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# Immunochemical characterization of wild-type and variant glucocorticoid receptors by monoclonal antibodies

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**Monoclonal antibodies raised against the rat liver glucocorticoid receptor were used to investigate receptors of wild-type and glucocorticoid-resistant variants of mouse lymphoma cells. Two of the variant types contained receptors of 'nuclear transfer deficient' ($nt^-$) and 'increased nuclear transfer' ($nt^i$) phenotypes, respectively, while the third was of the 'receptorless' ($r^-$) phenotype with negligible hormone binding activity. Three monoclonal antibodies of the IgM class and one of the IgG class reacted with both wild-type and $nt^-$ receptors but not with the steroid binding form of $nt^i$ receptors. Some of the antibodies bound the wild-type and $nt^-$ receptors more efficiently after activation at 20°C. By use of an immunocompetition assay we were able to detect cross-reacting material in considerable amounts in extracts of $nt^i$ and $r^-$ cell variants. This material was further characterized by gel filtration and immunoblotting. The immunoreactive material of wild-type, $nt^i$ and $r^-$ cells gave a major band of mol. wt. 94 000 upon SDS-gel electrophoresis while the steroid-binding polypeptides of wild-type and $nt^i$ receptors have mol. wts. of 94 000 and 40 000, respectively. The data show that in S49.1 mouse lymphoma cells the products of two receptor alleles can be distinguished.**

*Key words:* active domains/glucocorticoid receptors/monoclonal antibodies/mouse lymphoma cells/receptor variants

## Introduction

The initial events in the mechanism of steroid hormone action include the binding of the hormone to specific intracellular receptor proteins and activation of the receptor-steroid complexes to a form with high affinity for nuclear acceptor sites (for reviews, see Agarwal, 1978; Higgins and Gehring, 1978; Baxter and Rousseau, 1979; Katzenellenbogen, 1980; Sherman and Stevens, 1984). The essential function of such receptors became particularly clear through the isolation in cell culture of unresponsive variants of murine thymic lymphoma cells which in their wild-type state respond to glucocorticoids by growth inhibition and cell lysis (for a review, see Gehring, 1980a). Most abundant amongst these cell variants is the 'receptorless' ($r^-$) phenotype which is characterized by greatly reduced or negligible steroid-binding activity. This could result from one of two kinds of defects: the receptor molecule as such might be missing or the receptor protein might have a defect in the steroid-binding site. In addition, two types of variants have been found in which hormone binding is normal but interaction of the receptor-glucocorticoid complexes with cell nuclei, chromatin, or DNA is affected. Nuclear binding is decreased in the $nt^-$ type ('nuclear transfer deficient') while it is increased in the $nt^i$ variant type ('increased nuclear transfer').

Several biochemical investigations were aimed at characterizing these variant receptors and at comparing them with the wild-type (Yamamoto *et al.*, 1976; Pfahl *et al.*, 1978; Andreasen and Gehring, 1981; Dellweg *et al.*, 1982; Gehring and Hotz, 1983). In particular, the comparison of polypeptide mol. wts. and DNA-binding properties as well as partial proteolysis of these receptors led to a model for the wild-type glucocorticoid receptor that suggests three functionally distinct domains within the receptor polypeptide structure (Dellweg *et al.*, 1982; Gehring and Hotz, 1983).

In principle, there exist two types of glucocorticoid-sensitive mouse lymphoma cells: one of them, for example, line WEHI-7, expresses two active receptor alleles while others, for example, line S49.1, are hemizygous for the wild-type receptor (Bourgeois and Newby, 1977; Francke and Gehring, 1980; Gehring, 1980b). In S49.1 cells the second receptor allele may either be fixed in an inactive state or it may lead to the production of a defective receptor protein that is unable to bind the hormone. Mutation of the wild-type allele, for example, to the $nt^i$ type would therefore result in cells either expressing only the $nt^i$ receptor variant or synthesizing simultaneously two types of defective receptors. As outlined previously (Gehring, 1980a) the use of specific antibodies directed against the wild-type receptor should distinguish between these alternatives.

Glucocorticoid-specific receptors were highly purified from rat liver and rat thymus and antisera were produced in rabbits (Govindan, 1979; Eisen, 1980; Okret *et al.*, 1981; Tsawdaroglou *et al.*, 1980). Recently monoclonal antibodies directed against the rat liver receptor were obtained from spleen cells of immunized mice which were hybridized to mouse myeloma cells (Grandics *et al.*, 1982; Westphal *et al.*, 1982; Gametchu and Harrison, 1984). By a combination of biochemical and immunochemical techniques using some of these monoclonal antibodies it was possible to distinguish between the products of two receptor alleles in wild-type and variant S49.1 cells.

