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Correlation of Membrane Glucocorticoid Receptor Levels With Glucocorticoid-Induced Apoptotic Competence Using Mutant Leukemic and Lymphoma Cells Lines

Bahiru Gametchu[1]* and Cheryl S. Watson[2]

[1]Department of Pediatrics, Medical College of Wisconsin, Milwaukee, Wisconsin 53226
[2]Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, Texas 77555

Abstract We have studied the presence and functional implications of membrane glucocorticoid receptor (mGR) in several wild type (WT) and mutant mouse lymphoid cell lines (nuclear transfer decrease, NT^-; nuclear transfer increase, NT^i; and receptorless, R^-). Direct fluorescent antibody staining revealed large aggregates of mGR-specific fluorescing antigens in the plasma membrane of the WT and mGR-enriched (mGR^{++}) S-49 cells. While R^- cells totally lacked mGR, this receptor level was low in NT^- and NT^i groups. FACS analysis corroborated these results, showing a ~4–10-fold difference between the highest mGR levels (mGR^{++}) and the R^- and NT^i cells. Membrane extracts were analyzed for mGR by immunoblotting. Multiple receptor forms, ranging in M_r from 94,000 to > 200,000, were observed in the WT cells, while only smaller peptides (85,000–94,000) were found in NT^- cells. No detectable immunoreactive bands were identified in either membrane or cytosol immunoprecipitates of NT^i and R^- cell groups. Within 48 h post dexamethasone exposure > 98% of WT and mGR^{++} S-49 cells underwent apoptosis, compared to 0–30% in the mutant cells, albeit the total receptor number is two to three times higher in NT^i compared to WT. These results suggest a better correlation between the quantity and quality of mGRs (rather than total cellular GRs) and the ability of glucocorticoids (GCs) to lyse lymphoid cells. J. Cell. Biochem. 87: 133–146, 2002. © 2002 Wiley-Liss, Inc.

Key words: membrane glucocorticoid receptor; apoptosis; leukemia; lymphoma; steroid

The lymphocytolytic effects of glucocorticoids (GCs) have been used advantageously in the clinical treatments of certain kinds of leukemias and lymphomas. Although the mechanism of this induced cell lysis is not fully understood, the effect is known to be mediated by the glucocorticoid receptor (GR). The lack of a complete correlation of the cytolytic response with intracellular receptor concentration made us explore alternative or additional receptor forms. We identified membrane-associated glucocorticoid receptor (mGR) and produced evidence that this receptor form may play a significant role in GC-induced cell lysis [Gametchu, 1987; Gametchu et al., 1991a,b, 1993; Sackey et al., 1997; Chen et al., 1999b]. Several biochemical and immunological characterization studies have revealed that mGR includes some larger receptor forms, ranging in sizes up to 160 kDa while intracellular GR (iGR) is a single size of 94 kDa [Gametchu, 1987; Sackey et al., 1997]. The differences between mGR and iGR are limited to molecular size, cellular distribution, and affinity of steroid [Gametchu et al., 1999; Powell et al., 1999]; similarities between the two receptor types include the class of steroids bound, epitope recognition by both BUGR-1 and BUGR-2 monoclonal antibodies and a anti-peptide human GR antibody [Gametchu et al., 1995, 1999], many shared proteolytic enzyme cleavage sites [Gametchu et al., 1991b; Powell et al., 1999], an ability to be phosphorylated, and binding to heat shock proteins (HSPs)

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*Correspondence to: Dr. Bahiru Gametchu, Department of Pediatrics, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226.
E-mail: gametchu@mcw.edu

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70 and 90 [Gametchu et al., 1995; Powell et al., 1999].

As a member of the steroid receptor family, the iGR consists of three functional domains (regulatory, DNA binding, and hormone binding) [Evans, 1988; Wright et al., 1993]. Several classes of wild type (WT) and mutant lymphoid cell lines, both human and mouse, have been identified; most of these cell lines contain molecular lesions in one or two of these domains (see Fig. 1) and have GC resistant phenotypes [Gehring and Tomkins, 1974; Yamamoto et al., 1976; Bourgeois and Gasson, 1985]. Although this view is generally accepted, investigators have yet to agree upon the specific domain(s) responsible for mediating GC-induced cell lysis [Narareth et al., 1991; Dieken and Miesfeld, 1992]. To examine the significance of these domains and association with mGR more closely, we have collected several representative cell lines known for their GC lysis-sensitivity or -resistance. These cell lines were classified into five groups and are summarized in Figure 1: WT with fully functional receptor; those with abnormally low levels of nuclear translocation of steroid–receptor complex and with low or no affinity for DNA binding (NT^-); those with N-terminal truncated receptor and abnormally high level of steroid–receptor complex translocation into the nucleus (NT^i); receptorless (R^-) which lack detectable steroid-binding activity; and mGR-enriched S-49 cells [Bourgeois and Gasson, 1985; Gametchu, 1987]. A minimum of two representative cell lines from each group (with the exception of only one available mGR-enriched cell line) were used to test several characteristics of mGR including appearance of mGR staining, molecular size of mGR compared to its intracellular counterpart; expression levels of mGR; and dependency of the GC-induced cell lysis effect on the number and condition of nuclear and membrane GRs.

