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Original Article

Morphometric Studies of the Localization of the Glucocorticoid Receptor in Mammalian Cells and of Glucocorticoid Hormone-Induced Effects[1]

GUNNAR AKNER,[2] ANN-CHARLOTTE WIKSTRÖM, KARIN MOSSBERG, KARL-GÖSTA SUNDQVIST, and JAN-ÅKE GUSTAFSSON

Departments of Medical Nutrition (GA,ACW,JÅG) and Clinical Immunology (KGS), Karolinska Institute, Huddinge University Hospital, Huddinge, Sweden, and Department of Physics IV (KM), The Royal Institute of Technology, Stockholm, Sweden.

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We studied the subcellular distribution of the glucocorticoid receptor (GR) by light microscopy (LM) and confocal laser scanning microscopy (CLSM) in different mammalian cell types. The effect of added glucocorticoid hormones on GR distribution was investigated by photometric quantitation on optical sections obtained by CLSM followed by statistical analysis. In the control interphase cytoplasm, the distribution of GR was fibrillar in some and diffuse in other cell types. Fibrillar GR was distributed along cytoplasmic microtubules (MTs) with predilection for a subset of MTs. GR was also observed in the centrosomes. Nuclear GR was both diffuse and granular in distribution. During cell division, GR appeared in the mitotic apparatus at all stages of mitosis. These findings were not fixation-dependent. Glucocorticoid treatment increased both the nuclear and cytoplasmic GR signal. However, this was detectable only after precipitating but not cross-linking fixation. There was both intra- and intercellular GR heterogeneity in the absence and presence of hormone but no indication of a hormone-induced nuclear translocation of GR. We present a hypothetical model of two independent GR populations in the nucleus and cytoplasm, respectively, without any discernible ligand-induced nuclear translocation of GR. The extranuclear GR population may exert effect(s) on site in the cytoplasm without involving nuclear genomic transcription. (*J Histochem Cytochem 42:645–657, 1994*)

KEY WORDS: Glucocorticoid receptor; Localization; Nuclear translocation; Mammalian cells; Immunocytology; Fixation; Confocal laser scanning microscopy; Photometry; Microtubule; Heterogeneity.

Introduction

Present knowledge regarding the subcellular localization of glucocorticoid receptor (GR) indicates that it may be unique among the various receptors in the steroid receptor superfamily of genes (32). There is evidence that GR, in contrast to most other family members, exhibits both nuclear and extranuclear distribution (6,12,17, 22,44). Different immunolocalization results have been presented regarding the extranuclear GR pool; (a) the extranuclear and nuclear pools are stable, regardless of the presence or absence of glucocorticoid hormone (17,22); and (b) part of or the entire cytoplasmic pool moves to the nucleus in the presence of ligand (6,12,44).

Regarding the precise subcellular distribution, GR has been reported to be present in the plasma membrane (16), in the nuclear envelope (21), and distributed along cytoplasmic and mitotic microtubules (MTs) (2,3). However, it has also been claimed that GR is present solely in the nucleus both in the presence and absence of hormone (10,31). These reports suggest that the previously reported cytoplasmic signal is caused by artifactual leakage of GR due to inadequate fixation during sample preparation. Furthermore, overexpressed heterologous GR has been shown to immunolocalize only to the nucleus, regardless of hormonal state (39).

From the voluminous literature regarding localization of GR, there is no consensus concerning whether a ligand-induced nuclear translocation from cytoplasm to nucleus exists. Several nuclear localization signals in GR have been defined, and $t_{1/2}$ for the nuclear translocation of GR has been reported to be around 5 min (34). However, no mechanistic evidence exists as to how such a nuclear translocation could be accomplished. The cytoplasmic pool of GR has been assumed to constitute a storage form of GR, awaiting a proper activating signal to detach it from its putative multiprotein anchoring complex (36) and to move it into the cell nucleus, where it participates in regulation of transcriptional activity.

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[2] Correspondence to: Dr. Gunnar Akner, Dept. of Medical Nutrition, Karolinska Institute, Huddinge University Hospital, Novum F60, S-141 86 Huddinge, Sweden.

We have previously presented immunocytological evidence that GR is located in both the interphase cytoplasm and in the cell nucleus in human fibroblasts (2,3). In this study we further analyze the immunocytological localization of GR using various mammalian cell types, cell culture conditions, and detection techniques, with emphasis on the effect of added glucocorticoid hormones.

Materials and Methods

Cells

The following mammalian cell types were studied:

Primary Cultures or Isolated Cells. We studied: (a) male and female human gingival and skin fibroblasts from healthy donors aged 15–40, derived from explants of biopsies from the vestibular gingival mucosa or skin (abdominal and gluteal regions), used between serial passage 3–20; (b) human lymphocytes isolated from buffy coats; (c) human thymocytes, isolated from human thymus excised during open chest surgery; and (d) mouse spleen lymphocytes.

Cell Lines. The following cell lines were used: (a) 3T3; (b) L-929; (c) HeLa; (d) MCF-7; (e) CV-1; (f) H-4-IIE (HTC); (g) a human mesothelioma cell line established in our laboratory from a mesothelioma obtained by thoracocentesis; (h) CHO; and (i) C127I.

Cell Culture

All cells except the lymphocytic cell types were serially subcultured with a split ratio of 1:8 as monolayers on ethanol-washed, heat-sterilized, 18 × 18-mm glass coverslips (Chance Propper; Waple, UK) in incubators with a moist atmosphere of 5% CO_2 (L-929 in 10% CO_2) and 95% air in 35-mm wells in plastic culture cluster dishes (Nunc; Roskilde, Denmark). The standard medium was Eagle's minimum essential medium (MEM) containing 28 μM phenol red supplemented with 2 mM L-glutamine, 60 mg/liter (100,000 IU/liter) benzylpenicillin, 100 mg/liter streptomycin sulfate, and 8% (v/v) heat-inactivated (56°C, 30 min) fetal bovine serum (FBS). The cortisol content of the FBS was <2 nM (lower than the detection limit for the radioimmunoassay). After dilution of the FBS > 10 times to the final medium concentration of 8% (v/v), the concentration of bovine glucocorticoids to which the cells were exposed in the standard experiments was therefore at most 0.2 nM, well below the KD for GR in human fibroblasts (3,15). In some experiments the FBS was treated with dextran-coated charcoal (DCC). In other experiments the medium was changed to Dulbecco's modified Eagle's MEM (DMEM) containing 2 or 4.5 g/liter glucose, respectively, compared with 1 g/liter glucose in Eagle's MEM. Other changes were using phenol-free medium, no addition of serum and/or antibiotics, and changing FBS to human AB serum. Some cell types required special culture conditions: CHO cells were cultured in Ham's F12 medium without phenol red, supplemented with 2 mM L-glutamine, 10% (v/v) FBS, and 50 μg/ml gentamycin. HeLa cells were cultured in RPMI 1640 with L-glutamine, antibiotics, and serum as described above. MCF-7 cells were cultured in RPMI 1640 without phenol red, supplemented with the same three standard components and non-essential amino acids.

Manipulation of Cell Cultures

Cells were treated with five different glucocorticoid hormones, both lipophilic and hydrophilic: dexamethasone, dexamethasone phosphate, triamcinolone acetonide, cortisol, and cortisol Na-succinate. The dose range used was 10^{-3} to 10^{-9} M, and exposure times were 30 min–24 hr. Control cultures were exposed only to the vehicle (ethanol or water).