## Results

### *Immunoreaction with non-activated receptors*

The monoclonal antibodies used in the present study originated from spleen cells of mice which had been injected with activated rat liver glucocorticoid receptor of ~30% purity (Westphal *et al.*, 1982). Four of these antibodies were employed because they had previously been shown also to react with glucocorticoid receptors of other species than the rat. We first used an assay based on immunoprecipitation of radio-labelled receptor-steroid complexes. Table I shows the reactivity with wild-type and variant receptors of mouse lymphoma cells stabilized in the non-activated state by the addition of sodium molybdate (Leach *et al.*, 1979). In analogy to the rat liver receptor, the wild-type lymphoma cell receptors of lines WEHI-7 and S49.1 were bound most efficiently

**Table I.** Antibody binding of wild-type and variant receptors

| State of activation | Receptor type | % Receptor specifically bound by monoclonal antibody | | | |
|---|---|---|---|---|---|
| | | 25 | 49 | 57 | 98 |
| non-activated | wild-type (WEHI-7) | – | 48 ± 9 | – | – |
| | wild-type (S49.1G.3.5) | 5 ± 2 | 45 ± 1 | 19 ± 1 | 3 ± 3 |
| | $nt^-$-type (S49.1TB.4.41.22R) | 7 ± 2 | 55 ± 3 | 8 ± 1 | 3 ± 2 |
| | $nt^-$-type (S49.1G.3.83R) | 11 ± 3 | 55 ± 1 | 9 ± 4 | 4 ± 3 |
| | $nt^i$-type (S49.1TB.4.55R) | < 1 | < 1 | < 1 | <1 |
| | $nt^i$-type (S49.1TB.4.143R) | < 1 | < 1 | < 1 | <1 |
| activated | wild-type (WEHI-7) | – | 49 ± 1 | – | – |
| | wild-type (S49.1G.3.5) | 18 ± 2 | 47 ± 5 | 21 ± 1 | 9 ± 5 |
| | $nt^-$-type (S49.1TB.4.41.22R) | 19 ± 2 | 59 ± 4 | 18 ± 2 | 5 ± 2 |
| | $nt^-$-type (S49.1G.3.83R) | 19 ± 1 | 54 ± 5 | 22 ± 1 | 9 ± 4 |
| | $nt^i$-type (S49.1TB.4.55R) | < 1 | < 1 | < 1 | <1 |
| | $nt^i$-type (S49.1TB.4.143R) | < 1 | 1 | 1 | <1 |

Results are reported as means and ranges of 2–4 independent experiments.
Adsorption onto DNA-cellulose showed that ~50–60% of wild-type and 15–20% of $nt^-$ receptors bound to DNA after a 30 min incubation at 20°C.

by the IgG antibody mab 49. The antibodies of the IgM class, mab 25, 57, and 98 reacted to a considerably lesser extent. As mentioned previously (Westphal *et al.*, 1982) it is not yet clear why the receptors are incompletely bound despite the large excess of antibodies used in these experiments. It appears, however, that the IgG antibody mab 49 has a greater affinity for the lymphoma cell receptors than the IgM antibodies. Two independent cellular isolates of $nt^-$ variant receptors also interacted with the antibodies (Table I). The data show that mab 49 binds the $nt^-$ receptors slightly more efficiently and mab 57 somewhat less than the wild-type receptor. With these monoclonal antibodies we also tested variant receptors of the $nt^i$ type of two independent cellular isolates and we found that none was able to bind this type of variant receptor (Table I).

To obtain further evidence for the differences in immunoreactivity of wild-type and $nt^i$ receptors we used immunoaffinity chromatography. Monoclonal antibody 49 was covalently coupled to Sepharose. The affinity matrix retained 60% of the wild-type receptor complexes but <1% of the $nt^i$ receptors applied to the column. In some experiments the wild-type receptor complex with [$^3$H]triamcinolone acetonide was first subjected to photoaffinity labeling by exposure to u.v. light (Dellweg *et al.*, 1982; Gehring and Hotz, 1983) and subsequently applied to the immunoaffinity column. When the eluate with NaSCN was submitted to gel electrophoresis in SDS a major radiolabeled band of mol. wt. 94 000 was detected (data not shown). This shows that the antibody is able to recognize the receptor protein that carries a covalently cross-linked steroid. Also, degradation of the receptor turned out to be negligible during immunoaffinity chromatography. This method will therefore be useful for the purification of receptors.

