Journal of Cellular Biochemistry 49:157–165 (1992)

A New Monoclonal Antibody for Detection of EGF-Receptors in Western Blots and Paraffin-Embedded Tissue Sections

A. Fernandez, E. Spitzer, R. Perez, F.-D. Boehmer, K. Eckert, W. Zschiesche, and R. Grosse

Institute of Oncology and Radiobiology, Havana, Cuba (A.F., R.P.); Max-Delbrick-Centre of Molecular Medicine, Berlin-Buch, Germany (E.S., F.-D.B., K.E., W.Z., R.G.)

Abstract The prognostic significance of the epidermal growth factor receptor status (EGF-R-status) for certain human tumors requires the development of antibodies useful for clinical application. We used purified receptor preparations to generate monoclonal antibodies immunoreactive with the EGF-R purified from placenta membranes and A431 tumors. Four of the hybridomas contained antibodies (R2, R3, R5, and R9) which recognized both antigens. Antibody R3 was shown to display the following properties: it binds with a K_D value of about 10^{-9}–10^{-10} M to the receptor, a half maximal inhibition of EGF-binding is achieved at 5×10^{-8} M, and in Western blots of cell membranes R3 specifically detects the EGF-R at 0.1 μg/ml. R3 inhibits EGF-dependent clonogenic growth of NRK cells and completely blocks EGF stimulated autophosphorylation of the receptor. Moreover, R3 also detects EGF-R in paraffin-embedded tissue sections taken from human salivary gland, term placenta, and adult skin and mammary carcinomas. Thus, R3 can be used in retrospective diagnostic clinical studies and might help to develop new immunotherapeutic intervention.

Key words: epidermal growth factor receptor, breast cancer, immunohistochemistry, cell proliferation, receptor phosphorylation

EGF-R expression has been found in a variety of human malignancies [1–5]. Data reported by different groups about the expression of EGF-R in breast cancer indicate a relationship between EGF-R expression and bad prognosis [6–8]. Moreover, we showed that the rate of mitosis of breast cancer cells directly correlates with the number of EGF binding sites in sections of primary breast cancer biopsies [9,10]. It was shown that the EGF-R status correlates with typical clinical risk factors such as lymph-node incidence, estrogen-receptor status, and histological grading or size of primary tumor [6,7,10,11]. For patients with estrogen receptor positive primary breast tumors, the EGF-R status may have special prognostic significance as an independent risk factor indicating worse prognosis.

However, other authors [12–14] could not confirm these conclusions. The discrepancies may in part arise from methodological problems and from applying different criteria for evaluation of the data. For example, the EGF-R status was estimated by different methods including measurement of ^{125}I-EGF binding to cryostat sections [9] or to a microsomal fraction obtained from the primary tumor [6–8], and by immunocytochemical analysis of cryosections with a monoclonal anti-EGF-R antibody [12,13]. In addition, due to the limitations of the methods applied so far, in some of the prospective studies only a relatively small number of patients has been analysed. It is therefore desirable to define the role of EGF-R status by investigating larger patient groups. Here we introduce a new monoclonal antibody directed against the EGF-R. This antibody detects the receptor in tissue sections after formaldehyde fixation and paraffin embedding, and in Western blots after SDS-PAGE. It

Abbreviations used: EGF-R, epidermal growth factor receptor; OPD, o-phenylene diamine; 1Br2N, 1-Brom-2-naphthol; DAB, diaminobenzidine; POD, horseradish peroxidase; WGA agarose, wheat germ agglutinin agarose; HEPES, 4-(2-Hydroxyethyl)-1-piperazineethane-sulfonic acid; PBS, phosphate buffered saline; BSA, bovine serum albumin.

Received September 9, 1991; accepted January 13, 1992.

Address reprint requests to Prof. Dr. R. Grosse, Department of Cellular Biochemistry, Max-Delbrick-Centre of Molecular Medicine, Robert-Rössle-Straße 10, Berlin, 0-1115, Germany.

competes for EGF binding to the receptor and inhibits EGF-dependent autophosphorylation of the receptor and EGF-dependent growth of NRK cells in soft agar.

MATERIALS AND METHODS

Materials

Murine EGF was isolated according to Savage and Cohen [16] and radioiodinated by the chloramine T method [17]. Wheat germ agglutinin (WGA) agarose, protein A-sepharose, and activated sepharose 4B were obtained from Pharmacia (Sweden). EGF-sepharose was prepared following the protocol recommended by Pharmacia. Gamma-^{32}P-ATP, antimouse-IgG-conjugated horseradish peroxidase (POD), biotinylated mouse-IgG and the streptavidin-biotinylated POD complex were from Amersham (U.K.). The substrates o-phenylene-diamine (OPD) and 1-Br-2-naphthol (1Br2N) were from Sigma (St. Louis, MO) and diaminobenzidine (DAB) from Abbott (Chicago, IL). The anti-human interferon antibody alpha1mab D11 was kindly provided by Dr. F. Schneider, Department of Immunology, Central Institute of Molecular Biology. All other reagents were of analytical grade.

Cells

A431 cells were continuously grown in DMEM-F12 supplemented with 5% calf serum as described [18]. For development of solid carcinomas 3 × 10^6 A431 cells per athymic mouse (nu/nu) were inoculated subcutaneously. Approximately one month after inoculation the A431 tumors werde excised and used for EGF-R purification.

The Sp 2/O AG14 myeloma cells were maintained in RPMI-1640 supplemented with 10% calf serum, 26 mM sodium bicarbonate, 18 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate and antibiotics [19].

