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Enzyme-Linked Immunosorbent Assay for the Epidermal Growth Factor Receptor

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An enzyme-linked immunosorbent assay (ELISA) for the epidermal growth factor (EGF) receptor was developed using three different antibody preparations, one of which is commercially available. Using one of the antisera (986), the assay could detect as few as 200 $\times 10^6$ receptors. This is equal to 0.332 fmol. This sensitivity means that a minimum of 100 A-431 cells (human carcinoma) or 5,000 normal cells are needed to quantitate the number of EGF receptors. Two of the antisera (986 and 451) recognized EGF receptors from placental tissue. EGF receptors from as little as 667 ng of placental membrane protein were detectable. The assay is highly species specific, with the sensitivity for the EGF receptor from different species dependent on the antiserum used. The commercial antibody, 29.1, had especially strong reactivity against pig and dog EGF receptors. An ELISA using this antibody had the capacity to detect the number of EGF receptors in 10 μg of liver membrane protein. The assay is sensitive to receptor conformation. The binding of antisera 986 and 451 to 1% sodium dodecyl sulfate (SDS)-denatured receptor was reduced. The binding of antibody 29.1 was impaired by the presence of 1% Triton X-100 but not the same levels of Tween-20 or SDS. In addition to being a sensitive technique for the quantitation of the EGF receptor, this assay is very rapid, taking a total of 4 h. The microtiter dish format also allows hundreds of samples to be assayed at once. By using the appropriate antiserum and standards, the EGF receptor can be quantitated in tissues from humans, dogs, pigs, and mice.

Key words: ELISA, antiserum, receptor conformation, antibody 29.1, quantitation, detergents

Epidermal growth factor (EGF) receptors (EGFR) modulate the response of cells to EGF, transforming growth factor-α, amphiregulin, and certain viral proteins [1]. Altered expression of EGFR may play a role in diseases such as cancer, psoriasis, and pox virus diseases [2–4].

The determination of the number of EGF receptors per cell using ligand binding assays can be complicated by two factors. The first is that the affinity of the receptor can change with its phosphorylation state [1]. Scatchard analyses using very high ligand concentrations are necessary to obtain accurate assessment of receptor number. A second confounding variable is that a pool of EGFR can be intracellularly located and

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thus unavailable for binding assays to whole cells [5–7]. Even in broken cell preparations, membrane vesicles can be sealed, rendering a fraction of the receptors unavailable for binding. A binding assay has been described that avoids this second confounding variable [8]. Because of the requirement for very high ligand concentrations, for reasons previously described, this assay's sensitivity is limited to the nmol range.

An enzyme-linked immunosorbent assay (ELISA) method is described below that circumvents the problems associated with ligand binding assays. Furthermore, this assay has the advantages that the reagents are stable for long periods of time and that special safety and disposal precautions, necessary for radioactive techniques, are not required. The assay described below is more sensitive than previously described methods and can detect 2×10^8 EGFR.

MATERIALS AND METHODS

Antisera

Antisera 986 and 451 are rabbit polyclonal antisera produced against purified EGFR from human-derived A-431 cells as previously described [9]. Mouse monoclonal antibody against A-431 membranes [10,11] was purchased from Sigma (St. Louis, MO).

A-431 Membranes

A-431 cells were cultured as previously described [12]. The cells were grown to confluence in Dulbecco's modified Eagle's medium containing 5% calf serum. The cell surface was washed four times with saline, and the cells were scraped with a Costar cell scraper into ice-cold 5 mM EDTA in 20 mM Hepes (Research Organics, Cleveland, OH), pH 7.4. The cells were homogenized with six strokes of a motorized polyethylene tetrafluorethylene pestle of a Potter-Elvehjem homogenizer. Unbroken cells and nuclei were pelleted with a 5 min spin at 900*g*. The supernatant was centrifuged at 100,000*g* for 30 min. The pellet was resuspended in 0.5 M NaCl and homogenized and ultracentrifuged as described above. The pellet was resuspended in 20 mM Hepes, pH 7.4, containing 10% glycerol and then rehomogenized. The membranes were aliquoted and stored at −73°C. Protein was quantitated using the Coomassie blue dye reagent after adding 20 μl 1 M NaOH to all of the tubes including the standards [13].