*Immunoreaction with receptors after activation*

Receptor-steroid complexes need to be activated in order to bind tightly to the cellular genome (Milgrom, 1981). This can be achieved, for example, by warming soluble receptor complexes to 20°C, by increasing the ionic strength, or by dilution. The same is true for both the $nt^-$ and $nt^i$ variant receptors (Yamamoto *et al.*, 1976; Gehring, 1980a; Andreasen and Gehring, 1981); however, in the case of $nt^-$ receptors only a relatively small fraction of complexes is able to bind to DNA or nuclei even after activation. Table I shows the reactivity of monoclonal antibodies with wild-type and variant lymphoma cell receptors following their activation at 20°C. The antibodies bound the wild-type and both $nt^-$ receptor types but again did not react with the $nt^i$ receptor complexes. Interestingly, wild-type and $nt^-$ receptors were bound more efficiently by mab 25 after activation. Also mab 57 bound the $nt^-$ receptors better after 20°C activation than before.

*Cross-reacting material in variant lymphoma cells*

To be able to search for cross-reacting material in extracts of variant cells, we developed an immunocompetition assay. We chose WEHI-7 cells as a standard because these cells are known to express two wild-type receptor alleles (Bourgeois and Newby, 1977). In the assay we used limiting amounts of mab 49, in contrast to the previous experiments, and allowed non-labeled receptor complexes to compete with labeled complexes. An increase in the proportion of receptor complexes with unlabeled hormone over those with labeled ligand resulted in a hyperbolic decrease in the amount of radioactivity bound to the antibody; this was used for calibrating the assay.

In the experiments of Table II we used equal parts of variant and wild-type cytosols on a protein basis and calculated the amounts of immunocompeting material relative to WEHI-7 wild-type receptors. The data clearly show that extracts of $nt^i$ variant cells contain significant amounts of antigenic material which cross-reacts with the anti-receptor antibody mab 49. Also two $r^-$ variants were investigated and found to contain cross-reacting material, albeit to different extents.

Table II also compares the hormone binding activities of WEHI-7 and S49.1 wild-type cells and variants as determined by whole cell binding. In the $r^-$ cell clones steroid binding was negligible as compared with that in wild-type cells. Interestingly, the $nt^i$ clone S49.1TB.4.55R had significantly higher receptor levels than its progenitor wild-type cell S49.1TB.4.

*Molecular characterization of cross-reacting material*

Gel permeation chromatography was used in combination with an indirect competitive ELISA to characterize further the cross-reacting material in wild-type and variant lymphoma cells. Figure 1A shows for the S49.1 wild-type that hormone-binding activity and immunoactivity eluted from

**Table II.** Immunocompetition assays

| Cell type | Antibody-reactive material (relative to the WEHI-7 wild-type) | Specific steroid binding (molecules/cell) | (relative to WEHI-7 cells) |
|---|---|---|---|
| wild-type (WEHI-7) | = 100% | 36 500 ± 2500 (2)[a] | = 100% |
| wild-type (S49.1TB.4) | 68 ± 7% (4) | 13 000 ± 1000 (2)[a] | 36% |
| nt$^{i}$-type (S49.1TB.4.55R) | 33 ± 7% (3) | 24 800 ± 1700 (2) | 68% |
| r$^{-}$-type (S49.1TB.4.7R) | 36 ± 3% (3) | 200 ± 100 (2) | < 1% |
| wild-type (S49.1G.3.5) | 63 ± 3% (2) | 20 000 ± 1000 (2)[b] | 55% |
| r$^{-}$-type (S49.1G.3.1R) | 13 ± 6% (3) | 150 ± 100 (2) | < 1% |

Results are reported as means and ranges of 2–4 independent experiments.
[a]Data from Gehring *et al.* (1982a).
[b]Data from Gehring *et al.* (1982b).