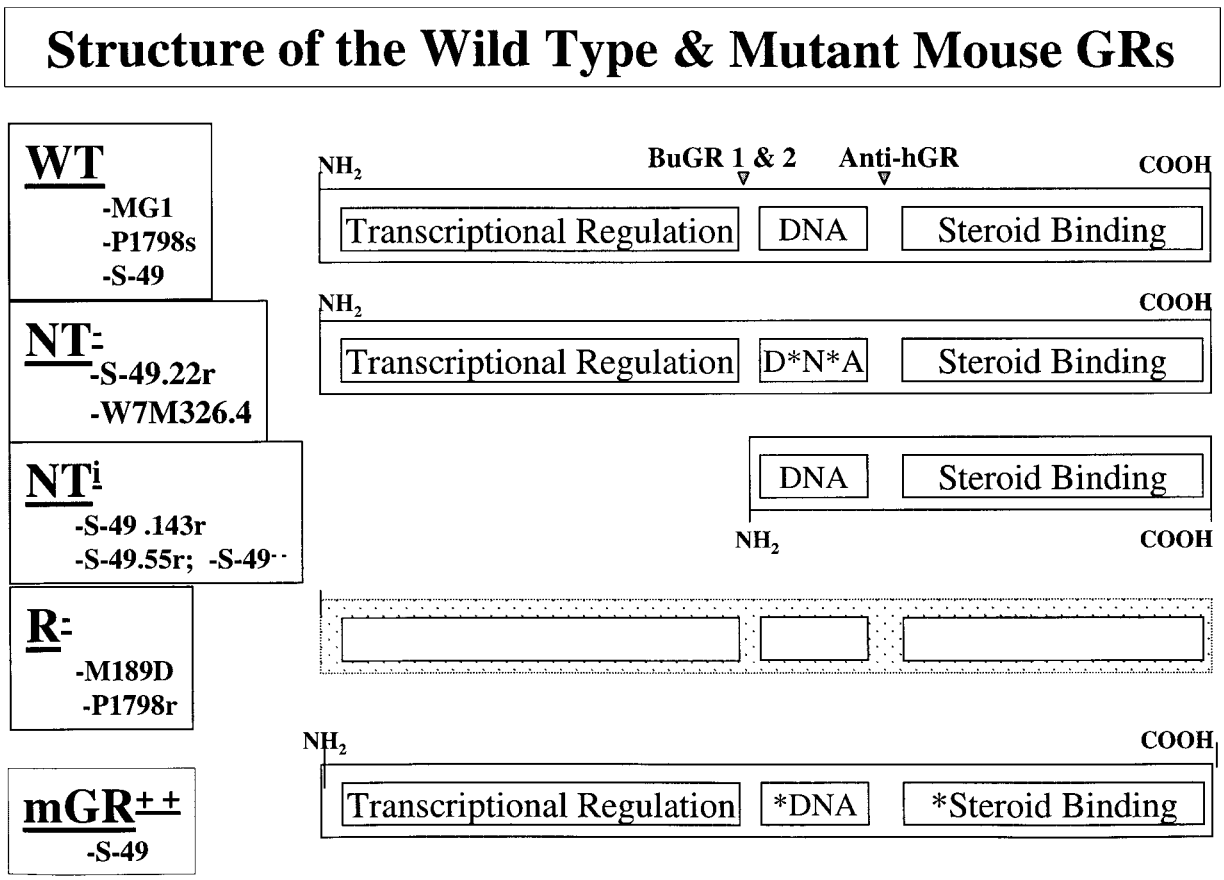

Fig. 1. Schematic representation of the glucocorticoid receptor structure from the different wild type (WT) and mutant mouse lymphoid cell lines. Asterisk (*) signifies mutations in the associated DNA and/or steroid binding domains of the NT^- and mGR^{++} cell groups. The dotted lines around the R^- group map indicate an absence of receptor. The position of the BUGR-2 and R1 human GR antibody epitopes are shown by the symbols (▽) on the WT receptor map.

MATERIALS AND METHODS

Cell Culture/Cell Selection/Antibody Preparation

S-49 cells were obtained from the ATCC (Rockville, MD) and grown either in RPMI 1640 (Biofluids, Rockville, MD) in the presence of 10% bovine calf serum (SM), or defined media (DM), which is phenol red-free RPMI 1640 supplemented with insulin, transferrin, and selenium [Thompson, 1991] (KC Biologicals, St. Louis, MO). Using sequential cell-separation techniques [immunopanning [Gametchu, 1987], fluorescent cell sorting [Gametchu et al., 1991a], and soft agar cloning [Koprowski et al., 1977]], we previously produced stable mGR-enriched and mGR-deficient mouse cell lines [Gametchu, 1987; Gametchu et al., 1991a,b]. For the present experiments, we used an additional immunopanning to further select from these populations, a subset of cells highly enriched (mGR^{++}) and very deficient (mGR^{-}) in mGR. Therefore, these cells were sequentially separated by these four steps, culminating in a second immunopanning. NT^{-} S-49.143r and NT^{i} S-49.55r were provided by Dr. R. Miesfeld [Dieken et al., 1990]. Derivatives of the WEHI-7 cell line, W7MG1, W7M326.4, and ADR6.M189D, known for dexamethasone sensitivity (W7MG1) and resistance, respectively, were obtained from Dr. M. Stallcup [Rabindran et al., 1987]. The mouse lymphosarcoma dexamethasone sensitive P1798s and dexamethasone resistant P1789r cells were provided by Dr. A. Thompson [Thompson, 1991]. Labeled steroids used to demonstrate GC binding were [^{3}H]triamcinolone acetonide (45 Ci/mmol), [^{3}H]dexamethasone, and [3H]dexamethasone 21-mesylate (48 Ci/mmol) which were obtained from Dupont-NEN (Boston, MA). Unlabeled steroids, Nonidet P-40 (NP-40), and RNase were obtained from Sigma Chemical Co. (St. Louis, MO). BUGR-2 anti-rat GR monoclonal antibodies were produced by culturing the hybridoma-producing cells in RPMI 1640 media containing 20% fetal calf serum, and harvesting the antibody-containing medium as described previously [Gametchu and Harrison, 1984]. Purification of IgG and direct conjugation with fluorescein isothyocyanate was carried out according to established methods [Lazarides and Weber, 1974], modified by us [Gametchu, 1987]. Rabbit antibodies (R1) raised against a peptide representing the human GR cross-react with mouse GR were prepared as previously described [Gametchu et al., 1993].

Buffers

The following buffers are referred to in the text by number: (1) 150 mM NaCl and 150 mM Na_2PO_4, 0.25% NP-40 (pH 7.2); (2) 10 mM Tris-HCl, 1 mM EDTA, 300 mM KCl, 12 mM thioglycerol, (pH 7.5); (3) 20 mM HEPES, 10 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol, (pH 7.6); (4) 10 mM Tris-HCl (pH 7.6), 0.5 mM $MgCl_2$, 10 μg/ml leupeptin, 10μg/ml aprotinin, 1 mM PMSF, 1.8 mg/ml iodoacetate, 100 μg/ml bacitracin, 125 μg/ml trypsin inhibitor, 2 μg/ml pepstatin, and 5 mM diisoproplyfluorophosphate (pH 8.2); (5) buffer 4 plus 0.6 M NaCl; (6) 10 mM Tris-HCl, 140 mM NaCl, 10 mM molybdic acid plus the protease inhibitors indicated in buffer 4; (7) 50 mM Tris-HCl (pH 7.6), 300 mM NaCl, 0.5% Triton X-100, and the protease inhibitors listed for buffer 4; (8) 62.5 mM Tris-HCl (pH 6.8), 1% $NaDoSO_4$, 10% glycerol (vol/vol), 25% mercaptoethanol (vol/vol), and 0.001% bromophenol blue; (9) 25 mM Tris-HCl, 192 mM glycine, and 20% methanol (vol/vol), (pH 8.5); and (10) 20% bovine calf serum (vol/vol) and 0.9% NaCl.

Direct Fluorescent Antibody Labeling and Fluorescent Antibody Cell Sorting (FACS) Analysis of mGR

Live cells (about 10^6) were incubated in 96-well microtiter plates with 20 μl (5.6 μg/ml) of FITC-conjugated BUGR-2 for 30 min at 4°C. The cells were then washed three times with buffer 1 by centrifugation at 500*g* for 10 min in cold. The cell pellets were then resuspended in fresh buffer, and an aliquot of the cells cytocentrifuged onto slides (10^5 cell/slide). To rinse off the salt left by the buffer, slides were then dipped into distilled water for 5 s, and the cells air-dried before processing for fluorescent microscopy. The remaining cells were analyzed by flow cytometry using a FACS analyzer (Benton/Dickeson) equipped with a FACS Lite laser [Gametchu et al., 1993; Sackey et al., 1997]. In both assays, the specificity of the receptor antibody interactions was established by control experiments in which only fluorescein-labeled secondary antibody (anti-mouse IgG) was used to establish background labeling.