Source of EGFR for Standards

A-431 cell number was determined using a hemocytometer. Replicate dishes were processed as described above except that the low-speed spin was omitted to ensure 100% recovery of membranes. The A-431 cells were assumed to have 2×10^6 receptors per cell [14,15].

Tissue Extracts

Tissues were homogenized in 10 volumes of ice-cold 3 mM EDTA, 3 mM iodoacetic acid, and 10 μg/ml leupeptin in 20 mM Hepes, pH 7.4, using a Potter-Elvehjem homogenizer. Livers were from mouse, rabbit, rat, pig, and dog. A human liver was not available for comparison. Placenta (blood type AB) was from a human. The homogenates were microfuged for 30 min at 16,000*g*. The pellet was resuspended in 2 volumes of the same buffer, and protein content was determined using the Pierce

bicinchoninic acid (BCA) protein assay (Pierce, Rockford IL). [13] The protein content was adjusted to 4 mg/ml, and Tween-20 was added to a final concentration of 1%. The tissues were incubated with the detergent for 30 min at room temperature before adding them to the assay.

The tissues for the experiment shown in Table III were processed as above except that the protein levels in the whole-cell extract, rather than the membrane fraction, were determined. Muscle was from the diaphram, and epidermis was from breast. The epidermis was separated from the dermis by incubation of the skin in 140 mM ammonium bicarbonate, pH 9.5, containing 10 mM dithiothreitol for 4 h at 4°C as previously described [16].

ELISA

Plates [96 well polyvinylchloride 2797 (PVC), Costar, Cambridge, MA, or 96 well polystyrene #25882-96 (PS), Corning, Houston, TX] were coated with 100 μl of a 20 μg protein/ml suspension of A-431 membranes in 0.1 M sodium bicarbonate buffer, pH 9.6, overnight at 4°C. Detergent was not used to solubilize the membranes prior to adding them to the wells because detergents inhibited the association of protein with the dish [17]. Several plates were prepared at once, and the ones to be used on later days were stored at −20°C after removing the membrane mixture and replacing it with 50% glycerol. No differences in results using fresh vs. plates stored up to 4 weeks was detected.

After overnight incubation or storage, the plates were washed twice with 0.05% Tween in PBS (twPBS). All solutions were removed by flicking them into a sink followed by rapping the plate upside down on a paper towel. Buffer (100 μl 5% Tween-20 PBS) was placed into each well and incubated for 30 min at 4°C. This step solubilizes unbound protein, which would interfere with subsequent steps. After the incubation, the plate was given four rinses with twPBS.

The solubilized standard or test solutions (50 μl) were placed into the washed plates, followed by 50 μl of a 1/10,000 dilution of antiserum 986 in twPBS. Both standard and test extracts were presolubilized in 5% Tween-20 in PBS containing 3 mM EDTA, 3 mM iodoacetic acid, and 10 μg/ml leupeptin for 30 min at room temperature (RT). Dilutions were made in this same solution. The covered plate was incubated for 1 h, with rocking. The plate was washed six times with twPBS and 1/3,000 Western blot-grade antibody against rabbit immunoglobulins conjugated to horseradish peroxidase (HRP; Bio-Rad, Richmond, VA) in twPBS was added. This was incubated for 30 min at RT, with rocking. The plate was washed six times with twPBS. The HRP substrate was incubated in the dish for 60 min, with rocking, unless otherwise specified. The substrate consisted of 1 mg/ml 2,2′-azino-di(3-ethybenzthiazoline-sulphonate) (ABTS) and 0.03% H_2O_2 in 0.1 M citrate buffer, pH 4 [17], or 3,3″,5,5″-tetramethylbenzidine (TMB) [18] as directed in the product specifications (Kirkegaard & Perry Laboratories, Gaithersberg, MD). When we used ABTS, the absorbance was read at 405 nm using a MR600 Microplate reader (Dynatech, Torrence, CA). When readings exceeded 2 absorbance units, the absorbance was read at 450 nm, which decreased readings 3.5-fold. All data points are the average of two determinations unless otherwise specified.