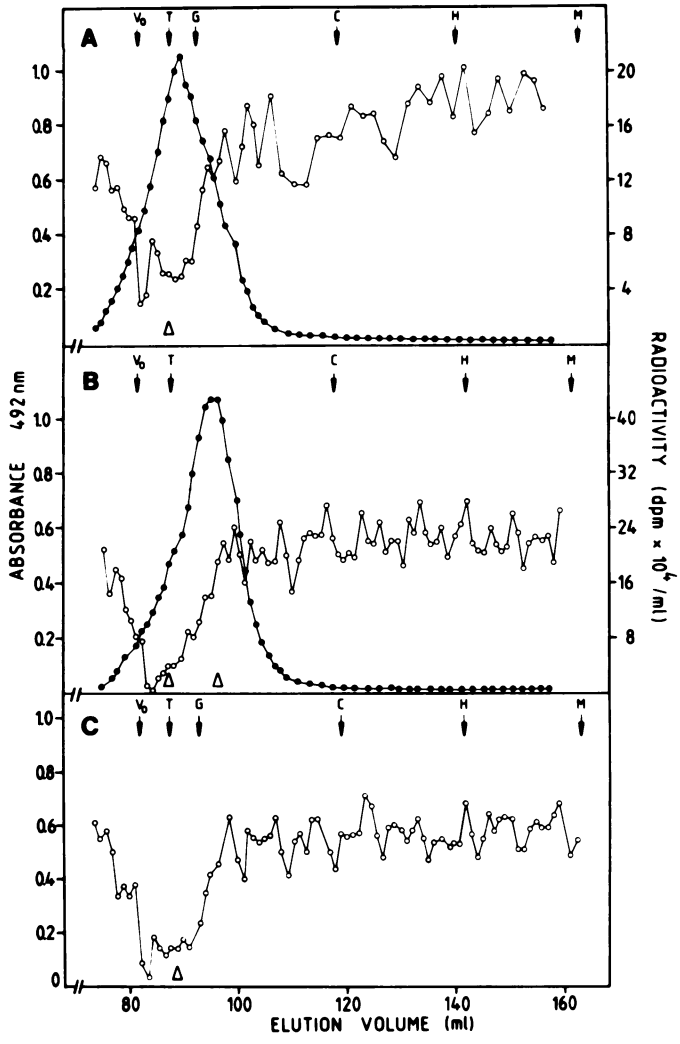


**Fig. 1.** Gel filtration of cytosols from wild-type and variant cells. Cytosols of S49.1TB.4 (**A**, wild-type), S49.1TB.4.55R (**B**, nt$^{i}$) and S49.1TB.4.7R (**C**, r$^{-}$) cells were incubated with $^{3}$H-labelled hormone (except for the r$^{-}$ cytosol that does not bind the steroid specifically) and chromatographed as described in Materials and methods. Fractions were analyzed for radioactivity (●) and immunoactivity by indirect competitive ELISA (○, absorbance at 492 nm). Fractions further analyzed by immunoblotting (Figure 2) are marked △. Elution volumes of markers are indicated: $V_o$ (void volume), T (thyroglobulin), G (β-galactosidase), C (catalase), H (hemoglobin), and M (myoglobin).

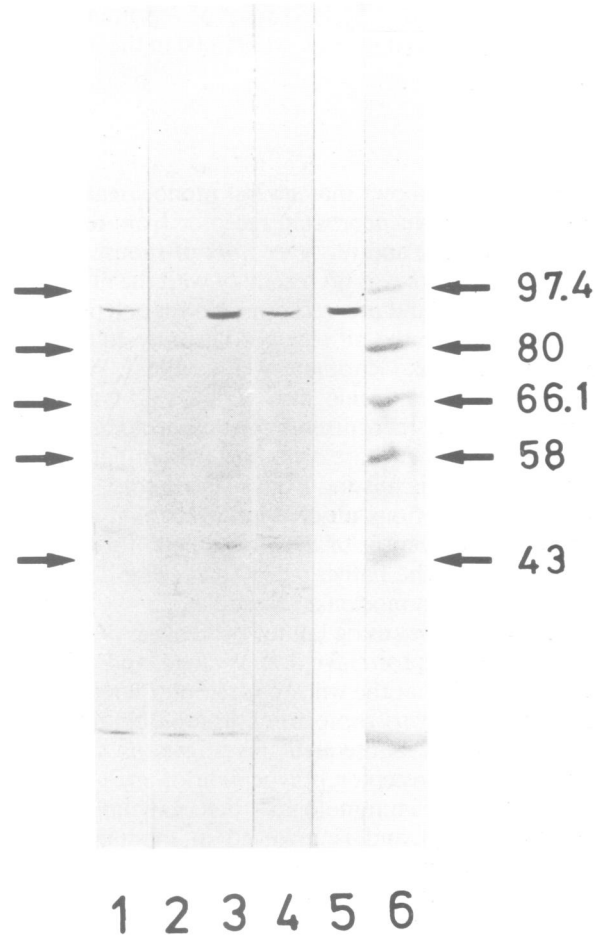


**Fig. 2.** SDS-gel electrophoresis and immunoblot. 150 μl of the peak fractions obtained after gel filtration (Figure 1, marked △) were analyzed by the immunoblotting procedure described under Materials and methods. **Lane 1**, wild-type ($R_S$ 8.1 nm); **lane 2**, nt$^{i}$ ($R_S$ 6.9 nm); **lane 3**, nt$^{i}$ ($R_S$ 8.1 nm); **lane 4**, r$^{-}$ ($R_S$ 8.1 nm); **lane 5**, 100 ng purified rat liver glucocorticoid receptor (~40% pure); **lane 6**, marker proteins: phosphorylase b, transferrin, BSA, catalase, ovalbumin (from top to bottom), mol. wt. (x 10$^{-3}$).

