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MONOCLONAL ANTIPEPTIDE ANTIBODIES TO THE GLUCOCORTICOID RECEPTOR

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Summary—The generation of monoclonal antibodies to synthetic peptides of the glucocorticoid receptor is described. Two antibodies to sequences from the DNA binding region are IgMs. Two other antibodies to sequences in the steroid binding region and the C-terminus belong to the IgG class. The specificity of the IgG binding to the receptor in an ELISA assay is demonstrated by competition with the relevant peptides. Both IgGs are able to recognize the receptor in Western blots, but do not form stable complexes in sucrose gradients. Steroid binding to the receptor is not influenced by preincubation with antibodies. This indicates that denaturation or distortion of the receptor is necessary for the accessibility of these antibodies to their epitopes. Both antibodies can be used to stain the glucocorticoid receptor in neoplastic cells of patients suffering from chronic lymphatic leukemia.

INTRODUCTION

Antibodies can be used to investigate specific regions of a protein. In the case of the glucocorticoid receptor there are three main functional regions: (1) the N-terminal modulating region; (2) the DNA binding region and (3) the C-terminal steroid binding region. Immunization with intact receptor protein yields antibodies which are directed predominantly against the N-terminal region. This part of the receptor seems to be more immunogenic than the others. In order to get antibodies to the other regions, it is possible to use peptides from the respective parts as antigens. There are several reports on the successful production of antisera against the progesterone [1] and the glucocorticoid receptor [2–4].

We have attempted to generate antibodies against the human glucocorticoid receptor which could be used as probes for the presence, intracellular distribution and possibly the integrity of the receptor in patients suffering from chronic lymphatic leukemia (CLL). These patients are treated with a combination of drugs, one of which is a corticoid. Some of the patients do not respond to the therapy; there is no tumor regression on the basis of the steroid-induced lymphocytolytic effect. The reason for this resistance could be a modified receptor, which can no longer perform vital functions because of missing or modified amino acid sequences. It has been shown in mouse lymphoma cell lines that resistance to glucocorticoids was always caused by a modified receptor.

In this communication we describe the generation of antibodies against defined epitopes by immunizing with peptides coupled to carrier proteins. In order to have an unlimited supply of reagents with a unique specificity we decided to generate monoclonal antibodies. Here we report for the first time that it is possible to get monoclonal antibodies directed against peptides from the glucocorticoid receptor which are able to recognize their epitope in the intact receptor molecule.

EXPERIMENTAL

Immunization and cell culture

Female Balb-c mice, purchased from Tierzuchtanstalt (Hannover, Germany) were immunized by i.p. injections with 10 μg peptide coupled to bovine serum albumin (BSA) or KLH with Freund's complete adjuvant during the first injection. Subsequent injections were performed with Freund's incomplete adjuvant for a period of 3–5 months at 2 weekly intervals. The last boost was given with 20 μg peptide–BSA or peptide–KLH in phosphate buffered saline (PBS) (0.02 M phosphate, 0.15 M NaCl, pH 7.2) one day before fusion. After aseptical

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removal of the spleen, lymphocytes were fused with Sp2/0-Ag14 myeloma cells at a ratio of 10:1 with 50% polyethylene glycol. After washing with serum-free RPMI-medium the cells were resuspended in RPMI medium, containing 10% fetal calf serum (v/v), 2 mM L-glutamine, HAT (0.1 M hypoxanthine, 4×10^{-7} M aminopterin, 1.6×10^{-5} M thymidine) and 100 U/ml PSNC (penicillin, streptomycin, neomycin).

The cells were then plated in 96-well Nunc culture plates (with a preseeded macrophage-monolayer culture) at a concentration of 6×10^2 cells per well.

Positive hybrids were cloned twice by limiting dilution and first grown in 50 ml culture flasks to confluence, followed by culturing in 250 ml flasks to confluence.