Later experiments were modified by increasing the incubation times to 2 h for the first antibody and to 1 h for the second antibody. This modification allowed the reaction to come closer to equilibrium and reduced variability caused by placing the reagents in

the wells at different times. The greatest source of variability was rapid HRP product formation. The concentration of the second antibody was adjusted to produce the desired amount of HRP product in no less than 1 h. With the reagent TMB, the assay could be stopped by the addition of 1 M phosphoric acid, which also resulted in a threefold intensification of colored product (at 450 nm rather than 650 nm).

Reagents

Chemical reagents were purchased from Sigma Chemical Company (St. Louis, MO) or Fisher Scientific (Springfield, NJ) unless otherwise specified. Tissue culture supplies were from Costar (Cambridge, MA) and media were from Gibco (Grand Island, NY). EGF was produced as previously described [19].

Effect of EGF on Antibody Recognition of the Receptor

A-431 membranes precoated on a polystyrene plate were incubated in the presence or absence of 50 ng EGF in 50 μl buffer containing 1 mg/ml bovine serum albumin (BSA) for 30 min at room temperature. Fifty microliters of a 1:20,000 dilution of 986 or 451 antiserum or 1:100,000 of 21.9 antibody was then added, mixed, and incubated 1 h on a rocker at RT. A 1:3,000 dilution of antirabbit Ig conjugated to HRP (aR/HRP) or antimouse Ig conjugated to HRP (aM/HRP) was added and incubated as described above. The enzyme substrate was allowed to incubate in the wells for 1 h. The absorbance was read at 410 nm.

RESULTS

Coating Plates

Three antisera were examined for their suitability for this assay. Two, 986 and 451, are rabbit polyclonal antisera [9] and one, 29.1, is a commercially available mouse monoclonal antiserum [10,11]. A-431 cell membranes were chosen as a source of EGFR since these cells have large numbers of receptors [15]. Whole membranes were used to coat the ELISA plates because the membranes are easy to prepare using the method described above and because solubilization necessitates the use of detergents that interfere with the association of proteins with plastic. The membranes were washed with detergent after allowing them to bind to the plastic to remove unbound proteins. Detergents were used in all subsequent steps in order to open all membrane vesicles so that a hidden pool of EGFR would not exist and because detergent lowers nonspecific interactions [17]. Bindings of different amounts (100, 20, 2, and 0.2 μg/ml) of A-431 membranes to PVC or PS plates were compared (data not shown). Maximum absorbance was obtained when 20 μg/ml membranes was used. The PVC and PS plates bound comparable amounts of membrane when antiserum 986 was used in the assay. When antiserum 29.1 was used, the PVC plates appeared to bind less EGFR than the PS plates. The PVC plates were found to have a background variation of up to 0.030 absorbance units. Transfer of the colored solution to PS ELISA plates reduced the well-to-well variation, indicating that the variation was due to an uneven optical surface.

The A-431 membrane-coated plates could be stored for future use. The best retention of immunoreactivity was observed for plates stored after placing 50% glycerol in each well and freezing at −20°C (data not shown). We have stored PS plates for as long as 4 weeks and still have not observed any appreciable loss of activity. Storage at

TABLE I. Effect of Detergent Concentration of EGFR ELISA*

Detergent	986	29.1	451
None	0.630	0.613	0.605
0.05% Tween	0.583	0.586	0.594
0.1% Tween	0.658	0.662	0.645
5% Tween	0.679	0.708	0.672
1% Triton	0.645	0.197	0.659
5% Triton	0.665	0.218	0.684
RIPA	0.398	0.145	0.435
0.1% SDS	0.088	0.127	0.091

*Fifty microliters of the detergents were added to a polystyrene dish precoated with 20 μg/ml A-431 membranes. Fifty microliters of a 1:10,000 dilution of 986 antiserum, 1:20,000 of 21.9 antibody, or 1:20,000 of 451 was then added and mixed. This mixture was incubated for 1 h on a rocker at RT. A 1:3000 dilution of aR/HRP was added and incubated as described in Materials and Methods. The enzyme substrate was allowed to incubate in the wells for 30 min. The absorbance was read at 410 nm.