Sucrose Density Gradient Analysis

Velocity sedimentation analysis of iGR and the interaction of this receptor with antibodies

was carried out on sucrose density gradients [4.8 ml of 5–20% (wt/vol), in buffer 2]. Cytosolic GR preparations from the different cell groups were labeled with 1×10^{-7}M [^{3}H]triamcinolone acetonide in the presence and absence of 100-fold excess concentration of unlabeled ligand according to established methods [Gametchu, 1987]. Following labeling, free steroid was removed by adsorption with dextran-coated charcoal and 120 μl of each preparation incubated overnight with either BUGR-2 monoclonal antibody [Gametchu and Harrison, 1984] or with cross-reacting R1 anti-human GR hinge region-specific antibody [Gametchu et al., 1993] at 4°C for 2 h. These preparations were layered on sucrose gradients (in buffer 2). After centrifugation in a Beckman SW 50.1 rotor at 120,000*g* for 18 h, the gradients were analyzed by puncturing the tube bottom and collecting 23 (0.2 ml each) fractions. Radioactivity in each fraction and that associated with the tube bottom were estimated by liquid scintillation counting [Gametchu and Harrison, 1984], and [^{14}C]BSA were centrifuged on separate gradients simultaneously and used as standards to estimate sedimentation values.

Preparation of Ligand-Labeled Cytosolic and Plasma Membrane-Extracted Proteins

For the preparation of intracellular receptor, the different lymphoid cell groups were harvested, washed three times with buffer 1 and homogenized in buffer 3 at 4°C using three bursts of a Tissuemizer (15 s each at setting 40; Tekmar, Cincinnati, OH) at a buffer to packed cell volume ratio of ~1:1. The homogenates were centrifuged at 120,000*g* for 60 min at 4°C. The upper fatty layer was discarded and the cytosol fraction containing GR was separated and labeled at 4°C for 2 h with 2×10^{-7}M [^{3}H]dexamethasone 21-mesylate or 2×10^{-7}M [^{3}H]triamcinolone acetonide plus or minus a 100-fold excess of its unlabeled counterpart, to estimate non-specific binding by charcoal assays [McBride, 1984].

For preparation of affinity-labeled membrane proteins, we made extracts of membrane vesicles isolated from sucrose step-gradient interfaces at 4°C [Eisen and Glinsman, 1978]. Briefly, cells were Dounce homogenized in 2 volumes of buffer 4 (30 strokes, B pestle). One forth volume of buffer 5 was added and the mixture centrifuged at 500*g* for 10 min. EDTA was added to the recovered supernatant at a concentration of 5 mM. This solution was then ultracentrifuged for 45 min at 150,000*g*. The resultant pellet was resuspended in 1 ml of buffer 6, loaded onto a 4 ml, 41% (in buffer 6) sucrose pad, and ultracentrifuged at 95,000*g* for 65 min. The opaque membrane band was recovered (between the buffer and sucrose pad), resuspended in 10 ml of buffer 6, and washed by centrifugation as above. The pellet was then resuspended in 0.5 ml of buffer 3 (with no protease inhibitors), and affinity-labeled as described above using 2×10^{-7}M [^{3}H]dexamethasone 21-mesylate in the presence and absence of 100-fold excess unlabeled dexamethasone. The preparation was then washed three times with buffer 3 (by centrifugation at 95,000*g* for 15 min each) and extracted with detergent by mixing the vesicles (with a magnetic stirring bar) for 3 h at 4°C in 750 μl of buffer 7. This solution was ultracentrifuged at 120,000*g* for 1 h at 4°C and the super- natant containing extracted mGR was recovered. Specific binding of extracted receptor was estimated by charcoal assay [McBride, 1984].

Western Blot Analysis of iGR and mGR From Various Mutant and WT Cells

To confirm differing mGR expression levels and molecular size variations within the WT and various mutant cell groups, we performed Western blot analysis; we also similarly analyzed iGR in these same cells. Extracted mGR (see above) was concentrated from an equal number of cells by immunoprecipitation [Gametchu, 1987]. Cytosol preparations or extracted membrane proteins from each cell group were incubated overnight with BUGR-2 (1:20 dilution in buffer 1) and then with protein A beads at 4°C and the receptor–antibody complex was captured on and eluted from protein-A Sepharose-4B beads. Samples were mixed with a 4 × volume of buffer 8, boiled for 5 min, and electrophoresed overnight at 50 V/SDS slab gel [Laemmli, 1970]. The proteins were then transferred to nitrocellulose filters by applying 60 V for 3 h in buffer 9. Nitrocellulose filters were soaked for 1 h in buffer 10 at room temperature and incubated for 16 h at 4°C with 1:50 dilution of BUGR-2. Subsequent washing and staining was done as described previously [Gametchu, 1987]. The immunoblot was air-dried, sprayed with Enhance (New England Nuclear, Boston, MA), and exposed to an X-ray film.

Whole Cell Binding Assays

Cells from each cell group were harvested, and washed in fresh RMPI 1640 medium. To estimate total binding, a 1 ml suspension containing 6×10^6 cells was added (in triplicate) to 1 ml glass test tubes (VWR Scientific, Inc., San Francisco, CA) containing [^{3}H]dexamethasone at concentrations ranging from 1.25 to 100 nM. To estimate non-specific binding, a 100-fold excess of unlabeled dexmethasone was included in another set of triplicate tubes. These cell suspensions were incubated at 21°C for 2 h with shaking. The cell suspensions were then washed with cold RPMI 1640 (three times, 1 ml each), by centrifugation at 800*g* in a Beckman GPR centrifuge. The final cell pellet was resuspended in 1 ml RPMI 1640 and 50 μl aliquots were used to estimate final cell number and radioactivity. Surviving cell numbers ranged from 1.80 to 4.27×10^6 (from an original 6×10^6 cells); this experimental loss in cell number was considered when calculating binding sites/cell. All binding data have been normalized to represent 3×10^6 cells. Specifically bound steroid was calculated by subtracting the non-specific binding from total binding. The steroid affinity and receptor number were estimated according to the method of Scatchard [1949] using the personal computer version of the program Ligand [Gametchu et al., 1991b] with minor modifications [Gametchu et al., 1991a; Powell et al., 1999].

Glucocorticoid Sensitivity Experiments

The various cell groups (9×10^5 cells/60 mm petri dish) were exposed to 10^{-6} M dexamethasone for 4 days and cell viability was estimated every 24-h by trypan blue dye exclusion assay [Gametchu, 1987]. Control experiments in which cells were not hormonally treated were carried out in parallel (not shown) and % cell survival numbers were based on the cells present on day 0. The kinetics of cell death appears to be faster for P1798 cells grown in DM compared to cells grown in serum-containing media (data not shown). For the other mutant groups, changes in growth media did not seem to affect the hormone sensitivity.

RESULTS

Fluorescent Antibody Staining

To compare mGR levels in the different cell mutants and WT cells, live cells with intact plasma membranes, were stained with FITC-conjugated antibody specific for the GR [Gametchu, 1987]; the rationale is that the IgG with a size of ~150 kDa is too large to traverse the living cell's plasma membrane to react with the iGR, therefore, interacting only with receptors at the cell surface using this staining approach. We have characterized and used this procedure in many of our past studies [Gametchu, 1987; Pappas et al., 1995; Sackey et al., 1997]. Figure 2 shows direct FA staining of these cells. Specifically fluorescing large patches of mGR antigens were detected at a much greater level in the plasma membrane of both the WT and mGR-enriched S-49 cells (panels A and B, respectively) compared to the mutant cell lines (panels C, D, and E). For WT and mGR^{++} cells the brightly fluorescing large aggregates of antigen appear to be accentuated on one pole of the cell in the plasma membrane. In mutant NT^- cells there were small granules of moderate fluorescence relatively evenly spaced around the cells periphery (panel C). NT^i cells show very dim and diffuse (non-aggregated) fluorescence (panel D). R^- cells contain negligible fluorescence (panel E). Panel F demonstrates that the background fluorescence due to the binding of non-specific IgG is absent at these exposure levels.

