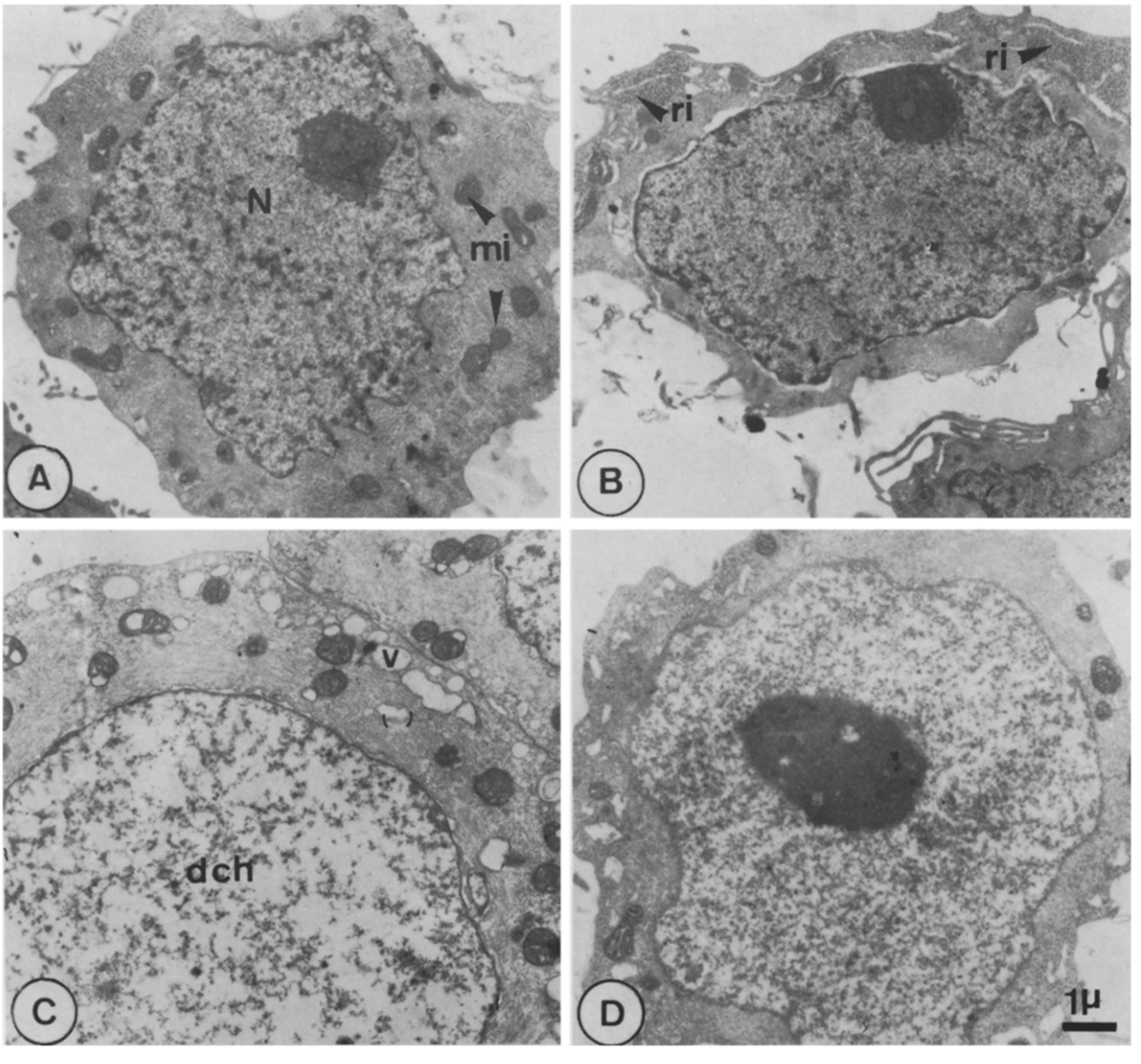

Fig. 3. General view in TEM of the R-27 cells after different treatments. (A) Non-treated cells; mitochondria (mi), nucleus (N). (B) Cells treated with estriol; ribosomes (ri). (C) Cells treated with estriol-3-sulfate; vacuoles (v), dispersed chromatin (d.ch). (D) The ultrastructure of the R-27 cells treated with estriol-17-sulfate appears similar to the non-treated cells. Magnification × 8100.