The supernatants were harvested and centrifuged (200 *g*) for 10 min prior to use.

ELISA

Fifty nanograms of purified rat liver glucocorticoid receptor in PBS was adsorbed per well overnight to 96-well Nunc ELISA-plates.

After washing with PBS–0.5% Tween (see above) unspecific binding was prevented by incubation of the plates with PBS–2% BSA (v/w) for 1 h at room temperature. The plates were then washed as described above. Incubation with the antibodies was performed for 1 h at room temperature at ratio from 1:200 to 1:1000 in PBS. Detection of bound antibody was performed with a peroxidase coupled second antibody, goat antimouse IgG, at a ratio of 1:1000 followed by reaction with H_2O_2 as substrate and orthophenylendiamine (OPD) as indicator (14 mg OPD in 25 ml citrate-acetate buffer 0.1 M, pH 6.0, 7 μl H_2O_2, 100 μl solution/well). Reaction was stopped with 50 μl 2 M H_2SO_4.

For a positive control an established monoclonal antibody (I GR 49/4; see Ref. [11]), for negative control RPMI media containing 10% fetal calf serum or mouse preimmune sera at a dilution of 1:200 in PBS, was used.

Competition ELISA

AntiGR 788-795 at a dilution of 1:200 in PBS (see above) was preincubated with increasing amounts of peptide GR 788-795 (0.001–10 μg) for 1 h at room temperature following incubation with 50 ng purified glucocorticoid receptor.

For the negative control the same incubations were performed with the irrelevant peptide GR 464-471 coupled to BSA. Subsequent steps were the same as described above.

Western blotting

Samples were prepared by heating in a boiling water bath for 2 min in sample buffer (62 mM Tris–HCl pH 6.8, 1% SDS, 10% glycerol, 0.001% bromophenol blue, 5% mercaptoethanol) and subjected to SDS–gel electrophoresis according to Laemmli [12] using a 7.5% running gel, 1.5 mm thick. Electrophoresis was done at 200 V for *ca.* 3 h.

After electrophoresis the gel slab was transferred to a nitrocellulose sheet in a semi-dry blotting devive from Biometra, for 30 min at 5 mA per cm^2. The nitrocellulose and the filter paper was wetted with 0.02 M Tris–glycine, 20% methanol.

The lane containing the marker proteins was cut off and stained for 20 s with 0.1% amido black in 45% methanol/50% acetic acid and destained with 10% acetic acid. The nitrocellulose sheet containing the antigen was soaked in blocking buffer (0.01 M Tris, 0.15 M NaCl, 5% nonfat dry milk) for 1 h followed by incubation with antibody (1:300–1:3000 diluted in blocking buffer) for 1 h. The antibodies were preadsorbed before dilution with an equal volume of mouse serum.

After washing 3 times with washing buffer (0.05 M Tris, 0.025 M NaCl, 0.03 M EDTA, 0.05% Tween, pH 7.5) the sheet was incubated for 1 h with peroxidase conjugated goat antimouse IgG, F_c fragment antibodies (Jackson Immunoresearch Lab.) at a dilution of 1:5000 in blocking buffer. All incubations were done at room temperature.

The detection was carried out by chemiluminescence. The ECL Western blotting reagents from Amersham were used and the procedure was performed according to the manufacturers recommendations. The films were exposed as necessary from 30 s to 3 min.

Receptor purification

Receptor was purified from rat liver as described by Wrange *et al.* [13], only the elution of the receptor from the second DNA–cellulose was performed with salt instead of pyridoxal phosphate. Briefly, the procedure involves the chromatography of crude cytosol first on phosphocellulose; the flow through is applied on DNA–cellulose. This flow through is heat-activated (30 min at 25°C) and applied on a second DNA–cellulose. The receptor is eluted with a

buffer containing 350 mM NaCl. About 20–30% of the total protein is receptor.