FACS Analysis

Plasma membrane-associated GR was analyzed in the different cell groups by flow cytometry and the results shown in Figure 3 illustrate varying intensities of specifically fluorescing receptor antigen in the plasma membrane of these cell groups. The non-specific antibody control profile was the same for each cell group. However, the GR antibody specific fluorescent peak was very bright in WT cells (an intensity peak centering around 800). NT^-, NT^i, and R^- cells had progressively dimmer fluorescent intensity peaks centering around values of 100, 20, and 11, respectively. To compare quantitatively the level of mGR in these cells (see Table I), the results were expressed in median fluorescence intensity index values [ratio of median fluorescence of experimental (with BUGR monoclonal Ab) to the median intensity of control (with non-specific goad anti-rabbit IgG)]. This value is the highest for the mGR^{++} S-49 cells followed by WT and NT^- cells. Finally, NT^i and R^- cell groups had much lower mGR values. Therefore,

expression of mGR in mGR^{++} S-49 is ~5–8 times higher when compared to the NT^{i} and R^{-} cells.

Western Blot Analysis of mGR and iGR in the Various Cell Groups

Immunoblot analysis was carried out in one representative cell line from each group to examine both the quantity of expression and molecular size variation in these receptors. The results shown in Figure 4 illustrate that the WT cells contained several immunoreactive peptides in the membrane ranging in M_r from 94,000 to >200,000. The intense labeling of these bands indicated that mGR concentration was highest in this cell group. Membrane preparations from NT^{-} cells produced smaller peptides ranging in size from 85,000 to 94,000. No detectable receptor immunoprecipitable with this antibody was present in membrane

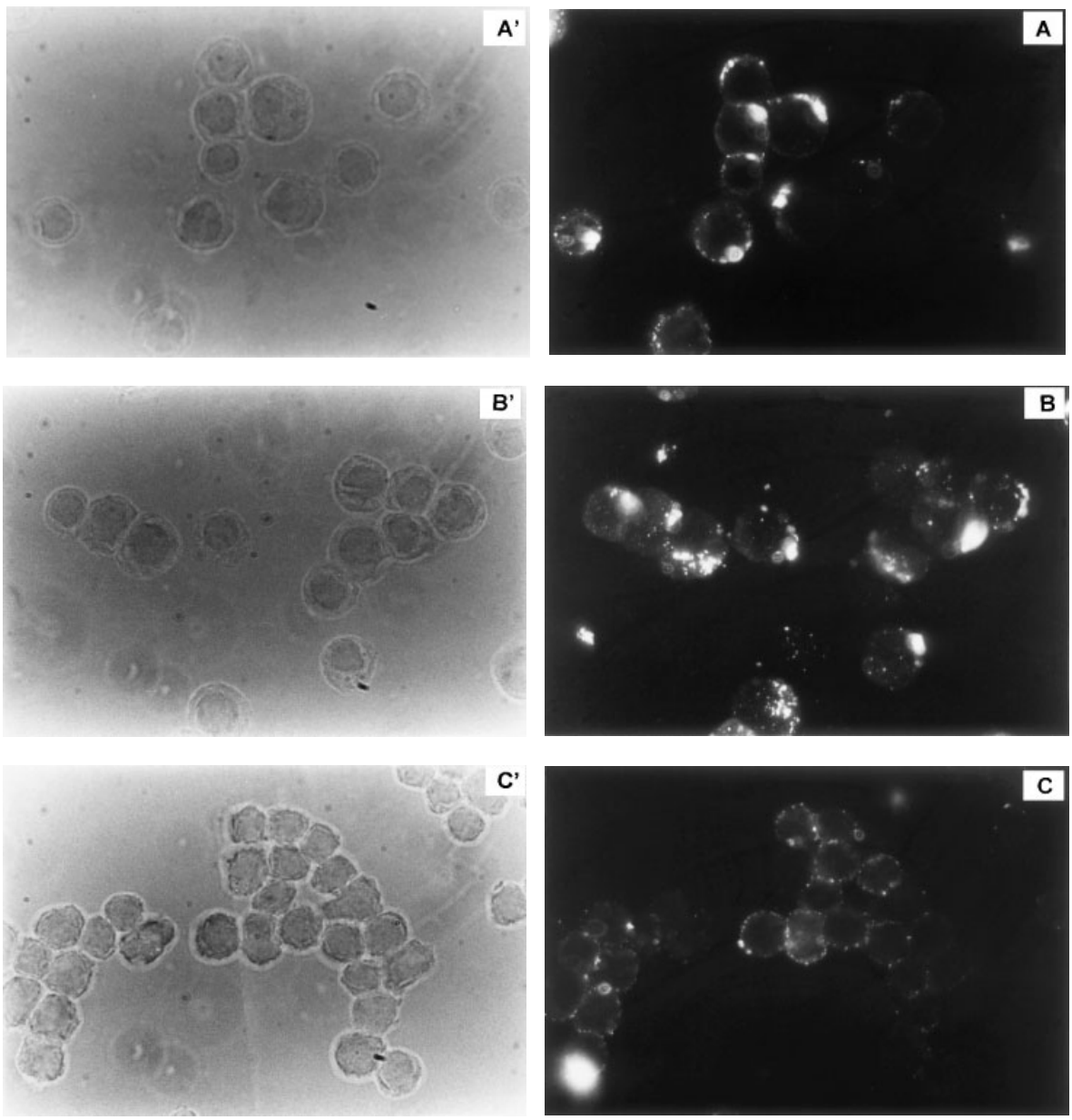

Fig. 2. Immunocytochemical localization of mGR in mutant and WT mouse lymphoma cell lines. Cells were stained with fluoresceinated BUGR-2 antibody, washed, and cytospun onto slides for dark and bright field micrography. Each figure is a representative of at least five photographs. Dark field micrographs: (**A**) WT (W7MG1); (**B**) S-49 mGR^{++}; (**C**) NT^{-} (S-49.22r); (**D**) NT^{i} (S-49.143r); (**E**) R^{-} (MRD6.M189D); (**F**) WT (W7MG1) with non-specific IgG (goat anti-rabbit FITC-conjugated IgG) staining. Bright field micrographs: **panels** (**A′**–**F′**), correspond to dark field micrographs.