the column at the same effluent volume, corresponding to a Stokes radius of 8.1 nm. Analysis of the peak fraction by SDS-gel electrophoresis and immunoblotting with mab 49 revealed a major band of mol. wt. 94 000 (Figure 2, lane 1) that co-migrated with the purified glucocorticoid receptor of rat liver (Figure 2, lane 5). As the same mol. wt. has previously been obtained by photoaffinity labeling of wild-type receptors (Gehring and Hotz, 1983) we conclude that this immunoreactive band corresponds to the native glucocorticoid receptor.

Gel filtration of an extract from nt$^{i}$ variant cells (Figure 1B)

resulted in separation of steroid-binding activity ($R_S$ of 6.9 nm) and immunologically reactive material ($R_S$ of 8.1 nm). Immunoblots of the corresponding peak fractions showed that no band was associated with the steroid-binding activity (Figure 2, lane 2) whereas a prominent band of mol. wt. 94 000 was seen for the immunoreactive material of $R_S$ 8.1 nm (Figure 2, lane 3). The same was observed in a gel filtration run in which we omitted molybdate from the buffer but instead added 300 mM NaCl; also in this case the immunoreactive material did not co-elute with the steroid-binding activity (data not shown). This suggests that the cross-reacting protein of mol. wt. 94 000 in the $nt^i$ variant is due to a receptor defective in steroid binding.

In a similar experiment with an extract of $r^-$ variant cells we found immunoreactive material that eluted from the gel filtration column with a Stokes radius of ~8 nm (Figure 1C) and gave a major band of mol. wt. 94 000 in the immunoblot (Figure 2, lane 4).

## Discussion

The present study shows that several monoclonal antibodies raised against the glucocorticoid receptor from rat liver also react with wild-type and $nt^-$ receptors of mouse lymphoma cells. However, there was no reactivity with the $nt^i$ variant receptors. Wild-type and $nt^-$ receptors have a polypeptide mol. wt. of 94 000 while the $nt^i$ receptor has only 40 000 daltons (Dellweg *et al.*, 1982; Gehring and Hotz, 1983). We therefore conclude that the antigenic sites within wild-type and $nt^-$ receptors which are recognized by the monoclonal antibodies used in our experiments are contained within that part of the polypeptide which is missing from $nt^i$ receptors. This is consistent with the previous observation (Westphal *et al.*, 1982) that the rat liver receptor of mol. wt. 40 000 formed by partial proteolysis of the native receptor likewise did not react with any of these monoclonal antibodies.

Biochemical studies using limited proteolysis of the rat liver glucocorticoid receptor have led Wrange and Gustafsson (1978) to propose that the wild-type receptor contains a third domain in addition to those for hormone binding and for nuclear interaction. Subsequent investigations showed that this region of the receptor polypeptide of mol. wt. 94 000 contains the main immunological determinants (Carlstedt-Duke *et al.*, 1982) and is involved in modulating DNA-binding affinity and nuclear interaction in such a way that specific genes can be expressed (Dellweg *et al.*, 1982; Gehring and Hotz, 1983). If the modulation domain is missing as in the partially degraded receptor of mol. wt. 40 000 and in the $nt^i$ variant receptor the hormone complexes might still bind to the biologically relevant sites on DNA (Scheidereit *et al.*, 1983) but without leading to transcriptional regulation.

Our immunochemical receptor studies with monoclonal antibodies agree with previous observations by others who used polyclonal antibodies. The rat liver receptor that was partially degraded by proteolysis did not react with these antisera (Okret *et al.*, 1981; Eisen, 1982); the cleaved-off immunoreactive fragment, however, could be recovered by gel filtration (Carlstedt-Duke *et al.*, 1982). Likewise, the receptor of glucocorticoid-resistant P1798 mouse lymphoma cells which is of the $nt^i$ type and has a polypeptide mol. wt. of 40 000 (Gehring and Hotz, 1983) was not bound by these polyclonal antibodies (Stevens *et al.*, 1981). By contrast, Govindan (1979) and Tsawdaroglou *et al.* (1981) described cross-reacting antisera against 90 000 and 45 000 mol. wt. forms of rat liver and thymus receptors. Nevertheless, it appears that the main antigenic determinants in glucocorticoid receptors are located within the modulation domain described above.