Fixation/Permeabilization

After removal of the culture medium, two standard fixation techniques were routinely compared: (a) 4% formaldehyde in PBS at 4°C for 10 min–16 hr, followed by 0.5% Triton X-100 for 30 min–16 hr, followed by wash with PBS. This process was designated "F/T." Triton X-100 was in some experiments exchanged for other detergents, such as 0.24% SDS, 0.017% Nonidet P40 (NP40), or 0.05% Tween 20. Various combinations of aldehyde/detergent concentrations were also tried, i.e., formaldehyde 2–4% and Triton X-100 0–0.5%; and (b) methanol at −20°C for 5–15 min, followed by rehydration with PBS for 30 min–2 hr. This process was designated "M." Other combinations of fixatives used were methanol followed by 80% acetone in water, or 4% formaldehyde followed by methanol and/or 80% acetone. Except for lymphocyte stainings, where samples were sometimes air-dried before fixation with formaldehyde, great care was taken not to allow drying of the cells at any time during the entire staining procedure.

Immunostaining Procedure

Cell cultures were regularly stained on the first to third day of subculture and the entire procedure was carried out in sequence during the same day. Both single and double immunostaining were used. All incubations and washes were performed at room temperature. PBS buffer (3) was used for dilution and washes and the substitute for primary or secondary antibodies. After fixation/permeabilization and PBS wash, the first antibody (or mixture of antibodies) was applied and incubated for 60–120 min, followed by a wash in PBS for 10 min and detection.

Three different detection systems were used: (a) Indirect immunofluorescence technique: the second, fluorochrome-conjugated antibody (or mixture of antibodies) was added and incubated for 45–60 min. After two PBS washes, the coverslips were mounted upside down on glass slides (Menzel Gläser; Braunschweig, Germany) in 50% glycerol in PBS. (b) Indirect immunoperoxidase technique [Vectastain ABC mouse kit (avidin–biotin–peroxidase)]: the substrate was diaminobenzidine tetrahydrochloride (DAB) diluted in PBS. Final concentration of DAB was 0.55 mg/ml and of H_2O_2 0.0045%. Substrate incubation time was 5–15 min, followed by two washes in distilled water and mounting. (c) indirect immunogold technique: this used a colloidal gold-labeled secondary antibody (particle size 1 nm) followed by washes and mounting as described above. Microscopic examination and microphotography of the immunofluorescence-stained specimens were performed as previously described (3). The film exposure times were 3–30 sec. All pictures were taken using immersion oil (Nikon; Tokyo, Japan). The immunoperoxidase- and immunogold-stained samples were examined with a Nikon Labophot microscope and photographed with a Nikon FE camera using Kodak Tri-X Pan 400 ASA black-and-white film.

Chemicals

Cell media and supplementary components were purchased from Gibco (Uxbridge, UK). Dexamethasone and triamcinolone acetonide were analytical grade products obtained from Sigma (St Louis, MO). Dexamethasone Na-phosphate (Decadron) was purchased from MSD (Rahway, NJ) and hydrocortisone Na-succinate (Solu-Cortef) from Upjohn (Kalamazoo, MI).

Antibodies

Primary Antibodies. The anti-GR antibodies are all produced in our laboratory (29) and are designated as: (a) monoclonal mouse anti-rat liver GR IgM (MAb1), used as protein A-purified ascites at a final protein concentration of 20 μg/ml; (b) monoclonal mouse anti-rat liver GR IgG_1 (MAb5), used as protein A-purified ascites at 20 μg/ml; (c) monoclonal mouse anti-rat liver GR IgG_{2a} (MAb7). This antibody was used as either

protein A-purified ascites at 100 μg/ml or (b) Diacult-produced (42), protein A-purified (MAb7) at 20 μg/ml; (d) monoclonal mouse anti-rat liver GR IgG_{2a} (MAb8), used as protein A-purified ascites at 20 μg/ml; (e) polyclonal rabbit anti-sea urchin egg tubulin heterodimer (Dakopatts; Glostrup, Denmark) in serum, diluted 1:100, yielding a final protein concentration of 0.51 mg/ml.

Secondary Antibodies. For fluorescence detection, we used: an FITC-conjugated goat anti-mouse antibody (Becton Dickinson; Mountain View, CA) diluted 1:10, yielding a final protein concentration of 25 μg/ml; and (b) a Texas Red-conjugated donkey anti-rabbit Ig antibody (Amersham; Poole, UK), diluted 1:25, 20 μg/ml. For peroxidase detection we used the Vectastain ABC mouse kit (Vector Laboratories; Burlingame, CA) according to the protocol provided by the manufacturer. For colloidal gold particle detection, we used AuroProbe One (Amersham) at a 1:50 dilution of the gold-labeled secondary antibody.

Confocal Laser Scanning Microscopy (CLSM)

CLSM was performed on immunofluorescence-stained cells as described (1,2). In short, the specimen was illuminated one point at a time by a focused laser beam and the fluorescent light was detected by a photomultiplier tube (PM tube). One optical section was recorded through the central part of each cell. The sections were oriented parallel to the plane of the coverslip (transversal) and had a thickness of 2 μm, dependent on the objective (×40/NA = 1.0) used in this study. After recording, the optical sections were represented by digital images with 512 × 512 pixels. The intensity value of each pixel lies between 0–255 and is proportional to the number of fluorescence photons emitted from the corresponding point in the specimen. The objective, the laser beam intensity, and the sensitivity of the detector were kept constant for those pairs of samples (with or without glucocorticoid treatment) where a comparison between the GR intensity values was to be made.

The digital images were shown on a TV display and the entire cell, the nucleus, and a part of the background were manually encircled. For each compartment (nucleus, cytoplasm, and whole cell), the number of pixels was taken as a measure of the area. After subtraction of the background level, the sum of the intensity values of all pixels in each compartment was calculated and represents a measure of the integrated fluorescence detected from each compartment. In several of the CLSM figures shown in this work, the gray scale was individually adjusted in a non-linear way to show the staining pattern as clearly as possible. Therefore, the presented CLSM images are valid only for qualitative interpretation. No such adjustments were performed in images used for subcellular quantitation.

Statistical Analysis

All cell cultures and immunostaining procedures were the same, as was the process of analysis by CLSM (see above). The major possible problem in objectively defining the data was the visual selection of microscopic fields of view to be scanned and analyzed by CLSM. This is a problem inherent to all morphological analysis unless automatic cell detection techniques are employed. However, this potential error was minimized by analyzing a relatively large number of cells for each variable.

The effect of glucocorticoid hormone treatment on the GR intensity in the nucleus, cytoplasm, and whole cell was studied for each of the two standard fixations. We analyzed the differences between the average intensity values in each pair (with or without hormone) by Student's *t*-test. In the diagrams, each single measurement is depicted by a vertical line, the length of which is proportional to its magnitude. The lines are then arranged in ascending order. By this simple device a complete portrayal of all data is achieved.

Gel Electrophoresis and Western Immunoblotting

SDS-PAGE electrophoresis was performed in 7.2% gels as previously described (1). The samples were prepared by boiling pellets of human gingival fibroblasts for 2 min in sample dilution buffer, followed by storage at −20°C until use. Proteins were transferred from the SDS-PAGE gel to Hybond-C membranes (0.45 μm) (Amersham) by electroblotting and were detected as described (1).

Results

Specificity Control

Human gingival fibroblasts contain around 100,000 GR-binding sites per cell (3). Figure 1 shows Western immunoblots of crude human gingival fibroblast (passage 6) cytosol, as well as a Coomassie-stained gel. All four anti-GR antibodies used in this study recognized a band around M_r 94 KD. One of the antibodies also reacted with a low M_r band, presumably a degradation product of GR. Similar results were observed when cytosol from gingival fibroblasts passage 20, L-cells, HTC cells, and HeLa cells were analyzed (not shown). The antibody MAb7 specifically recognizes GR in fixed cells (17,34,44). Controls, in which PBS was substituted for the first or the second antibody in Western immunoblotting or immunocytology experiments, were always negative. Fixation of the cells by any of the two standard fixation techniques, followed by addition of DAB substrate alone, resulted in no staining signal. Fixed but unstained cells showed essentially no autofluorescence after either of the two standard fixation techniques (2,3).