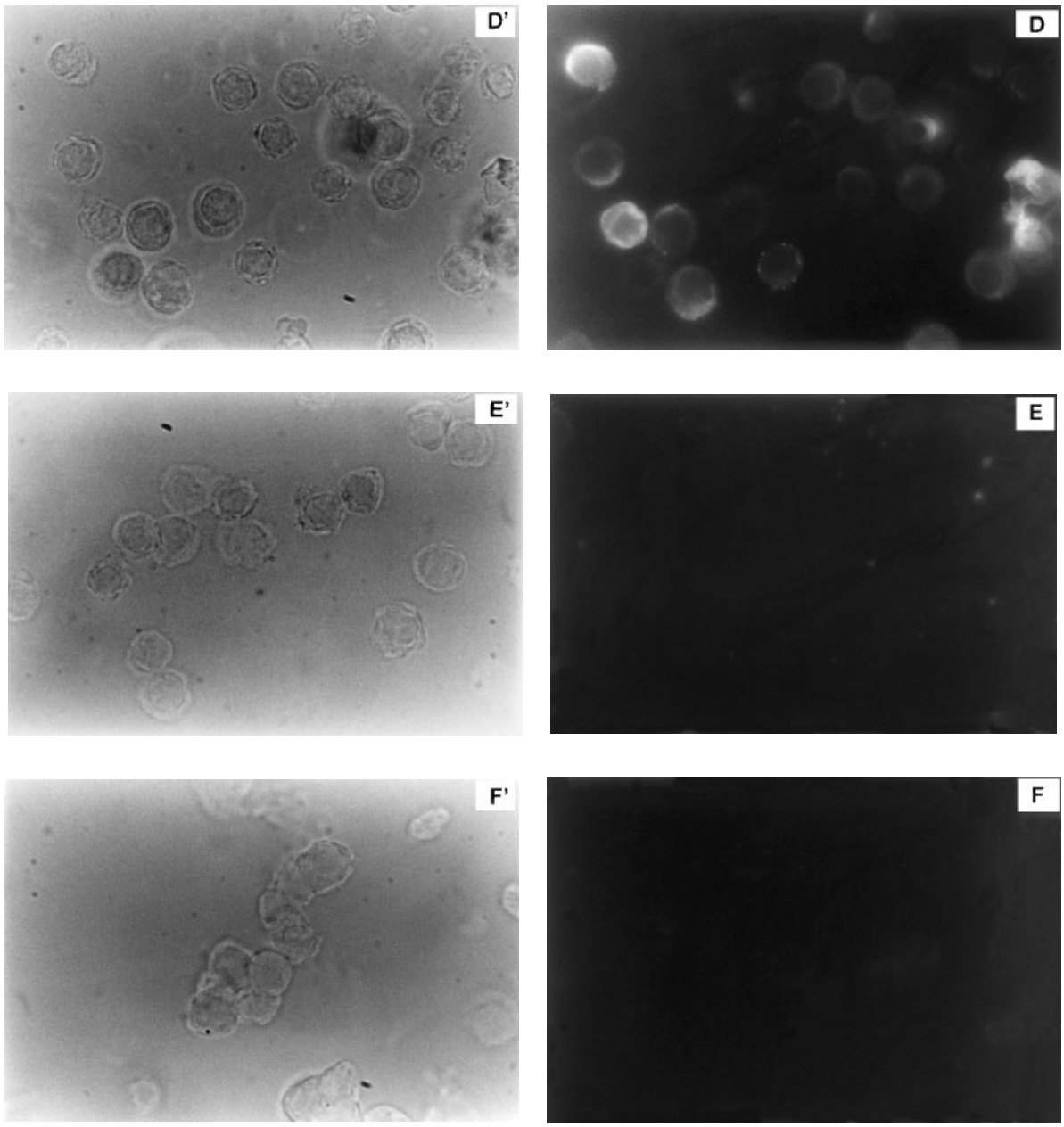

Fig. 2. (*Continued*)

extracts from either NT^i or R^- cell groups. As expected, cytosolic preparations from the WT cells produced only the classical 94,000 iGR, with no larger species present. While NT^- cells also produced a correctly sized iGR, neither NT^i nor R^- cells produced any precipitable cytosolic receptor. These results indicated that a portion of the mGR population varies in molecular structure from the intracellular receptor, confirming earlier observations made in WT, mGR-enriched (mGR^+), and-deficient (mGR^-) S-49 cells [Gametchu, 1987; Gametchu et al., 1991b].

GR-Binding Assays

Several hypotheses, dealing mainly with the quantity of GR protein in whole cells, have been proposed to explain the role of GCs in GC-mediated lymphocytolysis [Lippman et al., 1978; Quddus et al., 1985; Kato et al., 1993; Csoka et al., 1997]. To investigate this in comparison to our other findings on mGR, we performed whole cell GR binding-studies. The data presented in Table II indicate that the total GR binding sites significantly varied between

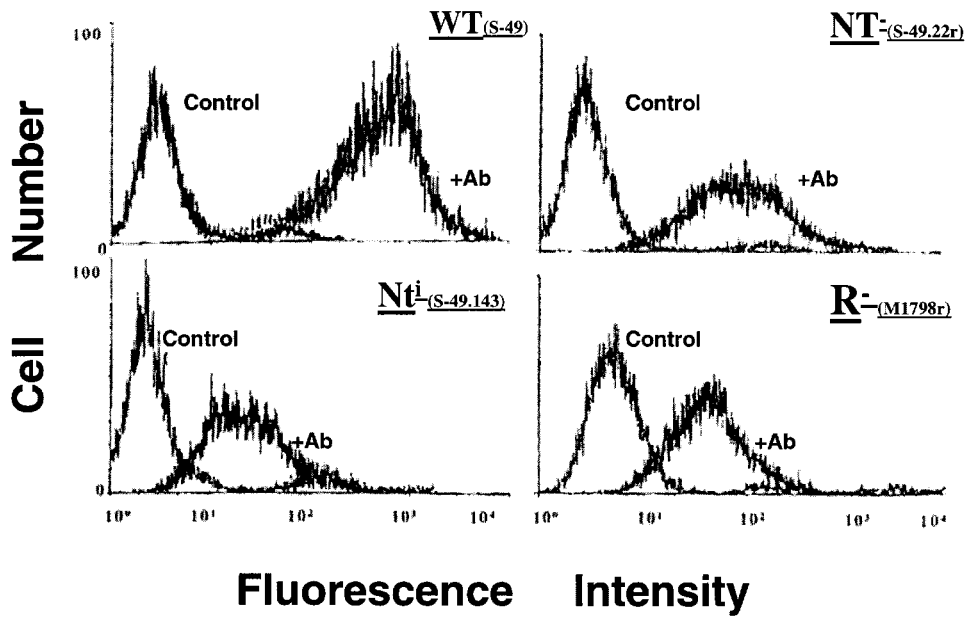

Fig. 3. Fluorescent histograms of the WT and mutant cells. Live cells were incubated with FITC-labeled BUGR-2 antibody (+Ab), followed by flow cytometry. Panels are labeled for cell types (WT, S-49; NT^-, S-49.22r; NT^i, S-49.143r; R^-, P1798r). Control samples were incubated with non-specific (goat anti-rabbit) FITC-conjugated IgG and are labeled CONTROL. Results are representative of cells analyzed from two to four separate experiments for each cell line.

the types of WT cells (~2,000–6,000 sites per cell) and types of NT^- cells (1,200–13,000 sites per cell). Although NT^i cells had low mGR shown by FACS analysis, they could contain plentiful total binding sites (as much as 165,000 sites per cell). As expected, the lysis resistant R^- MDR6.M189D cells contained a very low number (180 sites/cell) of total binding sites, although the 1789r cell group did not significantly vary in receptor number from most WT cells. The highly responsive mGR^{++} cells [Gametchu et al., 1994, 1995] did not contain especially high total GR. The binding affinity expressed in K_d values ranged from 1 to 8 nM for the WT group which appears to be slightly lower compared to the remaining three groups which range from 0.3 to 5 nM. However, all these values are within the range of those commonly reported [reviewed in [Gametchu et al., 1995, 1999; Powell et al., 1999]].