Immunocytochemistry

APAAP method [14]. A 100 μl cell suspension in PBS (10^2–10^3 cells/ml) was cytocentrifuged for 10 min at room temperature and air dried.

After 30 min of incubation with antibody at a ratio of 1:10 to 1:50 in PBS buffer (see above) at room temperature in a moist chamber, the slides were washed twice with TBS (0.1 M Tris–HCl, 0.15 M NaCl, pH 8.2) and incubated with a bridging antibody, rabbit antimouse Ig, at a dilution of 1:25 in TBS containing 25% heat inactivated human serum for 30 min at room temperature. After washing twice with TBS, an incubation with the APAAP complex (DAKO) was performed for 30 min at room temperature followed by washing twice with TBS.

Bound antibody complex was detected after incubation with detection buffer (20% naphtholphosphate, 2% dimethylformamide, 0.1% levamisol, 10 mg fast red salt in TBS at a final volume of 10 ml) for 20 min at room temperature.

RESULTS

Generation of antibodies

We were interested to obtain antibodies against parts of the DNA binding domain ("zinc finger") and the steroid binding region. There are several parameters such as hydrophilicity, secondary structure and flexibility which seem to contribute to the antigenicity of proteins [5]. We decided to select amino acid sequences with a hydrophilic character. This is based on the longstanding notion that polar amino acids are predominantly located on the surface of proteins were they can interact with the aqueous environment. We used the computer program of Kyte and Doolittle [6] to select the sequences. Figure 1 shows the profiles for the DNA binding region and the last 95 amino acids of the steroid binding region. GR 465-471 is a part of the sequence between the two zinc fingers; GR 472-495 comprises the whole second zinc finger. The two other peptides GR 721-727 and GR 788-795 are located in the center of the steroid binding region and at the C-terminus, respectively. It can be seen that all four chosen peptides have a pronounced hydrophilic character.

The peptides were synthesized with a N-terminal cysteine to allow coupling to the carrier

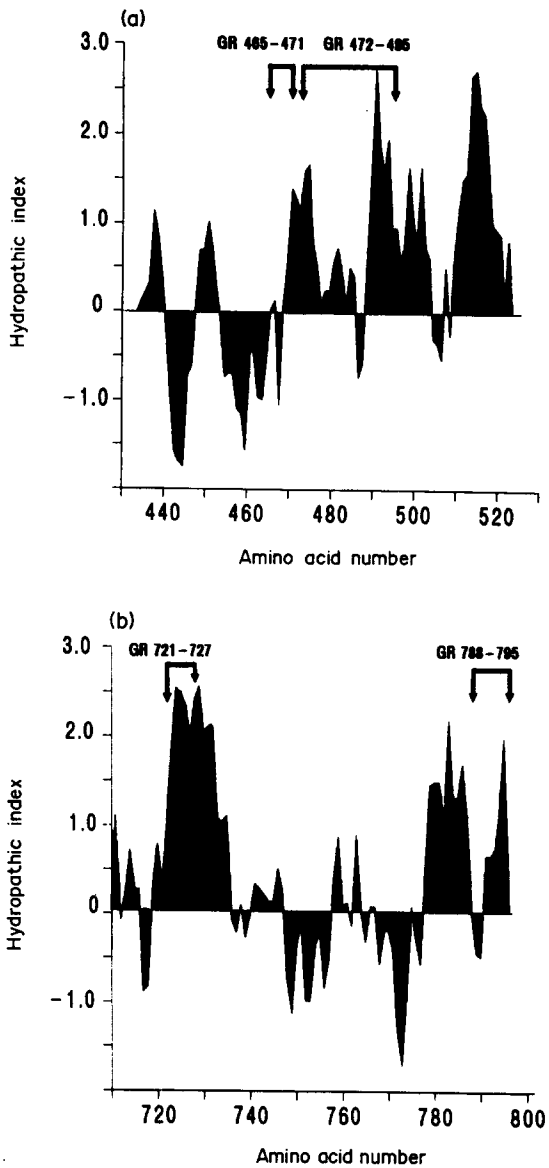

Fig. 1. Hydropathy profile of the peptides used for antibody generation. The amino acid numbering refers to the rat glucocorticoid receptor. (a) DNA binding region; and (b) steroid binding region and C-terminus.