Monoclonal Antibody Production

Purification of an EGF-R fraction from solubilized human placenta was performed according to Cohen et al. [20] using EGF-affinity chromatography with slight modification: Briefly, a crude placenta membrane fraction was solubilized in 20 mM Tris/HCl, pH 7.4, containing 10% glycerol/1% Triton X-100. After centrifugation at 100,000g for 1 h, the supernatant was applied to EGF-sepharose. The EGF-R fraction was eluted with 5 mM ethanolamine, pH 9.7, containing 10% glycerol/0.1% Triton X-100. This EGF-R fraction was used for immunization.

Enzyme-Linked Immunosorbent Assay (ELISA)

Immunoreactivities of hybridoma supernatants were tested with two ELISAs by coating the microtiter plates with EGF-R preparations purified either from placenta membranes or from A431 tumors. Initial screens of fusions were performed with the EGF-R fraction purified from human placenta. Positive clones were selected and then tested for reaction with the EGF-R preparation purified from solid A431 tumors as described above [20]. Immobilization of the EGF-R on polysterol plates was performed following the procedure of Yarden et al. [21]. Bound immunoglobulins were detected using a commercial anti-mouse POD conjugate [22].

Blocking of EGF Binding by Anti-EGF-R MAbs

To determine if monoclonal antibodies against the EGF-R (anti-EGF-R MAbs) purified by protein A-chromatography blocked EGF binding to its receptor, a radioreceptor assay was used. Displacement of ^{125}I-EGF by antibodies was monitored using EGF-R receptor rich crude membranes from human placenta as described earlier [11].

Dot Blot Analysis

Immunoreactivity of anti-EGF-R MAbs was tested with purified receptor protein immobilized on nitrocellulose. To this end, EGF-R protein was purified from cultured A431 cells by affinity chromatography on WGA agarose according to the procedure of Akiyama et al. [23]. Aliquots of the eluted fraction containing the EGF-R were then dotted directly onto prewashed nitrocellulose. The blots were blocked with 3% BSA in PBS/0.2% Tween 20 and incubated with different concentrations of anti-EGF-R MAbs in PBS/1% BSA/0.1% Tween 20 at 4°C for 20 h. Blots were developed by incubation with biotinylated anti-mouse IgG followed by the preformed streptavidin-biotinylated-POD-complex and visualized with 1Br2N in PBS (0.23 mg/ml) containing 0.01% H_2O_2.

Autophosphorylation and Western-Blot Analysis

A membrane fraction was prepared from A431 cells according to Akiyama et al. [23]. EGF-dependent autophosphorylation of the EGF-R was performed as outlined by Davies and Czech

[24]. Analysis for ^{32}P-labeled proteins was performed for membranes and lysates after SDS-PAGE in 7.5% gels and subsequent autoradiography. A corresponding part of the same gel was transferred to nitrocellulose and either processed for autoradiography or Western-blot analysis as described [25]. A transfer efficiency of 90% was calculated by counting ^{32}P-labeled protein bands in gel strips before and after electroblotting and analysed by Western blotting as outlined above. Cell lysates of A431 cells were obtained as described [26] and were analysed in parallel to the membranes.

Immunohistochemistry

Human placenta, sublingual salivary gland, skin, lymph node, and 13 primary mammary carcinomas of the invasive ductal type were used for immunohistochemical analysis. Tissue samples fixed in 4% formalin and embedded in paraffin were employed. Five micrometer sections were dried on defatted glas slides at 37°C and processed in xylene and ethanol. After washing in PBS for 30 sec, the sections were incubated for 5 min in 3% H_2O_2 at room temperature to block endogenous peroxidase activity. They were then washed again in PBS, and incubated for 20 min in 1% BSA/PBS to prevent nonspecific binding. Subsequently, the sections were treated with 20 μg/ml of the anti-EGF-R MAb R3 in presence of 1% BSA. The slides were washed three times in PBS, treated with biotinylated anti-mouse IgG (1:200) and, subsequently, with the streptavidin-biotinylated POD complex (1:200) according to the manufacturer's instructions. DAB was used as the substrate. Controls comprised either omission of the specific primary antibody or incubation with an unrelated primary antibody. Sections were counterstained with hematoxylin.

Inhibition of EGF Dependent Clonogenic Growth of NRK Cells

EGF-dependent clonogenic activity was estimated with NRK cells, clone 49F, as described before [27]. For testing anti-EGF-R MAb R3, 2×10^4 cells supplemented with fetal calf serum were preincubated with different amounts of antibodies for 3 h at 37°C in a humidified atmosphere at 5% CO_2. The mixture was then added directly to the clonogenic assay containing 1 ng/ml of human EGF. All values are corrected for the background values (about 0–20 cell aggregates per plate) using an appropriate buffer control or the unrelated monoclonal antibody D11 directed against human interferon.

Inhibition of Autophosphorylation of the EGF-R

Various amounts of antibody, dissolved in 15 μl of 50 mM Tris-HCl, pH 7.6, were incubated with 20 μl of the placenta membrane suspension (40 μg protein in a solution containing 71 mM HEPES, pH 7.5, 214 mM sodium chloride, 29 mM magnesium chloride, 4.3 mM manganese chloride, 143 μm sodium ortho-vanadate) for 30 min on ice. Then, 10 μl EGF were added (final concentration 100 mM) and incubation continued for another 20 min. For phosphorylation, gamma-^{32}P-ATP was added (5 μl, about 5 μCi, final concentration 15 μM) and after 2 min the reaction was terminated by addition of 10 μl of 6-fold concentrated electrophoresis sample buffer. Analysis was performed by SDS-PAGE, autoradiography, and liquid scintillation counting of the sections corresponding to the EGF-R band.