GR in Interphase Fibroblasts

We have previously described the cellular distribution of GR in human fibroblasts with conventional immunofluorescence (3) as well as CLSM (2). Similar results were obtained in the present study with fibroblasts from various locations, four different protein A-pur-

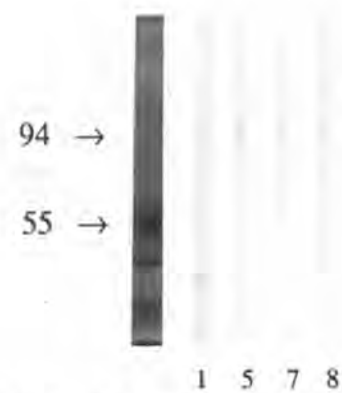


Figure 1. Antibody specificity on Western immunoblot. Western immunoblots of crude human gingival fibroblast (passage 6) cytosol and a Coomassie blue staining of the same gel. All four protein A-purified anti-GR MAb used in this study yielded one band around M_r 94 KD. MAb 1 also recognized one lower M_r band, presumably a degradation product of GR. Standard proteins × 10^{-3} are indicated by arrows. 1,5,7,8: MAb1, MAb5, MAb7, and MAb8, respectively (29).

ified anti-GR antibodies at low final concentration, a number of principally different fixation/permeabilization techniques, and three different detection systems. We focused on the indirect immunofluorescence technique, as it allowed the best subcellular resolution of GR and quantification of GR immunoreactivity on optical sections obtained by CLSM.

The various fixation/permeabilization techniques and anti-GR antibodies yielded similar immunolocalization of GR. There was a distinct cytoplasmic and nuclear GR signal in most cells, and neither of these signals could selectively be diluted away.

Cytoplasm. GR was distributed in a fibrillar pattern that colocalized well with cytoplasmic microtubules (MTs) as previously described (3). Neighboring GR stained fibrils often displayed a variation in staining intensity (Figures 2a and 2b). By serial sectioning, we ruled out that this was due to winding of individual MTs in different vertical planes within the cell (not shown). Furthermore, GR exhibited a beaded pattern along the individual fibrils (Figures 2a and 2b). The anti-GR antibodies also recognized a GR immunosignal at the centrosome, where the two individual interphase centrioles were often stained (Figures 3a and 3b). Cellular projections, both in interphase and dividing cells, usually stained positive for GR, as did the membranes of vesicles of various sizes often seen at the leading edge. The vesicle membranes also contained tubulin and hsp90, but not actin or vimentin (not shown). Some areas of the plasma membrane were often stained, usually the leading edge of the lamellipodium but also other parts (not shown). The entire circumference was never stained.

Nucleus. Nuclear GR was diffuse in all cells; however, approximately 30% of the cells displayed discrete GR-stained spots of varying number, size, and intensity. There were usually 20–40 such granules per nucleus, and CLSM sectioning showed that they were localized within the nuclei (Figures 3a and 3b). The granules disappeared during mitosis and reappeared in the reconstituted nuclei during late telophase. This granular GR staining was resistant to treatment with an MT-stabilizing buffer (not shown).

We observed a heterogeneous GR signal between closely growing cells, both regarding the average whole cell intensity (Figure 4) and the relative nuclear-to-cytoplasmic (n/c) intensities, more easily recognized after M (Figures 5a and 5c) than F/T (Figure 5e) fixation. Treatment with glucocorticoid hormones increased the relative n/c GR ratio. However, this was visually detectable only in ~10–20% of the cells in a monolayer and was observed only after M but not after F/T fixation (cf. Figures 5a, 5b and Figures 5e, 5f). The changes were similar independent of incubation time (30 min–24 hr), dose (10^{-3} to 10^{-9} M), or type of glucocorticoid (see Materials and Methods). The cytoplasmic fibrillar pattern was always discernible, regardless of short or prolonged hormone treatment, and there was no tendency towards reduced cytoplasmic staining in M-fixed cells that exhibited increased nuclear staining.

Photometric Quantitation of GR in Various Compartments Before or After Hormone Treatment

The observed inter- and intracellular heterogeneity of GR made

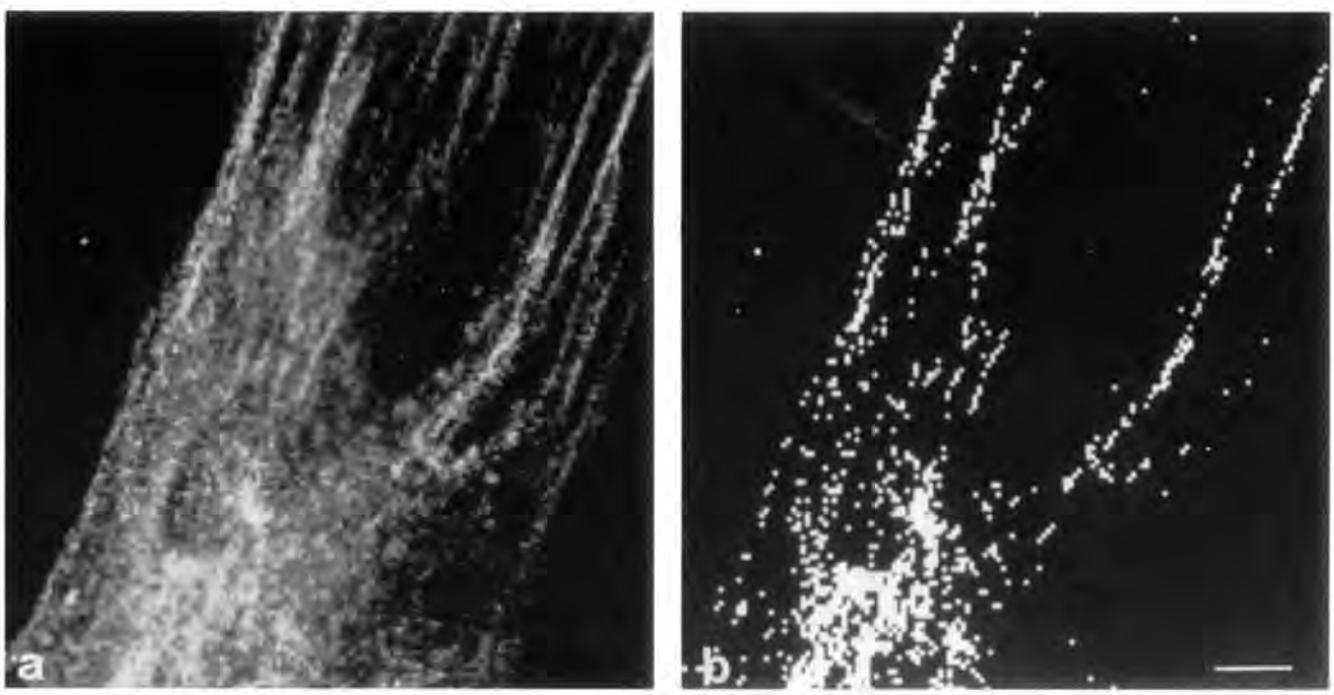


Figure 2. Stronger GR staining in a subset of MTs. CLSM-produced transverse optical sections showing indirect immunofluorescence monostaining of GR in human gingival interphase fibroblasts after M fixation and MAb1. (**a**) Individual MTs are stained with different intensity and exhibit a granular pattern. (**b**) High cut-off level, where pixel intensities below a threshold level were set to zero, leaving only the strongest GR staining along some of the fibrils. Bar = 8 μm.