−70°C with phosphate-buffered saline (PBS) was also satisfactory. Drying the plate severely reduced immunoreactivity, and care was taken not to allow the wells to dry during any step. The entire assay takes 4 h, assuming that the plates have been prepared ahead of time. Hundreds of samples can be assayed at once because of the 96 well microtiter plate format.

Effects of Detergents

Preliminary assays indicated that anomalous results were obtained with antibody 29.1 when using Triton X-100. This is the detergent usually used for solubilizing EGFR [20]. A careful assessment of the effect of different detergents was performed with the various antibodies (Table I). Triton X-100 interfered strongly with the association of antibody 29.1 with EGFR bound to the ELISA plate. In contrast, high levels of Triton X-100 slightly potentiated the interaction of antisera 986 and 451. Also, high levels of Tween-20 slightly potentiated the interaction of the antiserum with all the antibodies, including 29.1.

To determine whether Triton X-100 disrupted the binding site on the EGFR for antibody 29.1 (vs. disrupting the antibody itself or acting as a ligand), the membranes were pretreated with Triton X-100, after which the Triton was washed away and replaced with buffer containing Tween-20. The Triton X-100 interfered with the assay under this condition to the same extent as when the Triton was present in the primary antibody buffer (data not shown). This indicated that the Triton X-100 disrupted the EGFR rather than the 29.1 antibody. Triton X-100 and Tween-20 are closely related nonionic detergents [21], so it is interesting that these two detergents affect the interaction of antibody 29.1 to such different extents. The different sensitivities of the 29.1 antibody to EGFR bound to PVC vs. PS plates may also be related to conformational changes of the EGFR.

RIPA [9] and 0.1% SDS severely interfered with the assay. RIPA contains 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS. To differentiate the effect of the SDS on the antibodies and the EGFR, an experiment similar to the one described above was performed. EGFR coating the ELISA plate were treated with 1% SDS at 25°C for 15 min. The detergent was washed away, and the antibodies were added in the usual binding solution. The interaction of antibodies 451 and 986 but not antibody 29.1 with

the EGFR was inhibited by the SDS treatment (data not shown). SDS is known to denature proteins; thus these findings suggest that the three-dimensional structure of the EGFR is important for the binding of antibodies 451 and 986. All data had to be corrected for protein losses of bound membrane on the PS dish caused by the 1% SDS incubation.

Concentration of Antibodies

The optimal amount of antibody to use was determined by lowering the antibody concentration until the final absorbance after 1 h incubation of the substrate was approximately 2. Lower dilutions of antibody increased sensitivity on a percentage basis but actually decreased sensitivity on the basis of the difference in absorbance units (data not shown). The second antibody was also titrated for a 30 min incubation. A 1/3,000 dilution produced a near-maximal absorbance: When we increased the concentration of the second antibody threefold to 1/1,000, the absorbance increased only 60% (data not shown).

In a comparison of three different substrates, i.e., ABTS, o-phenylenediamine and TMB [17,18], ABTS was found to be the most sensitive. Increasing the concentration of ABTS decreased sensitivity and increased the background. Increasing the H_2O_2 concentration from 0.003% to 0.03% increased the sensitivity of the assay by 2.3-fold without increasing the background. The actual concentration of H_2O_2 in the solution may have actually been lower since this solution is highly unstable. We have subsequently found that TMB purchased from Kirkegaard & Perry Laboratories (Gaithersberg, MD), rather than from Sigma, was 10 times more sensitive than ABTS.

Effect of EGF

EGF receptors may have EGF bound to them under certain conditions. To determine whether bound EGF might alter the recognition by the antisera, EGF was first allowed to bind to the EGFR bound to the PS plate and binding of the antisera measured in the absence and presence of EGF (see Materials and Methods). No difference was observed between occupied and unoccupied EGFR for any of the antisera tested (data not shown). EGF is known to interfere with the recognition of at least one monoclonal antibody directed against the binding site of the receptor [10]. Thus the possible interference by EGF should be tested before an antibody is routinely used for this ELISA assay.