RESULTS

Characterization of Monoclonal Antibodies by ELISA

Four of the hybridomas contained anti-EGF-R MAbs subsequently designated as R2, R3, R5, and R9, reacting with both the partially purified EGF receptor from A431 cells and human placenta. All four monoclonal antibodies were of the IgG2a Kappa isotype. In all experiments described below, before using, the anti-EGF-R MAbs were purified by protein-A-sepharose-chromatography. The solid phase ELISA was used to estimate affinity constants of the selected antibodies R2, R3, R5, and R9 (Fig. 1). The apparent dissociation constant (Kd) was defined as the molar concentration of antibodies giving half maximal saturation in the ELISA. The Kd-value was estimated to 10^{-9}–10^{-10} M for R3 and did not differ if membranes from placenta or A431 tumors were used (Fig. 1). The Kd-values for antibodies R2, R5, and R9 were in the same range (not shown).

Characterization of Antibodies in a Radioreceptor Assay

The anti-EGF-R MAbs were tested for their ability to block ^{125}I-EGF binding to its receptor in human placenta membranes (Fig. 2). Half-maximal inhibition of EGF binding was achieved with 5×10^{-8} M R3. For control, murine EGF blocked ^{125}I-EGF binding by 50% at 10^{-8} M.

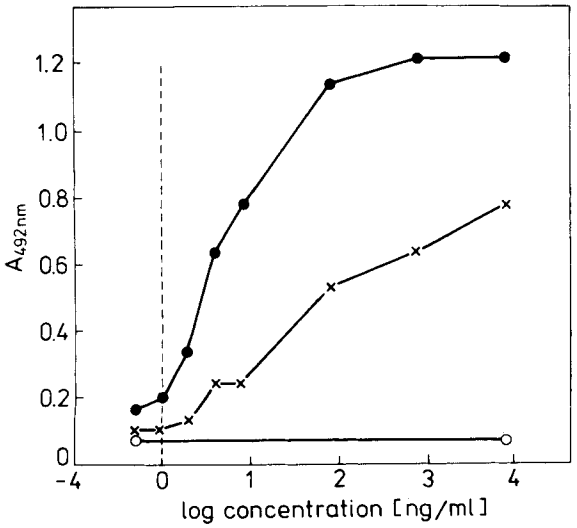

Fig. 1. Relative affinities of anti-EGF-R MAb R3 to membrane fractions from placenta (x) or A431 tumors (●). The solid phase ELISA was performed as outlined in Materials and Methods. Antibody concentrations 0.5–7,500 ng/ml.

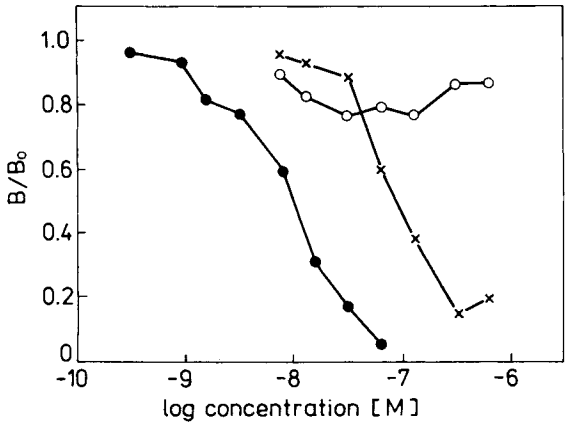

Fig. 2. Inhibition of ^{125}I-EGF binding to human placenta membranes by anti-EGF-R MAb R3. Microsomal fractions (200 μg/ml) were incubated for 60 min at 23°C with 0.2 ng ^{125}I-EGF per ml and with 1–100 μg/ml of anti-EGF-R MAb R3 (x). For control, unlabeled EGF (0–200 ng/ml) was used as competitor (●). A non-related anti-cytokeratin MAb did not block ^{125}I-EGF binding (○).

Immunodetection of Immobilized EGF-R

The immunoreactivity of antibodies to EGF-R immobilized on nitrocellulose was estimated (Fig. 3). At a concentration of 0.1 μg/ml the anti-EGF-R MAb R3 detected 9 ng of the EGF-R purified from A431 cells.

We next addressed the question of whether or not R3 would also recognize the denatured form of the EGF-R in Western blots after SDS-PAGE. To this end, A431 cell lysates and purified plasma

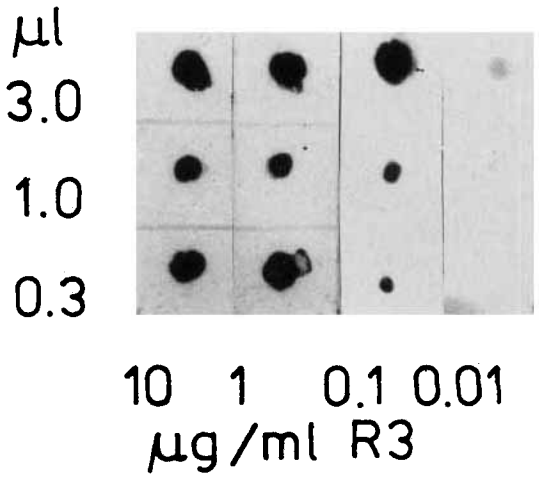

Fig. 3. Dot-blot analysis of reactivity of anti-EGF-R MAb R3 to the EGF-R purified from A431 cells. Aliquots (3.0, 1.0, 0.1 μl) of the WGA agarose-purified EGF-R (30 ng/μl) were dotted onto nitrocellulose and incubated with the indicated concentrations of R3.