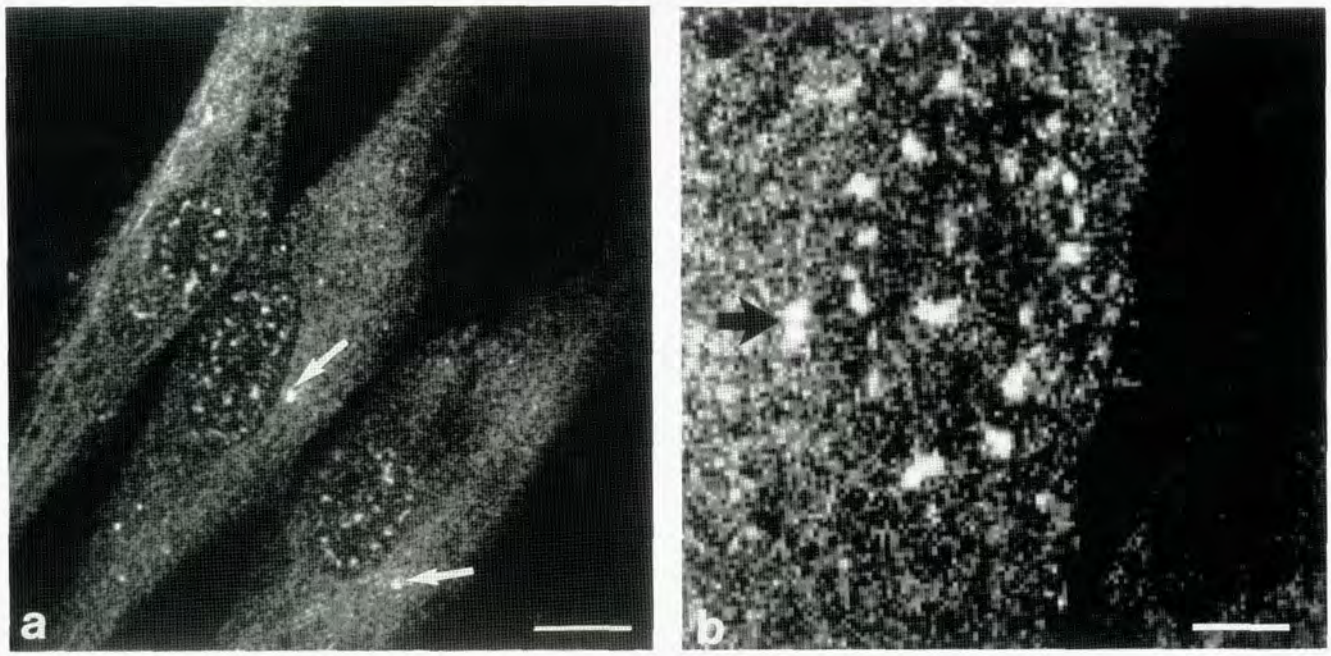


Figure 3. GR is present in nuclear granules and in the centrosome. (**a,b**) CLSM-produced transverse optical sections showing indirect immunofluorescence monostainings of GR in human gingival interphase fibroblasts after M fixation and MAb1. **b** is a higher magnification of **a**. Note the granular nuclear GR staining. The centrosomes in **a** are indicated by arrows. In **b**, the individual centrioles are separated and contain GR. Bars: **a** = 15 μm; **b** = 4 μm.

it difficult to visually judge the effect of hormone treatment. We therefore quantified the GR immunosignal photometrically in various cell compartments on CLSM-obtained optical sections and statistically analyzed the effect of the two standard fixations with or without treatment with dexamethasone (1 μM, 1.5 hr). The short hormone incubation time was chosen to avoid interference with the known ligand-induced up-regulation of GR protein peaking after ≈ 6 hr of treatment (28). We measured the total GR intensity in the nucleus, cytoplasm, and whole cell in 2-μm thin optical sections of the human fibroblasts and derived the quotient between the intensities in the nucleus and the cytoplasm as well as between the nucleus and the whole cell for each cell. There were no statistically significant changes in average cell or nuclear size after glucocorticoid treatment (not shown). Since such glucocorticoid-induced changes have been reported (41), we also calculated the mean GR intensity per pixel for the same three compartments ("pixel corrected values"), as well as the same ratios as indicated above ("pixeled quotients"). The results are shown in Table 1 and Figure 6. F/T fixation revealed a ~2.5-fold stronger average GR immunosignal than after M fixation in the nucleus, cytoplasm, and whole cell of non-hormone-treated cells, whereas the cellular distribution of GR was similar for both fixations (Figure 5), indicating a large difference in the degree of GR extraction between the standard fixations. Figure 6 shows the heterogeneous distribution of the individual GR intensities in the nucleus and cytoplasm, as well as the nucleus:cytoplasm intensity ratios for all cells, respectively. Hormone treatment did not change the average relative nucleus-to-whole cell GR distribution, which remained ~10–12%, similar for both fixations.