protein. Immunizations were performed as described in Experimental. The sera were tested by means of an ELISA with purified rat glucocorticoid receptor used as antigen. Thus, we were able to select only those antibodies which recognize the peptide in the intact receptor. Antibodies reacting only with the isolated peptide were excluded. All peptides yielded antisera which were able to react with the intact receptor. Fusion and cloning of the hybridomas were performed as described with the same screening test as for the sera. The class and sub-class determination showed that the antibodies against the two peptides in the DNA binding

region (GR 465-471 and 472-495) were IgMs. The two other antibodies (GR 721-727 and 788-795) both were of the IgG_1 type.

Specificity

In order to test whether the antibodies indeed recognize the peptide inside the intact receptor, competition experiments were performed. Increasing amounts of carrier-coupled peptide were preincubated with the antibody. It can be seen that the binding of antibody antiGR 788-795 to the receptor is inhibited by increasing amounts of carrier-coupled peptide GR 788-795 in a dose dependent manner (Fig. 2). As a control the same antibody was preincubated with an irrelevant peptide; as expected, this had no effect on the binding. A comparable competition effect was observed with peptide GR 721-727 and antiGR 721-727, too (data not shown).

Western blot

It has often been observed that monoclonal antibodies are not able to recognize their epitope in Western blots. This is in contrast to polyclonal antisera which rarely fail to react. Therefore it was of interest to test the antibodies in this regard. Figure 3 shows Western blots with the two IgG monoclonal antibodies. Glucocorticoid receptor from crude cytosol and after purification (*ca.* 20–30% of total protein was receptor) was electrophoresed and blotted. It can be seen that both antibodies indeed recognize the receptor selectively in crude cytosol, where the prominent band at 94 kDa represents the intact receptor. Additional bands with lower molecular weights most probably represent degradation products which are present in cytosol [Fig. 3(a)] or are generated during the purification procedure [Fig. 3(d)]. The affinity of the antiGR 788-795 is higher because a dilution of 1:3000 of ascitic fluid is sufficient whereas the antiGR 721-727 had to be used at a dilution of 1:300 after a purification with Protein G–Sepharose.

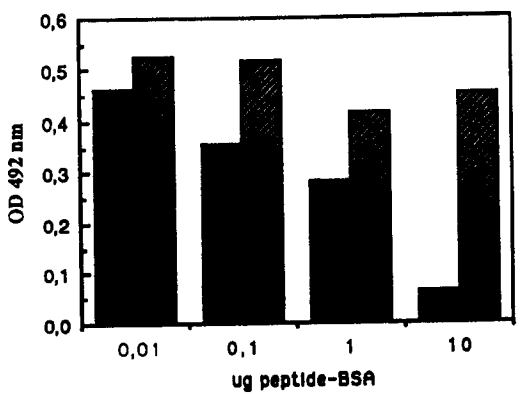

Fig. 2. Competition test. The indicated amounts of peptide coupled to BSA were preincubated with antiGR 788-795 and subsequently reacted with 50 ng purified receptor adsorbed to the ELISA plate. The amounts refer to the coupled peptide. Filled bar: peptide GR 788-795; and, hatched bar: irrelevant peptide (part of second zinc finger).

Influence of antibodies on steroid binding

The two IgG antibodies recognize epitopes which lie in the steroid binding region. Does a preincubation with receptor inhibit a subsequent steroid binding? We found no indication of an inhibition under the conditions employed (data not shown).