membranes were treated with the antibody R3 (Fig. 4). To identify the EGF-R, A431 cell membranes were phosphorylated in presence of EGF, then undergone SDS-PAGE and analysed by autoradiography (Fig. 4a). The other part of the same gel was electroblotted on nitrocellulose and then incubated with different concentrations of R3 for immunostaining the EGF-R in the membrane fraction obtained from A431 cells (Fig. 4, lanes b–d) or in A431 cell lysates (Fig. 4, lanes e–g).

As shown, 0.1 μg/ml of R3 detects one band which co-migrates with the phosphorylated receptor protein of 170 kDa (lane a). The specificity of immunostaining is further documented by the amidoblack staining of the nitrocellulose strip containing cell lysate proteins (lane h).

With the same Western-blot technique we were able to detect the EGF receptor also in human placenta membranes (not shown). In total cell lysates obtained from MCF-7 cells, which express only a low number of EGF-R [9] the EGF receptor band could not be visualized (not shown).

Inhibition of EGF-Dependent Clonogenic Growth and EGF-Stimulated Autophosphorylation

In order to test whether or not blocking of EGF binding by anti-EGF-R MAb R3 results in inhibition of cell proliferation, increasing amounts of R3 were added to NRK cells in presence of EGF (Table I). As shown, there was

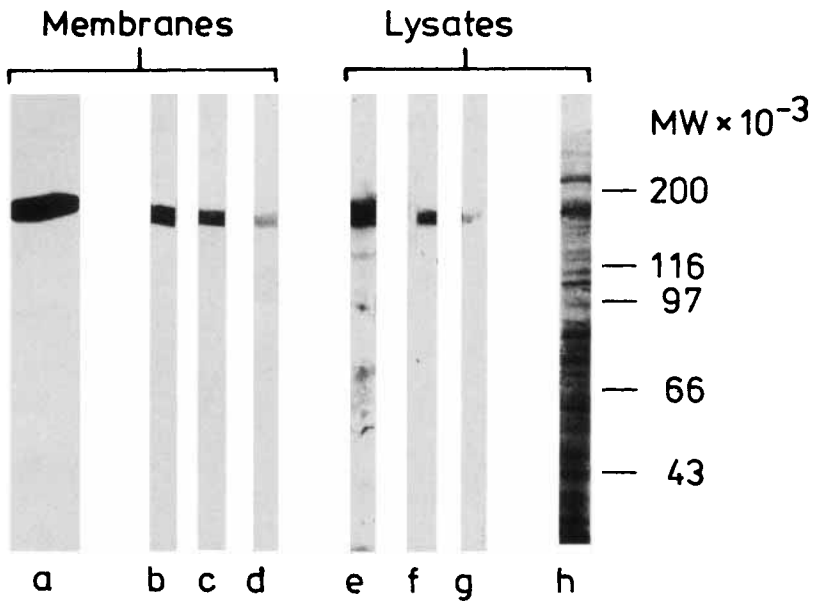

Fig. 4. Detection of EGF-R by Western blot analysis with anti-EGF-R MAb R3. A431 cell membranes (lanes **b–d,** 45 μg of protein per lane) or A431 total cell lysates (lanes **e–g,** 140 μg of protein per lane) were analysed with different concentrations of MAb R3: b, e 10 μg/ml; c, f 1 μg/ml; d, g 0.1 μg/ml. To identify the EGF-R, A431 cell membranes were phosphorylated in presence of EGF as outlined under Materials and Methods. The phosphorylated EGF-R was visualized by autoradiography (**a,** 40 μg of protein per lane). For control, cell lysate proteins were stained with amidoblack (**h**).

TABLE I. Inhibition of EGF-Dependent Clonogenic Growth of NRK-Cells by Anti-EGF-R MAb R3

Concentration of antibody (μg/ml)	% Inhibition[a]
0.04	7
0.4	10
4.0	38
10.0	65

[a]In the presence of 1 ng/ml EGF, 2,500 colonies per plate were estimated and set as 100%. Inhibition is expressed as the difference between this value and that obtained in presence of the antibody.

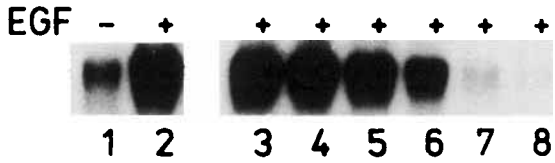

Fig. 5. Inhibition of EGF-stimulated autophosphorylation of the EGF-R in placenta membranes by anti-EGF-R MAb R3. Membranes were incubated with gamma ^{32}P-ATP in absence (**lane 1**) or presence of EGF (**lanes 2–8**) and analysed by SDS-PAGE and autoradiography. The effect of increasing concentrations of MAb R3 on ^{32}P-incorporation into the 170 kDa-EGF-R band is shown: lanes 2, 3, without R3; lane 4, 1.8 μg/ml; lane 5, 6 μg/ml; lane 6, 18 μg/ml; lane 7, 60 μg/ml; lane 8, 180 μg/ml R3.

a dose dependent inhibition of EGF-dependent clonogenic growth reaching a value of 65% at 10 μg/ml of R3.