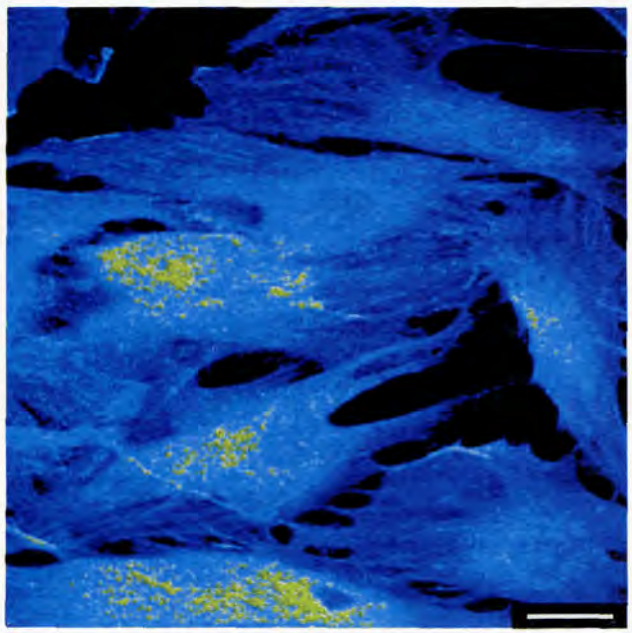
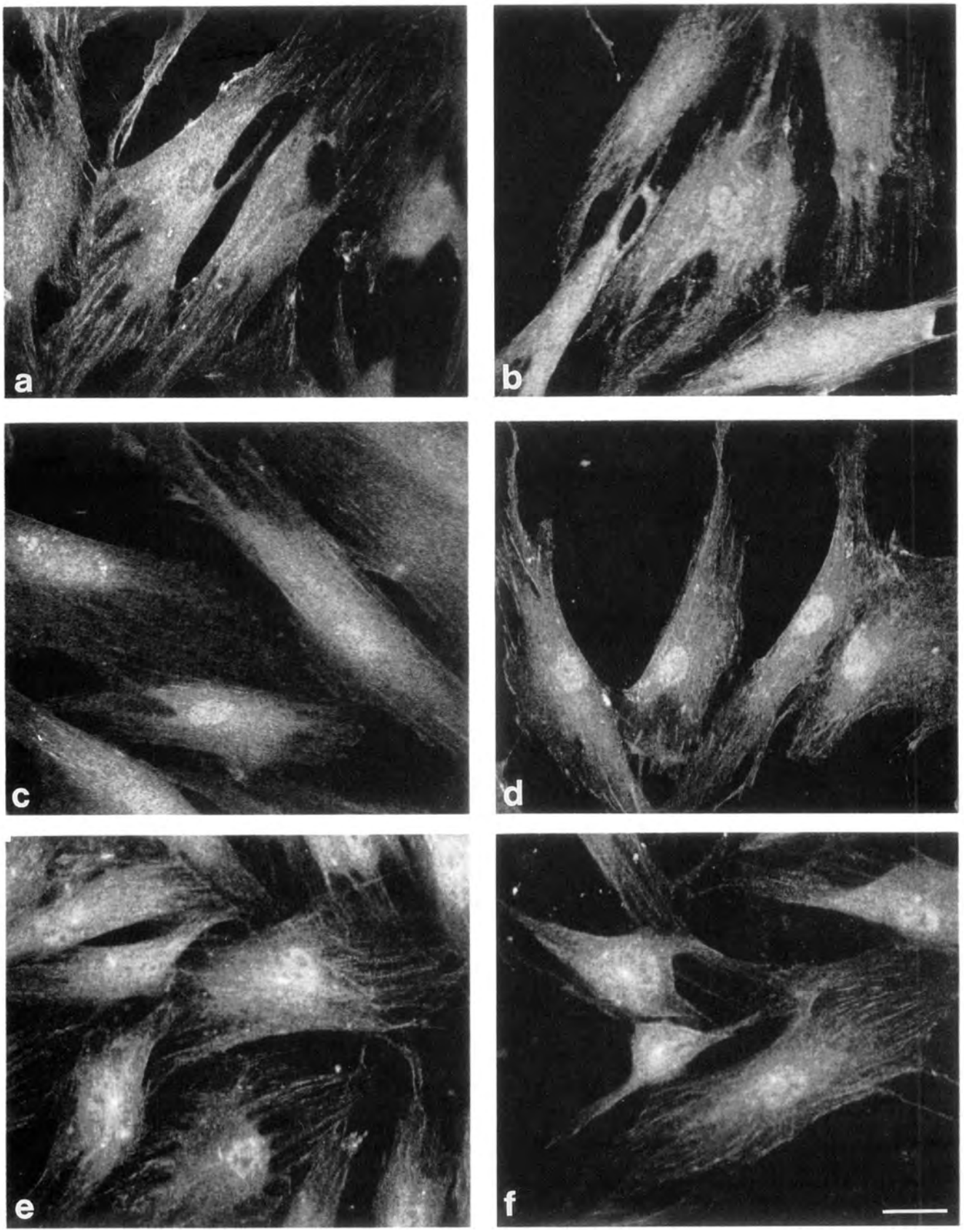


Figure 4. Intercellular GR heterogeneity. A CLSM-produced transverse optical section through M-fixed human gingival interphase fibroblasts monostained for GR by indirect immunofluorescence. The difference between the GR content in closely growing cells indicates intercellular GR heterogeneity. Bar = 30 μm.

Importantly, and in accordance with the visual analysis, statistically significant differences after hormone treatment were detectable only after M but not after F/T fixation. Glucocorticoid treatment induced significant increments in the average GR intensity in both nucleus, cytoplasm, and whole cell, but in different rela-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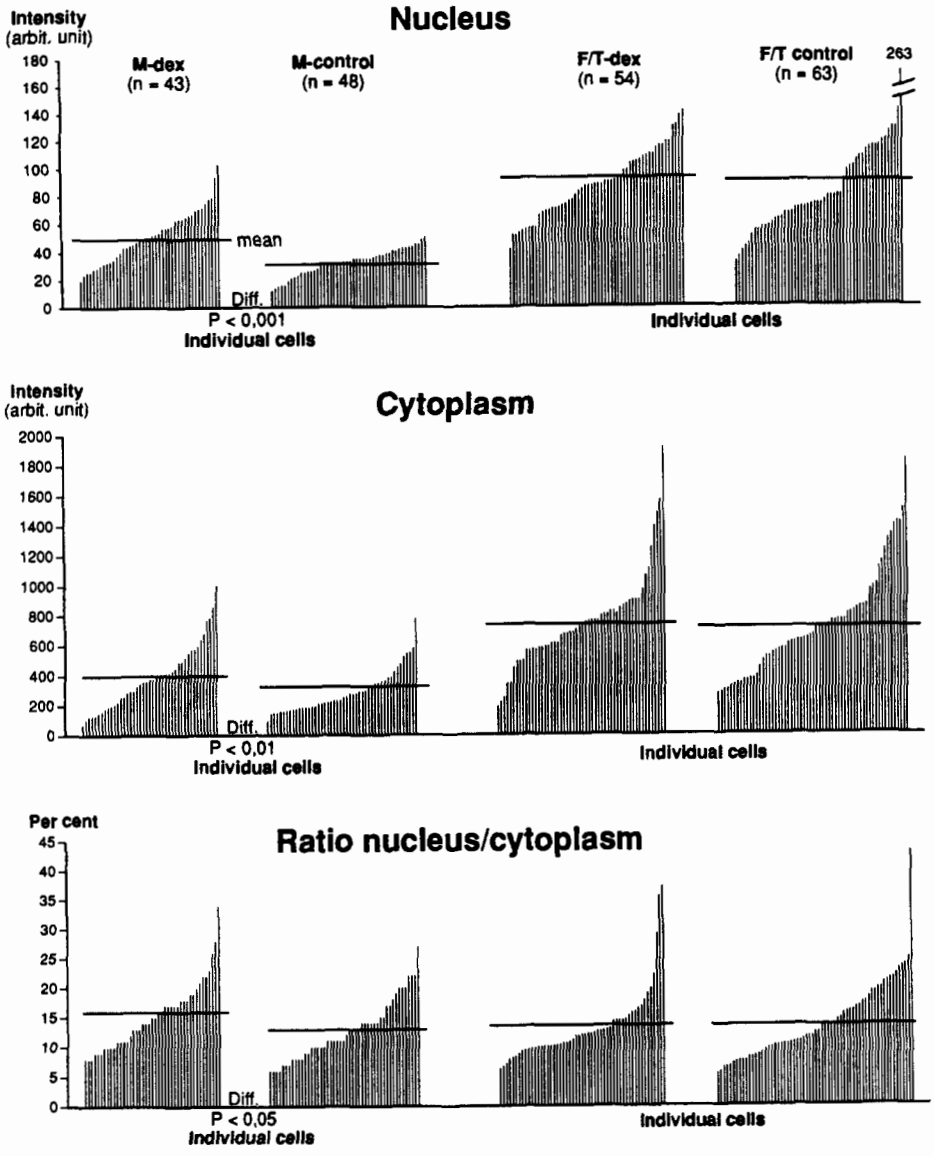

Figure 6. Graphic portraits of the distributions of individual GR intensities in the nucleus and cytoplasm after methanol (M) (left) and F/T (right) fixation and MAb7 in the presence (dex) or absence (control) of dexamethasone (1 μM; 1.5 hr) treatment. The lower part of the figure shows the corresponding distributions of the ratio between the GR intensity in the nucleus and the cytoplasm. The values are arranged in ascending order and for practical reasons are reduced by 10^{-3} for the nucleus and cytoplasm. The mean intensities and the statistical significances between M-dex and M-control are indicated. No statistical difference was found between F/T-dex and F/T-control.