Sucrose Density Gradient Analysis

The lack of immunoprecipitable receptor shown by Western analysis of NT^i cells was of particular interest, since this cell group contained the highest number of GR sites as shown by whole cell binding analysis and some diffuse staining by immunocytochemistry. The possibility that GR in these cells was either mutated or truncated at the BUGR epitope was further considered via density gradient analysis of the iGR. We used both BUGR-2 monoclonal antibody, specific to the DNA binding region [Gametchu et al., 1995], and a cross-reacting, anti-human GR antibody (R1) with epitope recognition in the hinge region, near the steroid binding site [Gametchu et al., 1993]. As illustrated in Figure 5, this analysis revealed that both the WT (S-49) and the NT^- (S49.22r) cells contained the classical GR (co-sedimenting with BSA in fractions 5–10 as a 4S protein) with recognition sites for both Abs, indicating the receptor was intact at the DNA-binding and hinge regions. As expected [Dieken et al., 1990], preparations from the NT^i (S-49.55r) cells resolved predominantly as a truncated receptor, with an estimated S value of ~2 [much like our previous finding for the GR of mGR^- S-49 cells [Gametchu et al., 1994]]. This receptor fragment reacted with anti-human GR Ab R1 only, and not with the BUGR-2 monoclonal Ab, showing that the truncation included the BUGR epitope, excluding from the mutant protein part of the DNA-binding and all of the N-terminal regions. Neither Ab reacted with preparations of R^- (MRD6.M189D) cells,

TABLE I. Median Fluorescent Intensity Index of mGR Staining in the Different Lymphoid Cell Lines

	Wild type			NT^-		NT^i		R^-		mGR^{++}
Experiment number	MG-1	P1798	WT S-49	S-49.22r	$S\text{-}49^-$ (mGR^-)	S-49.143r	S-49.55r	MRD6.M189D	P1798r	$S\text{-}49^{++}$
1	32	16	60	36.8	5.5	11.6	13.0	11.0	12.0	53.5
2	32	23	50	38.1	5.7	13.3	13.3	8.7	8.6	44.8
3	50		50	30.0		9.7	15.0	4.0	4.0	74.2
4				50.0		7.7				
Mean	38.0 ± 10.4	19.5 ± 5.0	53.3 ± 5.8	38.7 ± 8.3	5.6 ± 0.1	9.5 ± 3.2	13.8 ± 1.1	5.6 ± 5.0	8.2 ± 4.0	57.5 ± 8.7
Group mean		36.9		38.7		10.9			6.9	57.5

Variation in mGR levels in different mouse lymphoid cell lines measured by FACS laser analysis compared with values for controls. The mean fluorescence intensity index is the ratio of the median channel of fluorescence of the experimental to the median channel of fluorescence of the control (FITC-conjugated rabbit IgG). This assigns a relative value of intensity for mGR staining to each cell sample measurement. Therefore, a high number indicates that the cells have a high density of specific surface antigen. n = 2–4. NT^-, nuclear transfer increase; and R^-, receptorless.

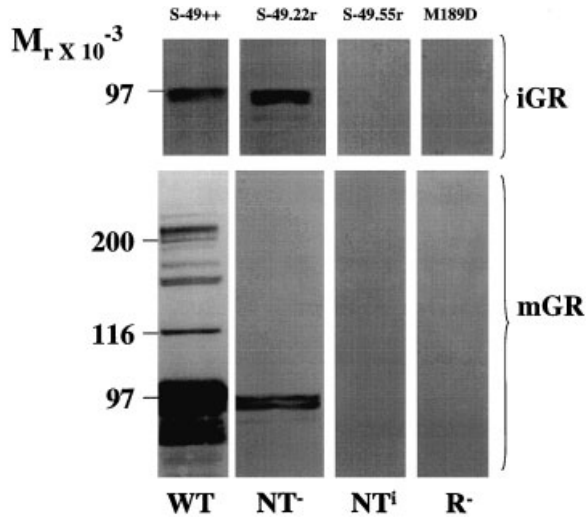

Fig. 4. Western blot analysis of membrane GR (mGR) and intracellular GR (iGR) from WT and three mutant cell lines. All preparations originated from an equal number of cells. Membrane extracts were immunoprecipitated prior to separation by SDS–PAGE and transblotting. Immunoblot analysis used BUGR-2 antibody. Lanes are: WT (S-49 mGR^{++}); NT^- (S-49.22r); NT^i (S-49.55r); and R^- (P198D). The proteins used as molecular weight (M_r) standards were myelin, 200 kDa; phosphorylase B, 116 kDa; β-galactosidase, 97 kDa; bovine albumin, 68 kDa; ovalbumin, 40 kDa and are marked on the left side of the figure. Results represents four to five separate experiments.

as shown by the radioactive label remaining on the top of the gradient, indicating absence of appreciable numbers of GR in these cells.

Glucocorticoid Sensitivity Studies

Previous studies in mGR-enriched and-deficient cells from our laboratory have correlated the presence of mGR with the cells' sensitivity to GC-induced cytolysis, suggesting a possible critical role for this receptor form [Gametchu, 1987; Gametchu et al., 1991b, 1994, 1995; Sackey et al., 1997]. We have now extended these studies to include these different lymphoma cell types shown to contain varying quantities and qualities of mGR. The results shown in Figure 6 indicated the WT P1798, W7MG1, and $S\text{-}49^{++}$ cells displayed substantial lysis within 24–48 h post dexamethasone exposure, much as in the mGR^{++} S-49 cells that we have shown previously [Gametchu, 1987; Gametchu et al., 1994; Sackey et al., 1997]. However, GCs cell-lysis sensitivity was either completely lacking, or the cells displayed moderate cell growth for the first 24 h, in the NT^- cell category; cells containing GRs (but not necessarily

TABLE II. Glucocorticoid Binding Sites in the Different Lymphoid Cell Lines

Cell group	Cell line	$K_d \times 10^{-9}$ M	Group mean for $K_d \times 10^{-9}$ M	Receptor sites ($\times 10^4$)	Group mean for receptor sites	Sensitivity
WT	W7MGR1	1.1	5.1	2.4 ± 0.2	3.1	+++
	P1798s	6.0		5.8 ± 0.3		+++
	S-49	8.1		1.9 ± 0.1		+++
NT^-	S-49.22r	$0.3 \pm .03$	2.7	13.2 ± 0.9	7.2	–
	W7M.326.4	5.1 ± 2.17		1.2 ± 0.2		–
NT^i	S-49.143r	2.6 ± 1.34	2.2	2.1 ± 0.2	10.4	–
	S-49.55r	0.3 ± 0.95		12.7 ± 0.9		
	$S\text{-}49^-$ (mGR^-)	3.2 ± 0.06		16.5 ± 2.0		–
R^-	MRD6.M189D	0.3 ± 0.32	0.5	0.18	1.2	–
	P1798r	0.4 ± 0.39		2.2 ± 0.2		–
mGR^{++}	mGR^{++}	0.2	0.2	2.2 ± 0.2	2.2	+++

Whole cell binding assays to determine total GR binding sites normalized to cell number for wild type (WT) and different lymphoma cell lines as described in Table I. Sensitivity to dexamethasone treatment is scored based on the data in Figure 6 and previously published data for mGR^{++} cells [Gametchu et al., 1991a,b, 1994, 1995]. Binding data were averaged for two to four separate experiments.

mGRs) have been shown to increase in cell number in response to this hormone. NT^i and R^- cells showed weak to moderate (~10–30%) lysis with no increase in cell number during the same period. After growth in dexamethasone for 9 days NT^i and R^- mutant cells eventually became completely lysis resistant and attained normal growth rates (data not shown). That cells with modified or absent mGR but plentiful iGR do not respond to GCs in the hormone-induced apoptosis assay suggests that mGR plays a significant role in hormone-mediated lymphocytolysis.