We next addressed the question of whether or not EGF-R MAb R3 would also inhibit EGF-dependent receptor autophosphorylation which is considered to be the crucial step in signal transduction leading to cell proliferation. As shown in Figure 5 and quantified in Table II, increasing amounts of the anti-EGF-R MAb R3 prevented EGF-stimulated autophosphorylation of the EGF receptor in placenta membranes.

Immunohistochemistry

Human sublingual salivary gland, term placenta, adult skin, lymph node, and primary mammary carcinomas were subjected to immunohistochemical analysis with the anti-EGF-R MAb R3 using paraffin embedded tissue sections.

In the salivary gland, positive staining was found in the granular convoluted tubules, being located linearly at the luminal surface and in granular form in the perinuclear region of the epithelial cells. Occasionally, groups of acinar cells displayed granular intracellular staining (Fig. 6A). In the skin, immunoreactivity was

TABLE II. Inhibition of EGF-Stimulated Autophosphorylation in Placenta Membranes

Concentration of antibody (μg/ml)	% Inhibition[a]
1.8	17
6	25
18	91
60	100

[a]Membranes were incubated with EGF and gamma-^{32}P-ATP and analysed by SDS-PAGE, autoradiography and counting of radioactivity incorporated into the 170 kDa EGF-receptor protein as described in Materials and Methods and shown in Figure 5. Inhibition is expressed as the difference between the EGF-dependent incorporation of radioactivity set as 100% and that obtained in the presence of the antibody.

most intense in keratinocytes of the basal and spinous layers (Fig. 6B). Langerhans' cells were not stained. Additionally, hair root sheat epithelia and basal sebocytes were stained (Fig. 6C). Likewise, smooth muscle cells of the arrector pilorum were immunostained with R3. An abundant reaction was regularly found at the outer surface of syncytiotrophoblastic cells of term placenta (Fig. 6D). Only slight and diffuse staining could be detected in the cytoplasm. Lymph node regularly failed to react with the MAb R3 (not shown). In all organs tested, connective tissue cells were negative, except for some occasional staining of vascular smooth muscle cells.

The immunohistochemical staining of two mammary carcinomas of the invasive ductal type is shown in Figure 6 (E,F). Both tumors were clearly stained with R3, though of different intensity. In general, the reactivity was located both at the surface and within the cytoplasm. Occasionally, nuclei were found positive. For 13 primary mammary carcinomas the intensity of immunostaining with MAb R3 was compared with the EGF-R status determined earlier [10] in frozen sections of the same tumors by use of the ^{125}I-EGF-binding assay (Table III). As outlined, a rough correlation between the results of both assays which had to be performed using different pieces of carcinoma tissue, is shown.

DISCUSSION

Overexpression of the EGF-R in primary breast, cervical, ovarian, head and neck, gastric and bladder cancer, comprising squamous and adenocarcinomas has been discussed in regard to molecular mechanisms responsible for growth and metastatic potential of human tumors [1–6]. By use of ligand binding assays, we [9,10] and other groups [6,28] described a statistically significant correlation between EGF-R status and clinically important risk factors for breast cancer such as lymph-node status, tumor size, or grading.

However, immunohistochemical evaluation of the EGF-R status which could be in practice the method of choice has not given unequivocal results. Sainsbury et al. [15], by comparing the immunohistochemical staining of cryosections with the monoclonal anti-EGF-R antibody R1 with the ligand binding data, estimated a similar receptor status, although the latter assay was more sensitive. Contrary to these studies, Bevilacqua et al. [13], as well as Wrba et al. [12], were not able to confirm a relationship between EGF-R status and clinically relevant prognostic markers using the anti-EGF-R antibody R1.

This antibody and other monoclonal antibodies produced with A431 cells as immunogen [29–31] have not been reported to recognize the EGF-receptor in paraffin-embedded tissue sections. By using MAb R3 we analysed paraffin-embedded sections from human adult skin, salivary gland, term placenta, and lymph node. The EGF-R distribution and tissue localization we demonstrated qualitatively corresponds with those of radioactive ligand binding results reported in the literature [10,32–35]. Here receptor expression of 120–150, 150, and less than 1.0 fmol/mg of protein were found in placenta syncytiotrophoblast, skin epidermis, and lymph nodes, respectively. These data even quantitatively fit in with those found immunohistochemically in our studies. The specificity and selectivity of MAb R3 staining in paraffin sections is also supported by our finding that tumors biochemically estimated as EGF-R positive were recognized by the antibody. With regard to the technical premises for the application of both methods (use of different samples of each tumor, arbitrary quantitative histochemical evaluation of the paraffin sections with respect to both intensity and distribution of reactivity), only a rough correlation between ^{125}I-EGF binding and immunohistochemical reactivity could be expected and was actually found as also reported by other authors [36]. The use of anti-EGF-R-MAb R3 thus allows inclusion of larger patient groups in retrospective clinical studies. We hope that this will help to further assess the