Table 1. *Photometric GR intensities in various compartments on optical sections of human fibroblasts*[a]

	Mean value of integrated intensity (arbitrary units, rounded off to thousands)			Mean ratio (%)		Mean intensity per pixel			Mean of individual ratios	
	n	c	w	n/c	n/w	n	c	w	n/c	n/w
M dex (n = 43)	52,000***	393,000**	445,000**	15.4*	13.2*	59***	17*	18**	3.6***	3.2***
M control (n = 48)	32,000	289,000	321,000	12.8	11.1	37	15	16	2.7	2.4
F/T dex (n = 54)	88,000	751,000	839,000	13.2	11.5	99	36	38	2.8	2.6
F/T control (n = 63)	86,000	716,000	802,000	13.8	11.9	102	37	40	2.8	2.6

[a] Effect of dexamethasone (1 μM; 1.5 hr) on the photometric fluorescence intensity of GR expressed in arbitrary units in 2-μm thin transverse optical sections of the nucleus (n), cytoplasm (c), and whole cell (w) of human fibroblasts, comparing different fixations [methanol (M) vs formaldehyde/detergent (F/T)], using anti-GR MAb7 at a final protein concentration of 20 μg/ml. Also included are the mean n/c and n/w ratios and the intensities corrected for the measured number of pixels in each compartment, as well as the mean of individual n/c and n/w ratios ("pixeled quotients"). Statistically significant differences between hormone treatment and control within each pair are indicated *** $p<0.001$, ** $p<0.01$, * $p<0.05$ (see also text). Values within parenthesis represent the number of observations for each variable. Identical settings were used in the CLSM scanning, so all the intensity values in the table are directly comparable.

Figure 5. Effect of various fixations and hormone treatment on GR distribution. CLSM-produced transverse, optical sections of human gingival interphase fibroblasts monostained for GR, (a,c,e) before and (b,d,f) after dexamethasone treatment (1 μM; 1.5 hr) using M (a–d) and F/T (e–f) fixation followed by anti-GR MAb7. Note the heterogeneous pattern in relative n/c GR signal in M-fixed cells (a,c) compared with F/T-fixed cells (e). Bar = 30 μm.

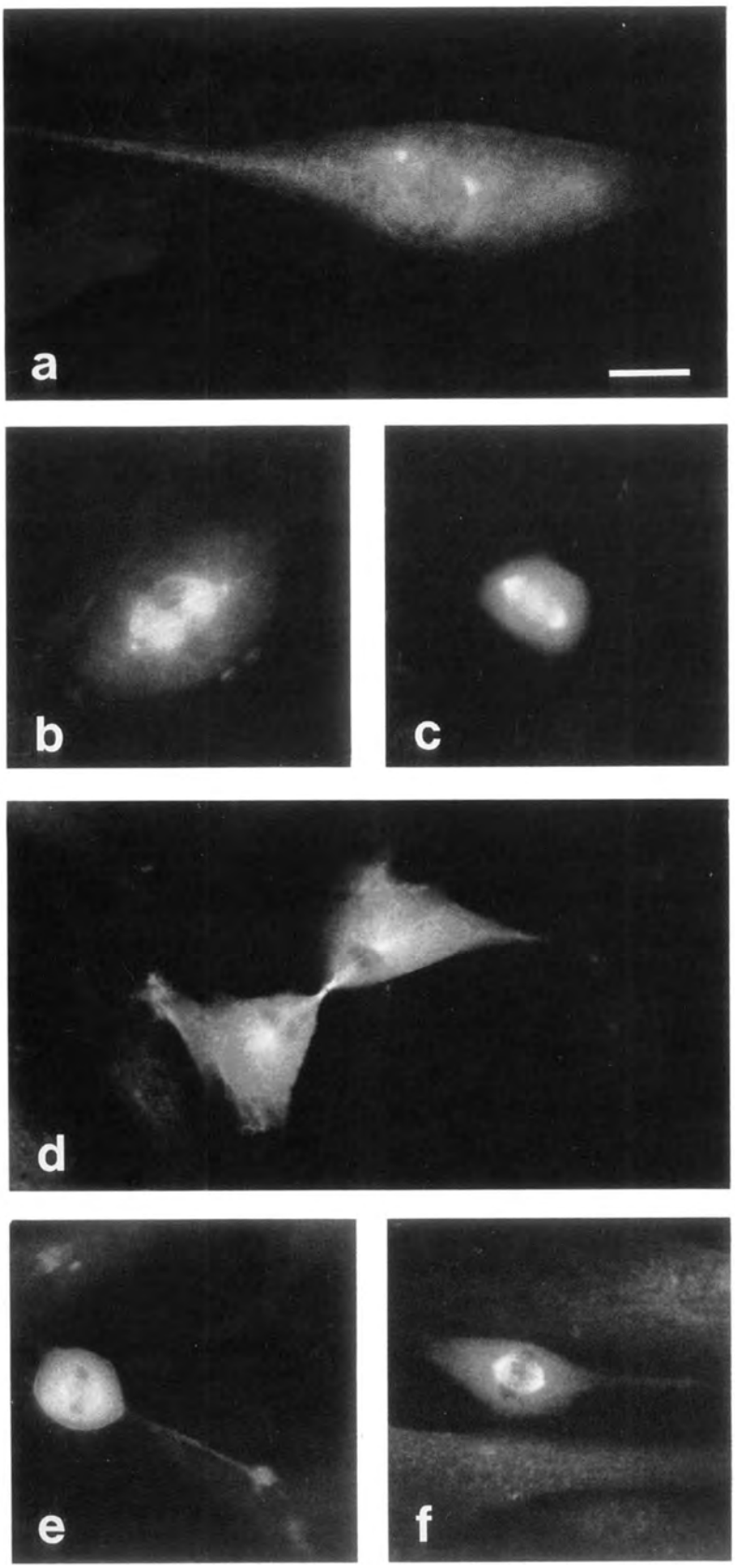

Figure 7. Localization of GR during cell division. (**a–d**) Conventional indirect immunofluorescence monostaining of GR in human gingival mitotic fibroblasts fixed by (**a,b**) M or (**c,d**) F/T, followed by MAb7 (**a,c,d**) or MAb1 (**b**) in various stages of mitosis. (**a**) Prophase; (**b**) metaphase; (**c**) anaphase; (**d**) telophase. (**e,f**) Conventional immunofluorescence monostaining of GR in the metaphase spindle apparatus comparing (**e**) M and (**f**) F/T fixation with MAb7. Note that M extracts less extra-spindle GR than F/T. Bar = 20 μm.

tive proportions compared with controls, i.e., 62% ($p<0.001$), 36% ($p<0.01$), and 39% ($p<0.01$), respectively. In addition, the nucleus-to-cytoplasm (n/c) and the nucleus-to-whole cell (n/w) intensity ratio increased significantly. Therefore, the nuclear GR exhibited a stronger average increase than cytoplasmic GR. Since F/T fixation did not reveal any statistical differences, hormone treatment reduced the difference in average GR intensity in both nuclei and cytoplasm between the two fixations from ≈ 2.5 to ≈ 1.9, indicating a lower general degree of GR extraction after hormone treatment and M fixation. In addition, the pixel-corrected values increased significantly after hormone treatment, as did the corresponding "pixeled quotients."

Mitosis

GR was present in the mitotic spindle apparatus through all stages of mitosis (Figures 7a–7d). There was usually a sharper GR staining of the mitotic apparatus after fixation by F/T compared with M, probably due to more extensive extraction of extra-spindle GR when detergent was used rather than alcohol (cf. Figures 7e and 7f). GR was found mainly in kinetochore but also in astral and probably polar MTs. The centrioles at the spindle poles usually contained GR immunoreactivity (Figure 7b).