In some of our experiments we used the monoclonal antibodies to probe for differences in receptors prior to and following activation. For example, antibody mab 25 reacted better with wild-type and $nt^-$ receptors after a 20°C incubation that is known to cause receptor activation to a form that is able to bind to DNA. This suggests that upon 20°C activation the $nt^-$ receptors, like the wild-type, undergo some structural changes that make both receptor types more accessible for some of the antibodies, despite the fact that the DNA-binding ability of $nt^-$ receptors remains quite limited. This is of interest in view of the fact that the antibodies are directed against the modulation domain of the receptors which appears to be intact in the $nt^-$ variants (Gehring and Hotz, 1983). Thus activation at 20°C causes some conformational or other changes within the modulation domain, the presence of which is not required for activation, since $nt^i$ receptors can also be activated. As mab 49 does not distinguish between the non-activated and activated receptor forms it probably recognizes a different epitope within the modulation domain. Our experiments show that monoclonal antibodies will become useful tools for detecting subtle changes in receptor structures that come about under activating conditions.

Even though glucocorticoid receptors of S49.1 $nt^i$ variants did not react with our monoclonal antibodies, these variant cells nevertheless were found to contain cross-reacting material (Table II). Gel permeation chromatography and immunoblotting experiments (Figures 1B and 2) showed that this immunoreactive material has a mol. wt. of 94 000 while the steroid-binding polypeptide is of mol. wt. 40 000 (Gehring and Hotz, 1983). Similarly, cross-reacting material was also detected in S49.1 variants of the $r^-$ phenotype (Table II) and likewise had a mol. wt. of 94 000 (Figure 2).

Quantitation of the immunoreactive material in several clones of S49.1 cells relative to wild-type glucocorticoid receptors of line WEHI-7 resulted in an interesting correlation (Table II). S49.1TB.4 cells contained about twice as much antibody-reactive material as expected from its steroid-binding activity. The $nt^i$ subclone S49.1TB.4.55R and the $r^-$ subclone S49.1TB.4.7R contained the same amount of immunoreactive material which was about half of that of the progenitor line S49.1TB.4. This clearly demonstrates that S49.1 wild-type cells which are known to be hemizygous for the receptor (cf. Introduction) produce, in addition to the wild-type receptor polypeptide, a defective protein that is unable to bind the steroid hormone but reacts quite normally with the anti-receptor antibody. This view is consistent with the previous observation that it was not possible to turn on wild-type receptor production in hybrid cells which contained only the S49.1 specific chromosome that carries the defective receptor gene (Gehring, 1984).

Different wild-type clones of S49.1 cells may express the wild-type and mutant receptor alleles at different levels. In S49.1G.3.5 (wild-type) and the subline S49.1G.3.1R the mutant receptor alleles appear to produce much lower levels of defective receptor protein than does line S49.1TB.4 and its subclones (Table II). Similarly, in the $nt^i$ clone S49.1TB.4.55R the $nt^i$ allele gives rise to increased expression as compared to the other allele.

One further observation deserves mentioning. Both $r^-$ variants of S49.1 cells investigated here appear to have the wild-type allele altered in such a way that no product is detectable with either steroid or antibody while they still express the other allele that produces an immunoreactive polypeptide devoid of hormone-binding activity. The wild-type allele might either be switched off in these cells or it might lead to a receptor-related polypeptide devoid of both steroid binding and immunoreactivity with mab 49. This suggests that the $r^-$ phenotype as originally defined may indeed be caused by several cellular events. Certainly more $r^-$ variants need to be analyzed by immunochemical methods as those described here in order to clarify this point further.

## Materials and methods

*Cell lines and cell culture*

Lymphoma line WEHI-7 and the S49.1 sublines S49.1TB.4 (wild-type), S49.1G.3.5 (wild-type), S49.1G.3.83R ($nt^-$ type), S49.1TB.4.41.22R ($nt^-$ type), S49.1TB.4.55R ($nt^i$ type), S49.1TB.4.143R ($nt^i$ type) were those previously used (Gehring, 1979; Gehring *et al.*, 1982a,1982b; Gehring and Hotz, 1983). Sublines S49.1G.3.1R ($r^-$ type), and S49.1TB.4.7R ($r^-$ type) were obtained by cloning the respective parental cell populations in soft agar containing $\mu$M dexamethasone and were characterized as described (Sibley and Tomkins, 1974). Cells were grown and harvested as previously described (Gehring and Hotz, 1983). Cell pellets were quickly frozen in liquid nitrogen and stored at $-90$°C.