Immunocytochemical reaction

The peptides used for immunization were chosen on the basis of two points of view: (1) they had to contain mainly hydrophilic amino acids and (2) they should be identical in the rat and human glucocorticoid receptor.

We have tested the IgG antibodies for staining neoplastic lymphocytes from CLL patients. Figure 4(a) shows a positive reaction of antiGR 788-795 with cells from a patient which had not been treated before with corticoids. A predominantly cytoplasmic reaction pattern is displayed. In Fig. 4(b) cells from a patient who had been treated with a combination of prednisolone and cyclophosphamide are shown. It can be seen that in this case the antiGR 788-795 stains predominantly the cytoplasm and only faintly the nucleus. The staining with antiGR 721-727 yielded essentially similar results.

The staining is strictly dependent on the presence of glucocorticoid receptor, because only a faint cytoplasmic staining is seen when the antibody is preincubated with purified glucocorticoid receptor [Fig. 4 (c and d)].

Immunocytology in the human lymphoblastic cell line IM 9 showed a predominantly cytoplasmic staining pattern before treatment with dexamethasone [Fig. 4 (e)]. After dexamethasone incubation for 2 h an intense nuclear and cytoplasmic staining pattern can be seen [Fig. 4(f)].

DISCUSSION

The presented results demonstrate that it is possible to produce monoclonal antibodies to synthetic peptides. Two of the four antibodies were IgMs. There is no obvious reason for this

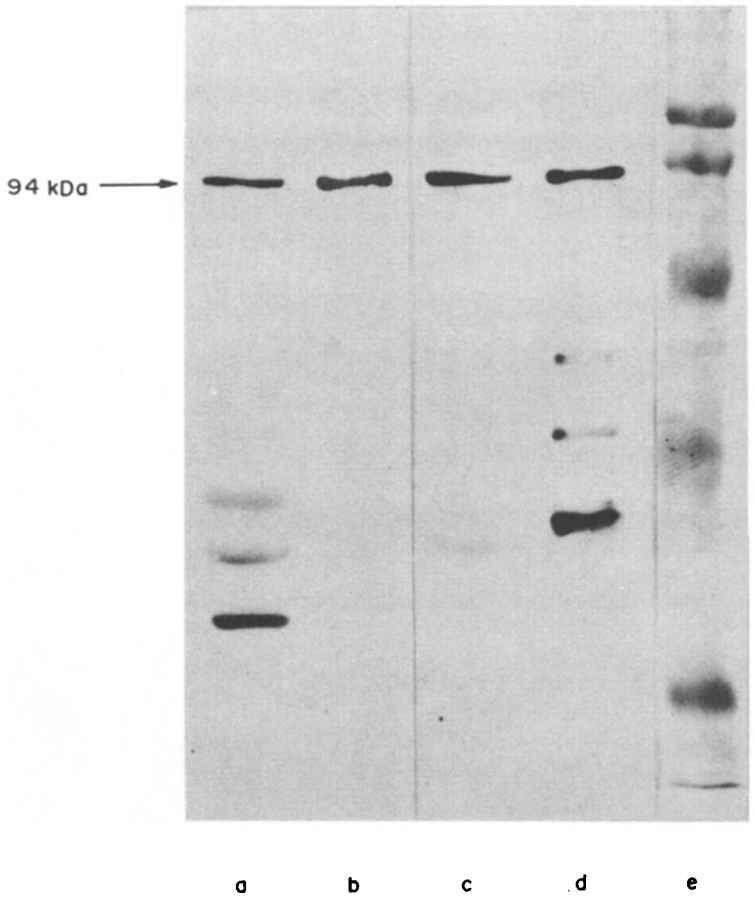

Fig. 3. Western blot of the glucocorticoid receptor. Crude cytosolic receptor: 8.5 ng receptor according to steroid binding test; total protein was 119 μg. Purified receptor: 170 ng according to steroid binding). Lanes (a) cytosolic receptor detected by antiGR 721-727; (b) purified receptor detected by antiGR 721-727; (c) cytosolic receptor detected by antiGR 788-795; (d) purified receptor detected by antiGR 788-795; and (e) marker proteins (molecular weights from top to bottom: 116, 97.4, 66, 45 and 29 kDa).

result. It may be that unknown features of the peptides favor IgM production although the immunization scheme was the same in all cases and should have led to IgGs. It is worth emphasizing, that one of the peptides leading to IgMs was applied without carrier protein because we expected it to be long enough (24 amino acids) to induce IgGs.