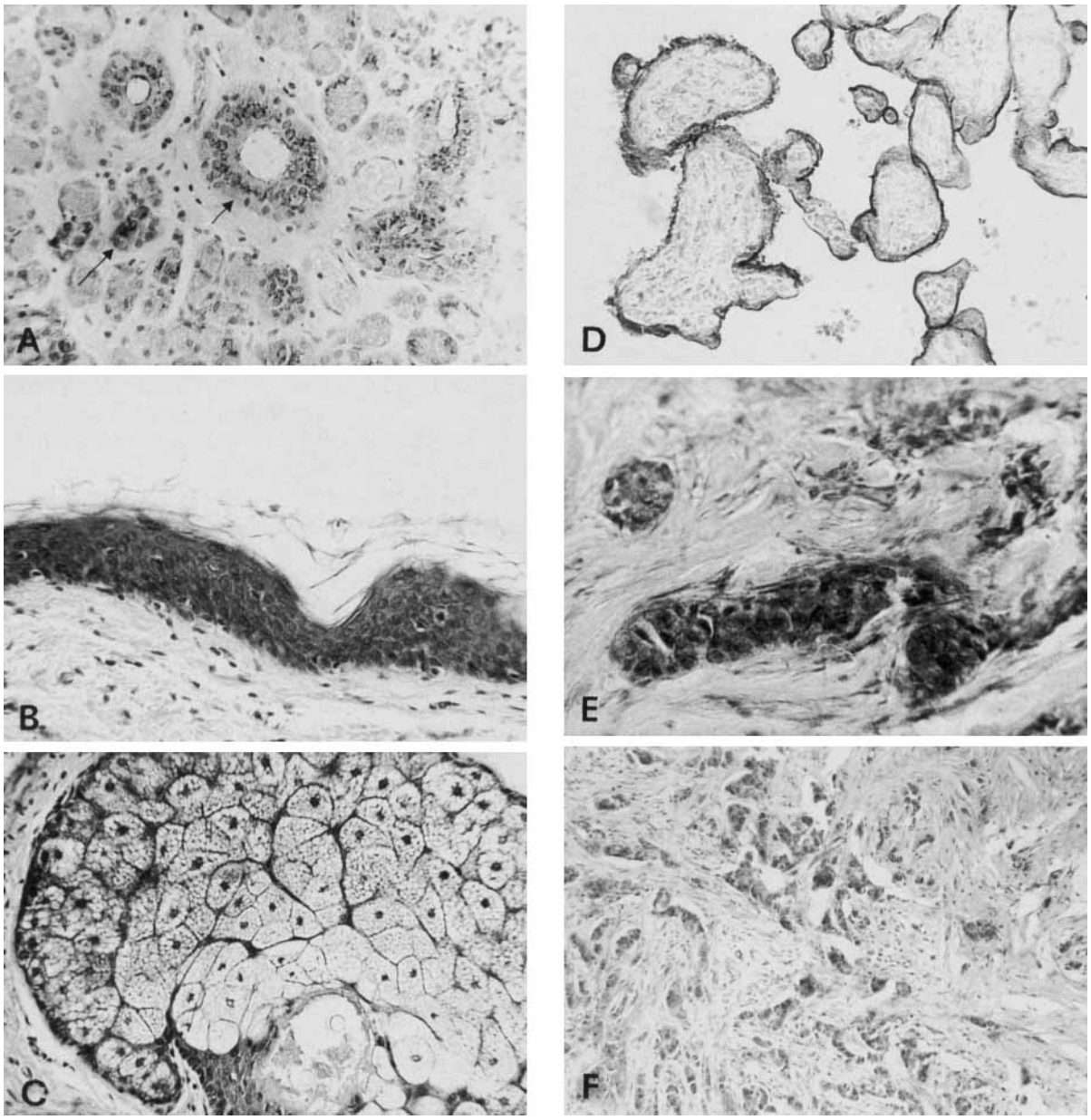

Fig. 6. Immunohistochemical analysis of paraffin embedded human tissue sections with anti-EGF-R MAb R3 (original magnification, 100×). **A:** Sublingual salivary gland. Epithelial cells of granular convoluted tubules (short arrow) and some acinar cells (long arrow) are immunostained. **B:** Adult skin. Keratinocytes of the basal and spinous layers are strongly stained. **C:** Sebaceous gland. Intensity of the immunohistochemical reaction in cells of the outer layer decreases towards the center of the gland. **D:** Placenta at term. Dense receptor staining of the superficial syncytiotrophoblastic cell layer. **E:** Lymphatic capillaries of mammary adipose tissue. Cells of an invasive ductal mammary carcinoma (tumor No. 6488 of Table III) show intense staining. **F:** Broad infiltration of connective mammary tissue by an invasive ductal mammary carcinoma (tumor No. 955 of Table III). Tumor cells are moderately stained.

prognostic value of the EGF receptor in human malignancies.

The heterogeneity of immunostaining we observed in paraffin-embedded sections is similar to the staining pattern described for sections incubated with the R1 antibody. It is widely known that breast cancer is comprised of various cell populations with different metastatic potential. Our preliminary immunohistochemical data give rise to hope that the new anti-EGF-R antibody will contribute to a better characterization of cell clones with high metastatic

TABLE III. Comparison of EGF-R Status Estimated by ^{125}I-EGF Binding in Frozen Sections to Immunostaining With Anti-EGF-R-MAb R3 in Paraffin Sections

Tumor No.	Ligand binding[a] (fmol/mg protein)	Immunohistochemistry[b]
3329	22.9	+ diffuse
6488	21.1	++ diffuse
6380	18.4	+ diffuse
2828	14.8	++ local
3173	11.7	+ diffuse
2106	11.5	+ diffuse
1156	11.1	+ local
1756	1.9	+ diffuse
955	0.0	+ local
2604	0.0	−
3965	0.0	−
3509	0.0	−
4009	0.0	−

[a]Data on binding of ^{125}I-EGF taken from an earlier publication [10].
[b]Arbitrary grading: −, negative; +, moderate reaction; ++, strong reaction.

potential by including larger patient groups in the analysis.