Comparison with Other Mammalian Cell Types

Under basal culture conditions we observed a similar inter- and intracellular heterogeneity of GR staining as in fibroblasts in several other cell types, before (Figure 8a) or after (Figure 8b) hormone treatment. A fibrillar staining pattern, as in human fibroblasts, was seen in mouse 3T3 cells. Sometimes we observed a fibrillar pattern in the cellular periphery, e.g., in MCF-7 cells and HeLa cells (not shown), especially after F/T fixation. However, the cytoplasmic GR signal in HTC cells (Figures 8a and 8b), mesothelioma cells (Figure 8c), L-929 cells (Figure 8e), and CV-1 cells (not shown) was predominantly diffuse, including peripheral areas close to the plasma membrane, even though the cells contained a typical MT network (Figure 8d). In mouse L-929 cells we observed weak, diffuse cytoplasmic GR staining, together with one intensely GR-stained cellular protrusion, on virtually all cells (Figure 8e). All lymphocytic cell types studied exhibited various degrees of diffuse extranuclear GR signal, with much less nuclear staining (Figure 8f). GR was present in the mitotic spindle apparatus in all cell types studied, except for lymphocytes, in which no dividing cells were seen. The effect of glucocorticoid treatment in the other cell types was similar to that in fibroblasts, as judged by visual analysis in the microscope. However, we did not undertake a corresponding statistical analysis on optical sections, as with the fibroblasts.

Comparison of Different Cell Culture Medium Compositions

The GR staining pattern did not show any significant changes in any of the studied cell types depending on the presence or absence of serum, DCC-treated or untreated serum, fetal bovine serum or human AB serum, antibiotics, phenol red, or increase of the glucose concentration in the medium from 1 to 4.5 g/liter (not shown).

Discussion

The four anti-GR antibodies used in this study (all developed against purified activated rat liver GR) recognize different epitopes in the N-terminal domain (immunodominant = transactivating domain) of both the non-activated and the activated GR (29). MAb7 has been further epitope-mapped to the amino acid interval 119–273 of the rat GR (38). The data presented here show that all four antibodies crossreact well with human gingival fibroblast GR.

GR staining was observed in fibroblasts, epithelial cells and lymphocytic cells, representing primary cultures or cell lines from two embryonic germinal layers (entoderm and mesoderm), derived from five mammalian species, both primates and non-primates.

Nuclear GR

Previously, the nuclear GR distribution has mostly been reported to be diffuse (17,22,44); however, in line with our findings, it has been reported that overexpressed heterologous mouse GR is distributed in a non-random, mottled pattern throughout all planes of the CHO cell nuclei in both the absence and presence of glucocorticoid hormones (26). Steroid receptor complexes have been reported to associate with RNP particles (23). Antibodies against several different small nuclear ribonucleoproteins (snRNPs) are known to give rise to a granular nuclear immunofluorescence pattern (37,43). It is therefore possible that GR is present in snRNP particles.

Extranuclear GR

There was strong extranuclear GR staining after both standard fixations. The average relative n/w GR distribution was about the same for both fixations. However, F/T yielded a much stronger GR signal, indicating a large degree of GR extraction by M. The similarity in GR distribution after both cross-linking and precipitating fixations strongly indicates that we observe the actual intracellular GR distribution and not an artifactual redistribution of receptor followed by recapture by cytoplasmic components, as suggested by Brink et al. (10).

The difference in cytoplasmic GR distribution between different cell types is in line with a previous investigation (44) demonstrating that the cytoplasmic GR pattern is fibrillar in NHIK cells and non-fibrillar in HTC cells. The observed difference in cytoplasmic GR distribution between fibroblasts of different origin, i.e., fibrillar in human primary culture fibroblasts and mouse 3T3 cells and non-fibrillar in mouse L-cells, may be explained by differences in MT resolution, MT development (11), or may reflect difference(s) in physiological function(s) of GR. In cells with a fibrillar GR distribution, the difference in staining intensity between neighboring GR-stained fibrils may indicate that GR exhibits a predilection for a subset of MTs. However, although the interphase cytoplasmic GR staining pattern could be either MT-like or diffuse, depending on cell type, there was always a conspicuous co-distribution between GR and mitotic MTs in dividing cells. The GR staining of lymphocytic cells agrees with previous results (16,24).

Our results indicate that there may exist an extranuclear pool of GR that remains quite constant independent of the glucocorticoid hormone status of the cell. This GR pool seems to be, at least