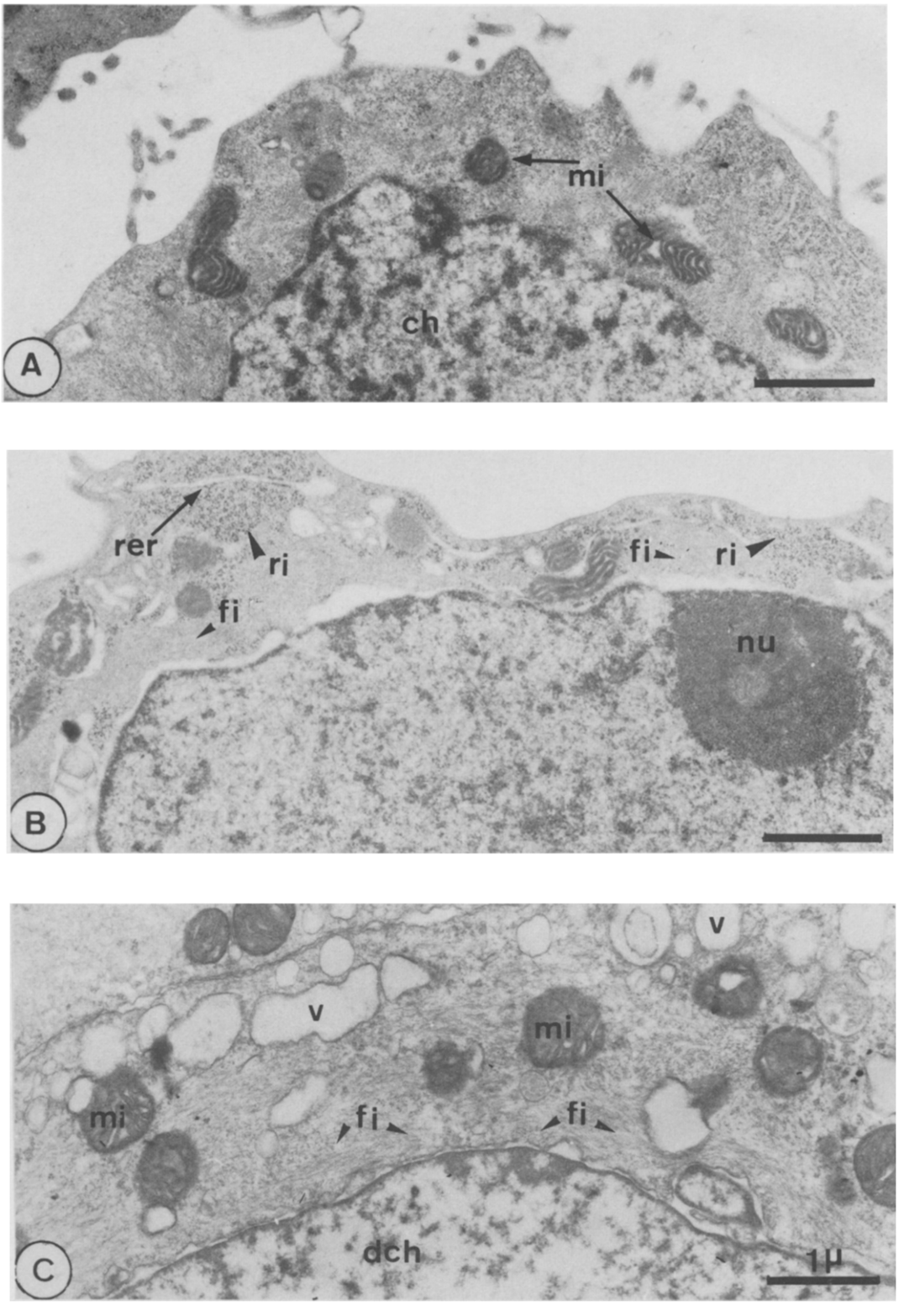

Fig. 4. Details of the morphological alterations of the R-27 cells after different treatments. (A) *Non-treated cells; mitochrondria (mi), chromatin (ch).* (B) *Estriol-treated cells; accumulation of ribosomes (ri), bundles of filaments (fi), nucleolus (nu).* (C) *Estriol 3-sulfate treated cells; large mitochondria (mi), vacuoles (v) and filaments (fi).*
Magnification × 19500.

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