The strategy we have chosen to generate monoclonal antibodies against the EGF-R was directed to select hybridomas immunoreactive to both the EGF-receptor from A431 cells and the "native" receptor partially purified from normal human placenta membranes, which was used as immunogen. This approach was successful in obtaining antibodies which recognize the EGF receptor in normal and transformed cells in its denatured form after Western blotting or formalin fixation and paraffin embedding. On the other hand, the anti-EGF-R MAb R3 completely blocked EGF binding, suggesting that a part of the unfolded denatured extracellular ligand binding site is still recognized by the antibody. In this way, the high-affinity-binding of R3 could lead to inhibition of EGF-dependent receptor autophosphorylation and clonogenic growth of NRK-cells in soft agar.

There have been several published reports about monoclonal and peptide directed polyclonal anti-EGF-R antibodies blocking EGF binding, receptor phosphorylation, or cell proliferation in vitro [31,37].

Fendly et al. [31] raised a panel of monoclonal antibodies inhibiting EGF binding and cell proliferation.

Polyclonal antisera were also produced to short synthetic peptides derived from the cytoplasmic domain of the EGF-R, which prevented receptor autophosphorylation [38]. More recently, these antibodies were also characterized with respect to immunohistochemical staining of fixed paraffin-embedded human tissues [36]. A future application of anti-EGF-R antibodies in immunotherapy employing their ligand binding characteristics seems to be possible. Also, use of cytotoxic antibody conjugates directed to cells overexpressing the EGF-R can be considered [39]. In order to approach a therapeutic application, more should be known about the EGF-R expression in human malignancies. The new antibody R3 allows us to conduct new retrospective and prospective clinical studies. A first retrospective study with a large group of patients is under way in our laboratory to further assess the value of the EGF-R as prognostic marker and potential therapeutic target.

ACKNOWLEDGMENTS

We are grateful to Dr. A. Frege (Central Institute for Cancer Research) for providing us with paraffin-embedded breast tumors, to S. Nowack and I. Suemnich for technical assistance, and to E. Hellmuth for excellent preparation of the manuscript.

REFERENCES

1. Gullick WJ: Prevalence of aberrant expression of the epidermal growth factor receptor in human cancers. Br Med Bull 47:87–98, 1991.
2. Gullick WJ, Marsden JJ, Whittle N, Ward B, Bobrow L, Waterfield MD: Expression of epidermal growth factor receptors on human cervical, ovarian, and vulval carcinomas. Cancer Res 46:285–292, 1986.
3. Ishitoya J, Toriyama M, Oguchi N, Kitamura K, Ohshima M, Asano K, Yamamoto T: Gene amplification and overexpression of EGF receptor in squamous cell carcinomas of the head and neck. Br J Cancer 59:559–562, 1989.
4. Bennett C, Paterson M, Corbishley CM, Luqmani YA: Expression of growth factor and epidermal growth factor receptor encoded transcripts in human gastric tissues. Cancer Res 49:2104–2111, 1989.
5. Neal DE, Sharples L, Smith K: The epidermal growth factor receptor and the prognosis of bladder cancer. Cancer 65:1619–1625, 1990.
6. Sainsbury JRC, Needham GK, Farndon JR, Malcolm AJ, Harris AL: Epidermal growth factor receptor status as a predictor of early recurrence and death from breast cancer. Lancet 1:1398–1402, 1987.
7. Rios MA, Perez R, Lage A, Skoog L: Receptors for epidermal growth factor and estrogen as predictors of relapse in patients with mammary carcinomas. Anticancer Res 8:173–176, 1988.
8. Costa S, Stamm H, Almendral A, Ludwig H, Wyss R, Fabbro D, Ernst A, Takahashi A, Eppenberger U: Predictive value of EGF receptor in breast cancer. Lancet 2:1258, 1988.