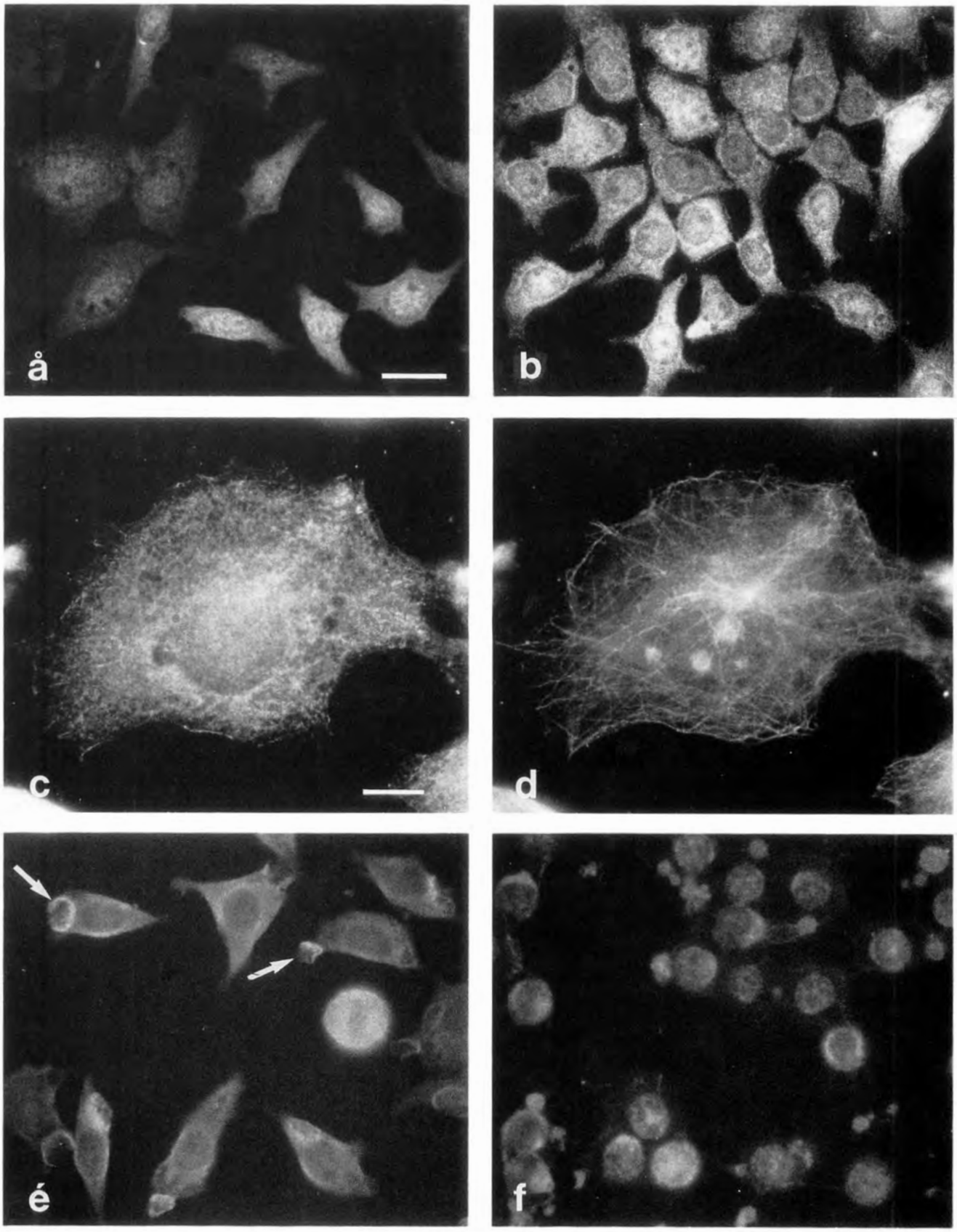

Figure 8. Conventional immunofluorescence micrographs showing GR in various mammalian cell types. (**a,b**) Monostaining of rat HTC cells fixed by M followed by MAb7 in the (**a**) absence or (**b**) presence of 1 μM dexamethasone for 2 hr before fixation. Note the inter- and intracellular GR heterogeneity and absence of fibrillar GR pattern in both situations. (**c,d**) Double staining of human mesothelioma cells fixed by M, using (**c**) MAb7 and (**d**) anti-tubulin antibodies. Much less fibrillar GR staining is found in these cells compared to, e.g., human fibroblasts (2,3). (**e**) Monostaining of mouse L-929 cells fixed by F/T, using MAb7. Note the diffuse staining of the cytoplasm and the intensely stained cell surface protrusions (arrows). (**f**) Monostaining of air-dried human lymphocytes fixed by 2% formaldehyde, using MAb7. GR is extranuclear and displays intercellular heterogeneity. Bars: **a,b,e** = 20 μm; **c,d,f** = 10 μm.

in some cell types, organized in a fibrillar manner that co-localizes well with cytoplasmic MTs. The physiological significance of this extranuclear GR pool is unclear. No specific function has been ascribed to a cytoplasmic non-activated or activated GR. There are data indicating that glucocorticoid hormones or GR may interact with MTs and possibly exert non-genomic effects (3,8,9,40). Hypothetically, there are also several other possibilities regarding the nature and function of the extranuclear GR pool. It might represent (a) newly synthesized GR, (b) a functionally inactive repository, (c) a modulator of extranuclear genomic activity, as mitochondria and also possibly the centrosome (14) contain DNA, (d) direct regulation of MT assembly without involvement of transcriptional regulation, or (e) GR might use MTs as intracellular transportation tracks (36).

The association of GR with the centrioles during the entire cell cycle and with the mitotic spindle apparatus throughout cell division represents the first demonstration of a hormone receptor localized in the mitotic spindle, suggesting that GR may participate in the regulation of the structure and function of the mitotic apparatus and thereby cell growth in vivo. Another possibility is that spindle association ensures equal distribution of GR to the two daughter cells.

Effect of Glucocorticoid Hormones

Photometry on optical sections revealed that glucocorticoids induced a statistically significant increase in both nuclear (60%) and cytoplasmic (35%) average GR intensity, as well as increased n/c and n/w ratios. However, these results were detectable only after M but not after F/T fixation. The findings that (a) precipitating and cross-linking fixation produced similar GR distributions but different GR intensities in the various compartments, and (b) glucocorticoid hormones followed by precipitating fixation induce a stronger GR intensity in all compartments and reduce the intensity difference compared to cross-linking fixation, indicate that these two standard fixations extract various amounts of GR from cells. The different increments in GR intensity in the two compartments may be due to a higher degree of M-induced extraction of GR from the cytoplasm than from the nucleus, indicating that cytoplasmic GR may be more loosely bound to its docking sites than nuclear GR. With F/T fixation it was not possible to detect any change in GR distribution or intensity, neither visually nor by statistical analysis of photometric data. This may be a consequence of 4% formaldehyde cross-linking nuclear and cytoplasmic GR equally strongly in the presence or absence of hormone, thereby preventing detergent-induced extraction. Taken together, these results argue against a hormone-induced nuclear translocation of GR. Fixation-dependent variations in relative nuclear to cytoplasmic distribution has previously been reported for other proteins, e.g., hsp90 and p59 (18).

On the basis of these observations, we propose the following hypothetical model. During basal culture conditions, GR is localized in both the cell nucleus and the cytoplasm. Treatment of cells with glucocorticoid hormones does not induce any major intracellular redistribution of GR but changes the affinity of GR for its intracellular docking sites, whether nuclear or cytoplasmic. This hormone-induced functional change in affinity is not detectable on cross-linking fixation. After precipitating fixation, however, more GR is retained in both nuclei and cytoplasm in hormone-treated cells compared with controls. Since the average increments are different in nucleus and cytoplasm, respectively (see above), this may give rise to a false visual impression of a hormone-induced apparent nuclear translocation of GR.

The hypothetical model implies the intriguing possibility that GR is independently and simultaneously activated on site in both the nucleus and the cytoplasm. This interpretation may be confounded by certain factors. (a) The anti-GR antibodies are produced against activated GR and may therefore display a higher affinity for the activated than the non-activated receptor form. We have, however, no evidence for such a difference based on Western immunoblotting of cytosolic GR. (b) Hormone-induced up-regulation (new synthesis) of GR is known to peak after around 6 hr (28) and may therefore already play a role after 1.5 hr. Assuming no difference in fixation-induced extraction of GR from the cells for various fixation techniques, both these putative confounding factors should give rise to increased GR intensity after hormone treatment, regardless of the fixation method employed. Since this was not the case, the extraction model presented above is favored.

The presented results were obtained with standard culture conditions in the absence or presence of glucocorticoid hormones. Similar results were obtained with a variety of culture conditions in fibroblasts and various other mammalian cell types. Therefore, the conclusions from the detailed analysis on fibroblasts regarding hormone-induced effects can probably be extended to the other mammalian cell types examined. We emphasize that we have never observed a complete glucocorticoid-induced cytoplasm-to-nucleus translocation of GR, as has been reported in several papers (19,30,34) regardless of mammalian cell type, medium composition, hormone dose, type of glucocorticoid, incubation time, fixation technique, or anti-GR antibody. The presented hypothesis on GR contrasts to the heat-induced reversible nuclear translocation of hsp90 during similar experimental conditions (1). This process occurs in virtually all cells in a monolayer and is accompanied by a simultaneous measurable decrease in cytoplasmic hsp90 staining intensity.

Heterogeneity

The observed inter- and intracellular heterogeneity of GR in the non-synchronous monolayer cell population under basal culture conditions are in line with previous immunolocalization data regarding GR (7,22,25), estrogen receptors (27,35), and progesterone receptors (33). Therefore, receptor heterogeneity seems to represent a general feature among steroid hormone receptors and may reflect various stages in the cell cycle (33) or genetic heterogeneity (13,20).

Comparison with Previous Studies on GR Distribution

There are several previous reports arguing against a glucocorticoid hormone-induced nuclear translocation. In some, there has been a predominantly or exclusively nuclear localization of both non-activated and activated GR (10,26,31); in others, similar cytoplasmic and nuclear GR distributions have been observed with or without hormone treatment (17,22). It is evident from several studies on the effect of hormone treatment that it has not been consistently possible to demonstrate an increased nuclear immunostain-

ing coupled to a diminution of cytoplasmic signal, as would be expected for a true translocation event. In several published reports, an increased average cellular GR intensity is seen after glucocorticoid treatment (6,12,44), without any convincing sign of actual compartment shift. A similar difference in intensities is also observed after adrenalectomy followed by substitution with glucocorticoids (5,31).

We believe that part of the controversy in the field of GR localization is due to the fact that results from immunocytology and immunohistology have been compared directly, even though these methodologies differ significantly in sample preparation techniques. Another problem is that sometimes only very few cells have been shown to illustrate the findings, which may represent selection bias. Several investigators have used artificial GR-overexpressing systems, full-length, various deletion mutants, or fusion proteins (4,34,39). Since these studies employed transfected cell lines with overexpressed heterologous GR, it is difficult to compare these studies with those analyzing endogenous GR.

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