*Steroid binding to intact cells*

The assay for specific steroid binding to whole cells was carried out as described before (Gehring *et al.*, 1982a,1982b). In the case of $r^-$ variants Scatchard analysis of the binding data was not meaningful; we therefore determined the specific binding at only one steroid concentration that saturated receptors of wild-type cells to $\sim 70-80\%$.

*Cytosol preparations*

Frozen cell pellets were thawed and homogenized in a Teflon-glass homogenizer with 0.5–1.0 ml buffer (20 mM sodium phosphate, pH 7.2, 50 mM NaCl, 2 mM mercaptoethanol, 1 mM EDTA, and 10% glycerol) per $10^9$ cells and centrifuged for 1 h at 100 000 *g* at 6°C. The supernatant fluid, called cytosol, was incubated with either 33 nM [$^3$H]triamcinolone acetonide (New England Nuclear, Boston; 30 Ci/mmol) or the same concentration of unlabelled triamcinolone acetonide (Sigma, St. Louis) at 6°C for 2–3 h. Unbound steroid was adsorbed by dextran-coated charcoal (Beato and Feigelson, 1972) and radioactivity was assessed by liquid scintillation spectrometry at 40% efficiency. Sodium molybdate (10–20 mM) was added to cytosols prior to the steroid in all experiments in which receptor complexes were to be preserved in the non-activated state (Leach *et al.*, 1979). For activation, receptor-steroid complexes were incubated at 20°C for 30 min; subsequently sodium molybdate was added. DNA binding of activated complexes was checked by chromatography on DNA-cellulose (prepared from calf thymus DNA) using step-wise elution (Gehring, 1980b). Protein concentrations were determined by the Biuret method.

*Gel filtration*

Cytosol preparations containing 39 mg protein were pre-incubated with [$^3$H]-triamcinolone acetonide and chromatographed at 6°C on a column (192 ml bed volume; 100 cm length) of Sephacryl S300 (Pharmacia, Uppsala). The column was equilibrated and developed with 20 mM phosphate buffer pH 7.4 containing 20 mM sodium molybdate, 2 mM mercaptoethanol, 1 mM EDTA and 10% glycerol. The flow rate was 8 ml/h. Fractions of 1 ml were collected and aliquots were used to determine radioactivity and immunoreactivity by an indirect competitive ELISA. For calibrating the column (Niu *et al.*, 1981) we used Blue Dextran, thyroglobulin ($R_S$ 8.6 nm), $\beta$-galactosidase ($R_S$ 6.85 nm), catalase ($R_S$ 5.23 nm), hemoglobin ($R_S$ 3.21 nm), and myoglobin ($R_S$ 2.0 nm).

*Antibody binding assay*

Rabbit anti-mouse IgG (RAM) antibodies were coupled to Sepharose (Pharmacia, Uppsala) as described (Westphal *et al.*, 1982) at a final concentration of 2 mg antibody/ml packed Sepharose.

Except for minor modifications the antibody binding assay was that of Westphal *et al.* (1982). Cytosol preparations containing 0.1 pmol non-activated or activated receptor-[$^3$H]triamcinolone acetonide complexes were added to a 20–50-fold excess of monoclonal antibodies and incubated in the presence of 10 mM sodium molybdate overnight at 0°C in a total volume of 30 $\mu$l. 50 $\mu$l RAM-Sepharose (25% v/v suspension) were added and the mixture was further incubated for 2 h while shaking gently. After collecting the immunoadsorbent by centrifugation in a table-top centrifuge it was washed three times with 750 $\mu$l each of 20 mM sodium phosphate, pH 7.2, containing 150 mM NaCl and 10 mM sodium molybdate. Receptor-antibody complexes were eluted by washing twice with 300 $\mu$l each of 3.5 M NaSCN; radioactivity was determined in the combined eluates. The percentage of receptor complexes bound to the antibodies was expressed relative to the amount of complexes left after the overnight incubation (61 ± 10% of the input). Radioactivity unspecifically bound to RAM-Sepharose was determined in parallel incubations without monoclonal antibodies and was subtracted.

*Immunocompetition assay*

The antibody binding assay described above was modified in order to allow to test for cross-reacting material. 0.5–0.55 pmol of monoclonal antibody mab 49 (Westphal *et al.*, 1982) were incubated in the presence of 10 mM sodium molybdate overnight at 0°C in a total volume of 100 $\mu$l with 0.55–0.6 pmol of WEHI-7 wild-type receptor complexed with [$^3$H]triamcinolone acetonide and various amounts of competing wild-type or variant receptors complexed with unlabeled steroid. Protein concentrations were adjusted to that of the WEHI-7 cytosol used in the competition assay (7–12 mg/ml). Receptor-antibody complexes were detected by adding 75 $\mu$l RAM-Sepharose (25% v/v suspension) and using the procedure described above. All assays were in triplicate.