9. Spitzer E, Grosse R, Kunde D, Schmidt HE: Growth of mammary epithelial cells in breast cancer biopsies correlates with EGF binding. Int J Cancer 39:279–282, 1987.
10. Spitzer E, Köpke K, Kunde D, Grosse R: EGF binding is quantitatively related to growth in node-positive breast cancer. Breast Cancer Res Treat 12:45–49, 1988.
11. Perez R, Pascual M, Macias A, Lage A: Epidermal growth factor receptors in human breast cancer. Breast Cancer Res Treat 4:189–193, 1984.
12. Wrba F, Reiner A, Ritzinger E, Holzner JH: Expression of epidermal growth factor receptor (EGFR) on breast carcinomas in relation to growth fractions, estrogen receptor status, and morphological criteria. Path Res Pract 183:25–29, 1988.
13. Bevilaqua P, Gasparini G, Fior SD, Corradi G: Immunocytochemical determination of epidermal growth factor receptor with monoclonal EGFR1 antibody in primary breast cancer patients. Oncology 47:313–317, 1990.
14. Hawkins RA, Killen E, Whittle JR, Lack WJL, Chetty U, Prescott RJ: Epidermal growth factor receptors in intracranial and breast tumours: Their clinical significance. Br J Cancer 63:553–560, 1991.
15. Sainsbury JRC, Malcolm AJ, Appleton DR, Farndon JR, Harris AL: Presence of epidermal growth factor receptor as an indicator of poor prognosis in patients with breast cancer. J Clin Pathol 38:1225–1228, 1985.
16. Savage Jr RC, Cohen S: Epidermal growth factor and a new derivative. Rapid isolation procedures and biological and chemical characterization. J Biol Chem 247: 7609–7611, 1972.
17. Hunter WM, Greenwood FC: Preparation of iodine-131 labeled human growth hormone of high specific activity. Nature 184:495–496, 1962.
18. Barnes DW: Epidermal growth factor inhibits growth of A431 epidermoid carcinoma in serum-free cell culture. J Cell Biol 93:1–4, 1982.
19. Shulman M, Wilde CD, Kohler G: A better cell line for making hybridomas secreting specific antibodies. Nature 276:269–271, 1978.
20. Cohen S, Fava RA, Sawyer ST: Purification and characterization of epidermal growth factor receptor/protein kinase from normal mouse liver. Proc Natl Acad Sci USA 79:6237–6241, 1982.
21. Yarden Y, Harari I, Schlessinger J: Purification of an active EGF receptor kinase with monoclonal antireceptor antibodies. J Biol Chem 260:315–318, 1985.
22. Nakamura RM, Voller A, Kidwell DE: In "Immunochemistry," Weir DM (ed): Vol. I, Oxford: Blackwell Scientific Publ., 27.1–27.20, 1986.
23. Akiyama T, Kadooka T, Ogawara H: Purification of the EGF-receptor by tyrosine-sepharose-affinity chromatography. Biochem Biophys Res Commun 131:442–448, 1985.
24. Davies RJ, Czech MP: Amiloride directly inhibits growth factor receptor tyrosine kinase activity. J Biol Chem 260:2543–2551, 1985.
25. Burnette WM: Western blotting: Electrophoretic transfer of proteins from sodium dodecyl sulfate—Polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and Protein A. Anal Biochem 112:195–203, 1981.
26. Tandon AK, Gary MC, Chamness GC, Ullrich A, McGuire W: HER-2/neu oncogene protein and prognosis in breast cancer. J Clin Oncol 7:1120–1128, 1989.
27. Eckert K, Granetzny A, Fischer J, Nexö E, Grosse R: A M_r 43.000 epidermal growth factor-related protein purified from the urine of breast cancer patients. Cancer Res 50:642–647, 1990.
28. Battaglia F, Scambia G, Rossi S, Panici PB, Bellantone R, Pollizi G, Querzoli P, Negrini R, Iacobelli S, Crucitti F, Mancuso S: Epidermal growth factor receptor in human breast cancer: Correlation with steroid hormone receptors and axillary lymph node involvement. Eur J Cancer Clin Oncol. 24:1685–1690, 1988.
29. Waterfield MD, Mayes ELV, Stroobant P, Bennet PLP, Young S, Goodfellow PN, Banting GS, Ozanne B: A monoclonal antibody to the human epidermal growth factor receptor. J Cell Biochem 20:149–161, 1982.
30. Kawamoto T, Sato JD, Le A, Polikoff J, Sato GH, Mendelsohn J: Growth stimulation of A431 cells by epidermal growth factor: Identification of high affinity receptors for epidermal growth factor by an anti-receptor monoclonal antibody. Proc Natl Acad Sci USA 80:1337–1341, 1983.
31. Fendly BM, Winget M, Hudziak RM, Lipari MT, Napier MA, Ullrich A: Characterization of murine monoclonal antibodies reactive to either the human epidermal growth factor receptor or HER2/neu gene product. Cancer Res 50:1550–1558, 1990.
32. Damjanov J, Mildner B, Knowles BB: Immunohistochemical localization of the epidermal growth factor receptor in normal human tissues. Lab Invest 55:588–592, 1986.
33. Real FX, Rettig WJ, Garin Chesa P, Melamed MR, Old LJ, Mendelsohn J: Expression of epidermal growth factor receptor in human cultured cells and tissues: Relationship to cell lineage and stage of differentiation. Cancer Res 46:4726–4731, 1986.
34. Nanney CB, Stoschek CM, Magid M, King Jr, LE: Altered /125I/ epidermal growth factor binding and receptor distribution in psoriasis. J Invest Dermatol 86:260–265, 1986.
35. Maruo T, Mochizuki MD: Immunohistochemical localization of epidermal growth factor receptor and myc oncogene product in human placenta: Implications for trophoblast proliferation and differentiation. Am J Obstet Gynecol 156:721–727, 1987.
36. Gullik WJ, Hughes Ch M, Mellon K, Neal DE, Lemoine NR: Immunohistochemical detection of the epidermal growth factor receptor in paraffin-embedded human tissues. J Pathol 164:285–289, 1991.
37. Ennis BW, Valverius EM, Bates SE, Lippman ME, Bellot F, Kris R, Schlessinger J, Masui H, Goldenberg A, Mendelsohn J, Dickson RB: Anti-epidermal growth factor antibodies inhibit the autocrine-stimulated growth of MDA-468 human breast cancer cells. Mol Endocrinol 3:1830–1838, 1989.
38. Gullick WJ, Downward J, Waterfield MD: Antibodies to the autophosphorylation sites of the epidermal growth factor receptor protein-tyrosine kinase as probes of structure and function. EMBO J 4:2869–2877, 1985.
39. Hirota N, Ueda M, Ozawa S, Abe O, Shimizu N: Suppression of an epidermal growth factor receptor hyperproducing tumor by an immunotoxin conjugate of gelonin and a monoclonal anti-EGF R antibody. Cancer Res 49:7106–7109, 1989.