DISCUSSION

We examined the connection between quantity and size of the membrane and intracellular forms of the GR, and the sensitivity of lymphoma and leukemic cells to GC-induced lymphocytolysis, by examining a number of established mutant cell lines. Fluorescent antibody staining and FACS provided corroborative results with respect to the level of mGR expressed in the different cell groups, although immunocytochemistry provided the added information that the staining for mGR on WT and mGR^{++} cells was qualitatively different from the other cell types showing membrane staining. This additional visual information could suggest an inability of the mGR in NT^- cells to cluster, as has been shown for the mGR and other membrane steroid receptors [Gametchu, 1987; Pappas et al., 1995; Watson and Gametchu, 1999] and could be related to the ability of such receptors to be organized into membrane raft domains [Chambliss et al., 2000]. The fact that reduced or altered mGR could best be detected by the live cell staining techniques of immunocytochemistry or FACS suggests that this protein, or at least this epitope, is vulnerable to membrane preparation techniques.

Quantified mGR levels by FACS analysis varied considerably between the cell groups, with the mGR^{++} [Gametchu et al., 1995] expressing the highest concentrations, followed by the WT and NT^- cells, and finally the NT^i, and R^- groups expressing the lowest amounts. Western analysis demonstrated immunoprecipitable mGR only in WT and NT^-, although NT^- cells had only a 94 kDa form and not the higher M_r forms that we have previously reported for mGR-enriched GC-lysis competent cells [Gametchu, 1987; Gametchu et al., 1991a,b; Powell et al., 1999]. As the identity of the high M_r mGR bands has been established by competitive steroid binding studies before [Gametchu, 1987], it is unlikely that these are non-specific cross-reacting peptide bands. Additionally, computer analysis of the BUGR epitope failed to reveal any BUGR-related sequence in the database. Our other recent studies did not reveal any additional coding sequence in the mGR-encoding 1A splice variant [Chen et al., 1999a,b]. Therefore, this increase in size must be due to a post translational modification of mGR. Since NT^- cells are GC-lysis resistant, it is tempting to suggest that whatever the modification of mGR that is responsible for making it bigger, it must be necessary for the function of the apoptosis competent mGR protein.

The correlation of intact mGR with the ability of cells to undergo GC-induced cell lysis was suggested by our previous work which produced mGR-enriched and mGR-deficient cells using

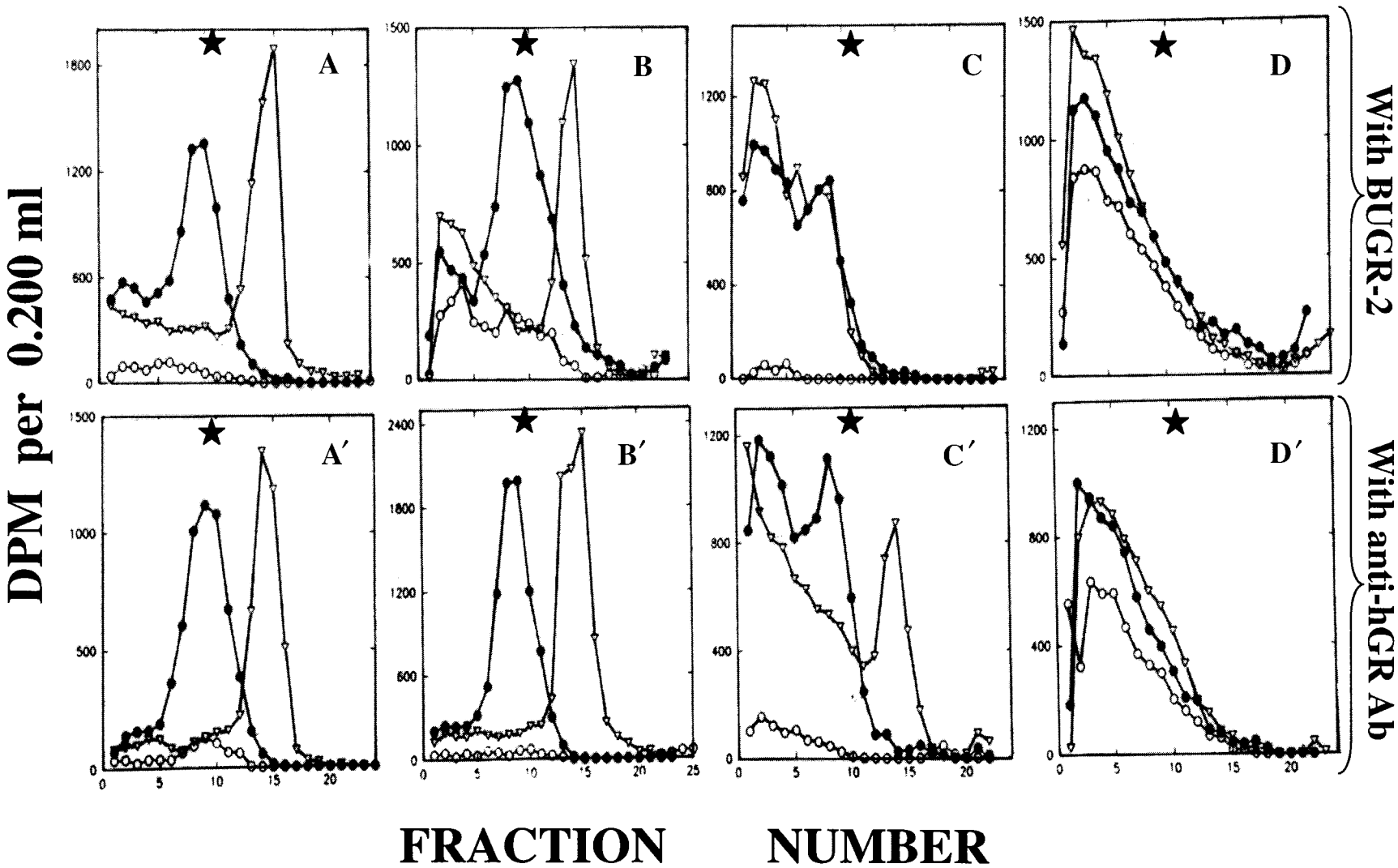
DPM per 0.200 ml
A
B
C
D
A′
B′
C′
D′
With BUGR-2
With anti-hGR Ab
FRACTION NUMBER

Fig. 5.