*Immunoaffinity chromatography*

Antibody mab 49 was chromatographed on Sephacryl S300 and coupled to BrCN-activated Sepharose 4B (Pharmacia, Uppsala) at a final concentration of 3 mg/ml Sepharose according to the procedure of Axén *et al.* (1967). Cytosols pre-incubated with [$^3$H]triamcinolone acetonide were chromatographed on DEAE-cellulose (Dellweg *et al.*, 1982) in the presence of 10 mM sodium molybdate. The eluates containing 30 pmol receptor complex in a volume of 1–1.5 ml were incubated with 0.5 ml immunoaffinity absorbend for 3 h in the cold with gentle shaking. The material was transferred into a column, washed with 25 ml 20 mM sodium phosphate, pH 7.2, containing 150 mM NaCl and 10 mM sodium molybdate and finally eluted with 3.5 M NaSCN.

*Indirect competitive ELISA*

The method used was modified from that described by Okret *et al.* (1981). 100 $\mu$l samples of the fractions obtained after gel filtration were incubated overnight at 4°C with 10 $\mu$l of mab 49 (ascitic fluid diluted 1:2000 with PBS-Tween).

In parallel, polystyrene micro-ELISA plates (Nunc, Roskilde, Denmark) were coated with 50 $\mu$l containing 60 ng purified rat liver glucocorticoid receptor (Westphal *et al.*, 1982) per well overnight at 4°C. The wells were then washed with PBS-Tween and incubated with 100 $\mu$l bovine serum albumin (BSA; 20 mg/ml) in PBS-Tween for 1 h at 37°C in order to saturate all unspecific protein binding sites. After washing, the sample-antibody mixtures (110 $\mu$l) were pipetted into the wells and incubated for 2 h at 4°C. After washing, 100 $\mu$l of peroxidase-conjugated goat anti-mouse IgG + IgM antibodies (Tago, Inc., Burlingame, CA) diluted 1:1000 with PBS-Tween was added per well and incubated for 1 h at 37°C. The wells were washed and incubated with 100 $\mu$l of 40 mM Tris-HCl, pH 7.6, containing 150 mM NaCl and 14 mg *o*-phenylenediamine dihydrochloride (Sigma, St. Louis) and 7 $\mu$l 30% $H_2O_2$ per 25 ml. This solution was prepared during the last washings of the plate. After 10–15 min the enzyme reaction was stopped by addition of 50 $\mu$l 2 M $H_2SO_4$ per well and the absorption was measured using a Titertek-Multiskan photometer (Flow Laboratories) at 492 nm.

*Immunoblotting*

Samples were prepared by heating in a boiling water bath for 2 min in sample buffer (62.5 mM Tris-HCl pH 6.8, 1% SDS, 10% glycerin, 0.001% bromophenolblue, 5% mercaptoethanol) and subjected to SDS-gel electrophoresis according to Laemmli (1970) using a 7.5% running gel, 3 mm thick. Electrophoresis was done at 75 V for ~15 h.

After electrophoresis the gel slab was applied to nitrocellulose paper (Schleicher and Schüll, BA 85; 0.45 $\mu$m) and placed in a Bio-Rad Trans-Blot cell, filled with 20 mM Tris/150 mM glycine/20% methanol (pH 8.3). The transfer was performed at 4°C using 60 V for 6 h. The lane containing the marker proteins was cut off and stained for 30 s with 0.1% amido black in 45% methanol/10% acetic acid and destained with 10% acetic acid. The paper was then soaked in 0.9% NaCl containing 10% newborn calf serum for 1 h at room temperature and then incubated overnight at 4°C with mab 49 (ascitic fluid diluted 1:500 with 100 ml washing buffer containing 0.2% SDS, 0.5% Triton X-100, 0.5% BSA and 0.9% NaCl). After washing three times (10 min each) with washing buffer at room temperature, the paper was incubated with peroxidase-conjugated goat anti-mouse IgG antibodies (Tago Inc., Burlingame, CA) diluted 1:500 in 40 ml washing buffer for 2 h at 37°C

followed by 1 h at 4°C. The paper was washed twice each with washing buffer and Tris-buffered saline (50 mM Tris-HCl pH 7.4, 200 mM NaCl) 10 min each. Staining was done with 4-chloro-1-naphthol according to the procedure of Hawkes *et al.* (1982). Within 15–30 min sufficient colour intensity was obtained. The paper was then washed twice with Tris-buffered saline and water, dried on filter paper and photographed.

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