Most important, however, all four antibodies are capable of recognizing the peptide in the context of the folded intact protein. It is known that monoclonal antibodies often fail to react with their antigen after Western blotting. Interestingly, the two IgGs analyzed in more detail, bind to the receptor after SDS–PAGE and blotting. Unexpectedly, we were unable to observe a complex of the antibodies with the steroid-labeled receptor free in sucrose gradients, regardless of whether the receptor was in the unactivated (8S) or in the activated (4S) form. It is possible that some unfolding or distortion of the polypeptide chain as it occurs during Western blotting is even necessary to allow access of these two antibodies to their epitopes. The finding that the antibodies work in the ELISA is consistent with this assumption since the adsorption of the receptor protein to polystyrol plates probably leads to some distortions of the protein, as well. In addition, other authors also found that even some polyclonal antibodies against peptides failed to form complexes with the steroid receptor complex in solution [2].

We found that binding the antiGR 788-795 to the receptor did not inhibit a subsequent steroid binding regardless of whether the receptor was in the nonactivated or in the activated DNA-binding form. Therefore, the interaction of heat-shock protein 90 with the receptor does not explain this result. It could mean, however, that the last C-terminal amino acids are not necessary for steroid binding. In accordance with this

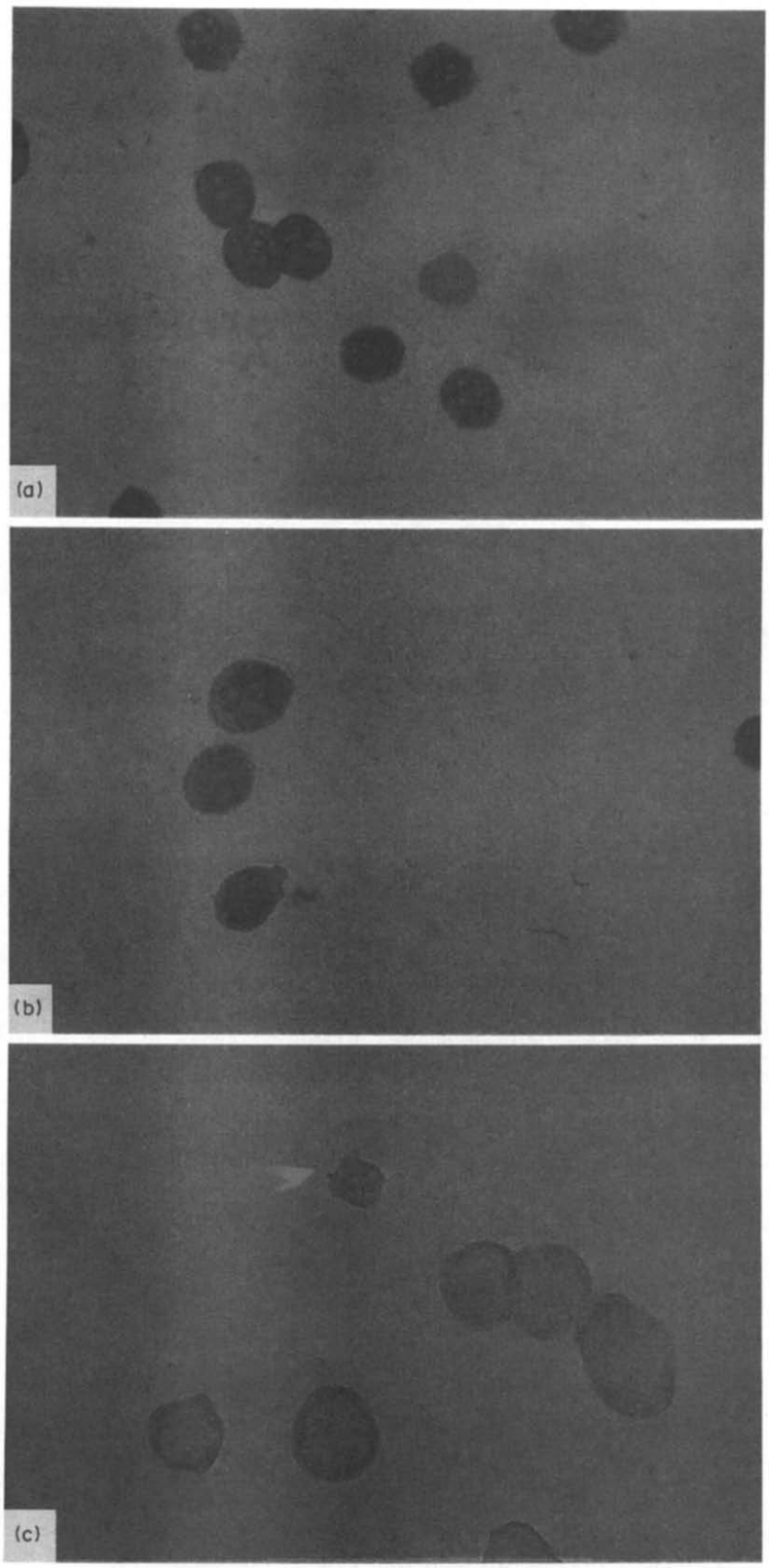

Fig. 4(a–c)—*legend opposite.*

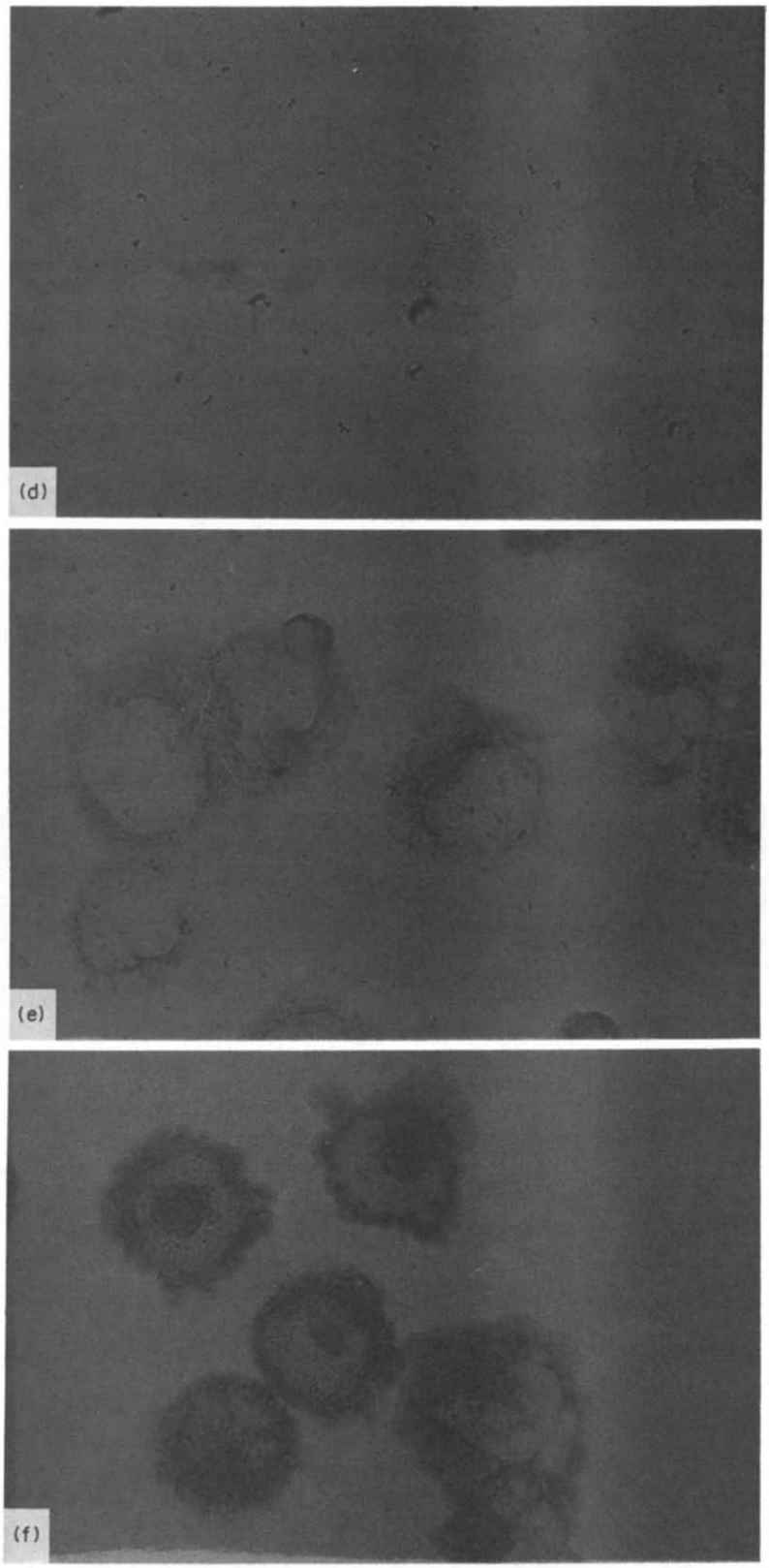

Fig. 4(d–f)

Fig. 4. Staining the glucocorticoid receptor in human and mouse lymphoma cells with the antiGR 788-795 at a dilution of 1:20 with the APAAP technique. Magnification × 120. Counterstaining with hematoxylin was performed only in (a) and (b). (a) CLL. No therapy. (b) CLL. Combination chemotherapy with prednisolone (200 mg i.v.) and cyclophosphamide (750 mg i.v.). (c) Mouse lymphoma cell line S 49. (d) Mouse lymphoma cell line S 49. Preincubation with 100 ng purified glucocorticoid receptor. (e) Human lymphoblastic cell line IM 9. (f) Human lymphoblastic cell line IM 9. Preincubation with 10^{-5} M dexamethasone for 2 h.

interpretation it has been reported that receptor mutants lacking as many as 14 C-terminal amino acids could be labeled specifically [7]. Alternatively, the inability of the antibody to inhibit a steroid binding results simply from the inaccessibility of the epitope when the receptor is in solution. This interpretation seems more probable (see above).

The immunocytochemical tests show that it is possible to prepare antipeptide monoclonal antibodies to investigate the receptor in lymphoma cells. It is known that simple steroid binding tests are necessary but not sufficient to define a fully functional receptor. Thus, it has been demonstrated that in glucocorticoid-resistant variants of the nt^- type ("nuclear transfer decreased") point mutations are responsible for the nonfunctional receptor conferring the steroid resistance while steroid binding is normal [8]. The nt^i type ("nuclear transfer increased") also shows normal steroid binding, but this receptor variant is nonfunctional because the N-terminal transactivation modulating half of the protein is missing [9]. The reason for this is an aberrant splicing of the primary transcript [10]. The receptor variants which are nonfunctional because of point mutations or deletions could be detected if antibodies against the respective sequences are available. Our results show that it is generally possible to prepare such antibodies.

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