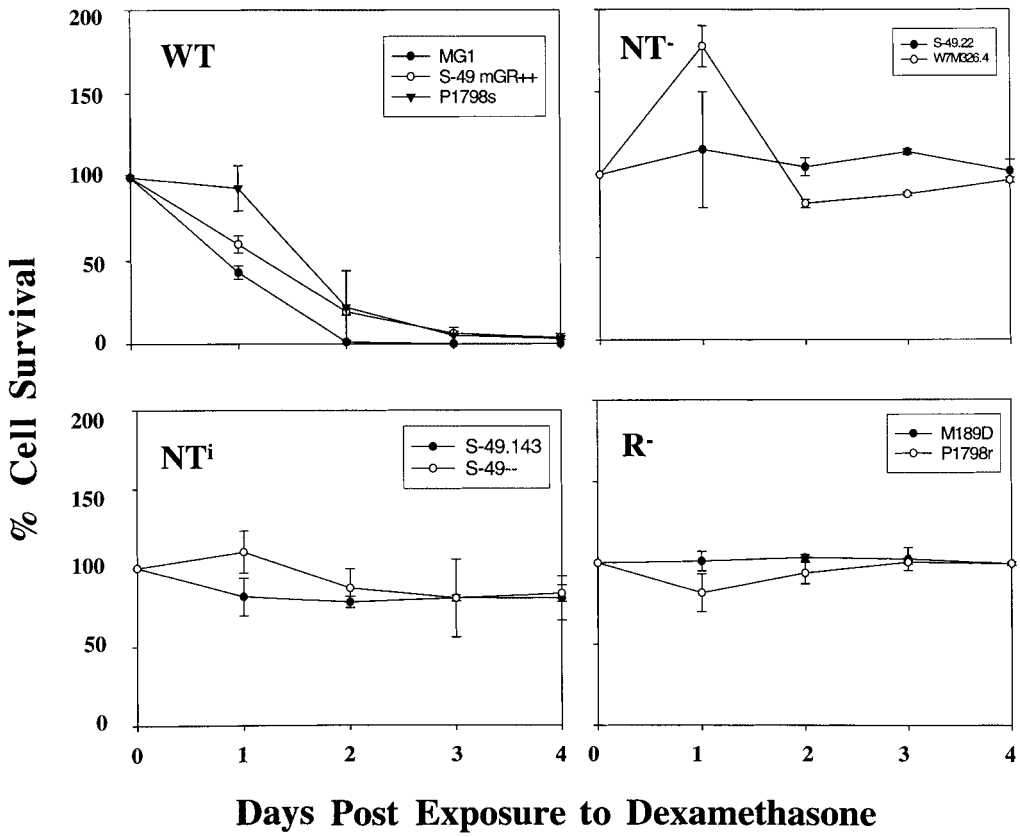

Fig. 6. Glucocorticoid sensitivity assay of the various types of mouse lymphoid cell lines. Following exposure to dexamethasone, cell viability was monitored by trypan blue exclusion as described in text and expressed as % cell survival compared to cells present on day 0. WT (P1798s, W7MG1, and S-49^{++}); NT^{-} (S-49.22r and S49 W7M326.4); NT^{i} (S-49.143r and S-49^{-}); R^{-} (M198D and P1798r). Results represent three to five separate experiments.

sequential immunoselection (immunopanning, fluorescent cell sorting, and soft agar cloning) techniques [Gametchu, 1987; Gametchu et al., 1991b, 1994; Sackey et al., 1997; Chen et al., 1999]. These findings are now validated in other commonly used lymphoid cell lines by the current studies. The fact the 4S iGR is present in both the GC-sensitive, WT, and GC-resistant NT^{-} cell groups also argues that the mGR is necessary for cell lysis. Since we have never found and analyzed a cell which has mGR but no iGR, we cannot determine if mGR alone could mediate the lysis effect. However, we believe that this is doubtful, as later genomic actions of GCs are probably required to sustain a continuing apoptotic response.

Density gradient analysis of the intracellular receptor preparations preserved mutant receptor signals as long as the epitope was present. Thus this is another method of assessing overall size of the receptor variant. From this analysis the NT^{i} cells appeared to have a truncated 2S version of the iGR (vs. 4S for the WT). The differences between the recognition of two closely spaced epitopes (see Fig. 1) suggests that this truncation occurred near the BUGR epitope.

Fig. 5. Sucrose density gradient analysis of iGR from the various cell groups with antibodies recognizing two different epitopes. [^{3}H]triamcinolone acetonide-labeled cytosol mixed with non-specific Abs (●) or with DNA-binding region-specific BUGR-2 antibody or hinge region-specific R1 anti-human GR (anti hGR) antibody (_) were subjected to sucrose density gradient analysis. Specific antibodies were used to shift iGR downward in the gradient via binding. Samples competed with 100-fold excess of unlabeled steroid were run in parallel to establish the specificity of GR labeling (○). Results are representative of two to three separate experiments performed for each cell line. **Top panels**: first from left, cytosol from WT (S-49); second from left, NT^{-} (S-49,22r); third from left, NT^{i} (S-49.55r); fourth from left, D. R^{-} (ADR6.M189D) cell groups after incubation with BUGR-2. **Bottom panels** (**A′**–**D′**), cytosol from the corresponding cell groups after incubation with anti-hGR Abs. * = position of ^{14}C-BSA ran as marker standard and sedimenting as a 4S protein.

The reduced size is consistent with a truncation, which spans the N-terminus region and the DNA binding domain, removing the entire amino terminus half of the receptor molecule. These findings corroborate other studies on the NT^i receptor mRNA sequence [Dieken et al., 1990], which also described the resistance of such cells to GC-mediated cell death [Dieken and Miesfeld, 1992].

It is always possible that other protein factors that are crucial for GC signaling through the mGR may be additionally absent from these mutant cell lines, and we have made previous observations relating to this scenario. Earlier comparative analysis of WT, mGR^{++}, and mGR^- S-49 cells by differential cDNA display revealed that while most genes were commonly expressed, more than one cDNA was distinctly expressed in one or the other cell group (Gametchu, unpublished results). Some of these additional products are unique to mGR^{++} cells, and, therefore, may interact with this protein in the signal transduction cascade. Additionally, we have observed that some mouse cell surface marker antigens (Thy-1,2, H-2, and LFA-1) typically expressed T lymphoma cells were conspicuously absent from the mGR^- cells [Gametchu et al., 1994] and we hypothesized that the highly selected minus cells had also been selected on the basis of some component of the membrane protein targeting system being mutated or missing [Gametchu et al., 1991a].

Although the presence of GR is necessary for the hormone effect to be elicited, this cannot necessarily be predicted by just examining whole cell receptor number as previously thought [Lippman et al., 1978; Quddus et al., 1985; Kato et al., 1993; Csoka et al., 1997]. Despite resistance to GC-induced cell lysis, the NT^i cells contain three times more whole cell binding sites than WT cells. Also, there was no significant difference in the whole cell number of receptor sites between the GC-lysis sensitive WT and GC-lysis resistant NT^- cells. Since mGR is either absent, diminished, or modified in lysis-resistant mutant cells, the variations in whole cell receptor number in these cells appear to be due to the iGR. Our studies support the view that high whole cell receptor numbers do not necessarily predict the hormonal response of cell lysis, and offer an explanation for why some tumors have high receptor number by clinical assays (usually whole cell assays) but fail to respond to hormonal therapy.

In summary, using different WT and mutant mouse lymphoma cell lines, we have found a correlation between the quantity and integrity of mGR and the GC-mediated lymphocytolysis response, corroborating our previous findings in S-49 cells [Gametchu, 1987; Gametchu et al., 1991a,b, 1993; Sackey et al., 1997]. It appears that cellular GC-sensitivity is not only influenced by the presence of mGR, but also by the receptor's molecular size. This suggests that a fully-modified mGR is necessary to carry out part of the receptor-mediated apoptotic cellular response, and that the utility of clinical assessments will improve if this receptor form is taken into consideration in receptor measurements.

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