Species Specificity

An important question is whether this assay can be used for EGFR from human cells other than A-431 and species other than human. Liver was used as the tissue source of EGFR since this tissue contains comparatively high levels of this receptor [22]. For this analysis, I made the assumption that the livers from different species have approximately the same number of receptors per milligram of protein. Since human liver was not available, human placenta, which is also a rich source of EGFR [23], was used. Each antiserum had a unique order of specificity between the different species. In Table II, the most reactive tissue source produced the lowest absorbance, since this indicates that the EGFR are competing successfully for the antibodies. For both of the antisera made in rabbits against human EGF receptors (986 and 451), human EGFR was the most potent and rabbit the least. The order of sensitivity for the EGFR of antiserum 986 was human

TABLE II. Species Specificity of the ELISA*

Tissue	986	451	29.1
None	1.698	1.069	0.991
Human	0.293	0.184	0.969
Mouse	1.318	0.676	0.968
Rabbit	1.569	1.078	0.865
Rat	1.386	0.819	1.035
Pig	1.127	0.719	0.308
Dog	1.039	0.649	0.220

*The solubilized extracts (50 μl) were added to a polystyrene dish coated with 20 μg/ml A-431 membranes. Fifty microliters of a 1:10,000 dilution of 986 antiserum, 1:50,000 of 21.9 antibody, or 1:40,000 of 451 was then added and mixed. This mixture was incubated for 1 h on a rocker at RT. A 1:3,000 dilution of aR/HRP or aM/HRP was added, as appropriate, and incubated as described in Materials and Methods. The enzyme substrate was allowed to incubate in the wells for 30 min. The absorbance was read at 410 nm.

≫ dog > pig >mouse >rat ≫ rabbit. The order of sensitivity of antiserum 451 was human ≫ dog >mouse >pig—rat ≫ rabbit. For the mouse monoclonal antibody, which was made in mice against human EGFR, dog and pig EGFR were the most potent and mouse and rat the least. The order for 29.1 was dog >pig ≫ rabbit ≫ human, mouse, and rat.

Calibration Curves

Using antiserum 986, a plot of log micrograms protein against absorbance was sigmoidal and operationally linear from 0.067 to 6.67 μg (Fig. 1). Placenta has 53-fold fewer receptors on a per nanogram membrane protein basis as indicated by the displacement of the curve on the x-axis at higher protein concentrations. The parallel

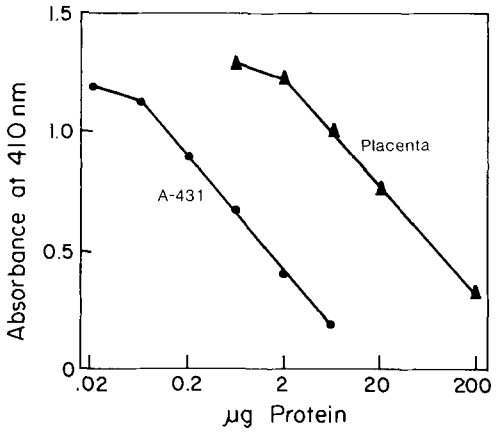

Fig. 1. Competition curves of human placenta and A-431. The solubilized extracts (50 μl) in 5% Tween-20 were added to a polystyrene dish coated with 20 μg/ml A-431 membranes. Fifty microliters of a 1:10,000 dilution of 986 antiserum was then added and mixed. This mixture was incubated for 1 h on a rocker at RT. A 1:3,000 dilution of aR/HRP was added and incubated as described in Materials and Methods. The enzyme substrate was allowed to incubate in the wells for 2 h. The absorbance was read at 410 nm. The x-axis indicates the amount of membrane protein from A-431 cells or placenta as determined in the BCA assay [9]. Circles represent the data obtained from A-431 membranes, and triangles represent the data obtained from placenta membranes.

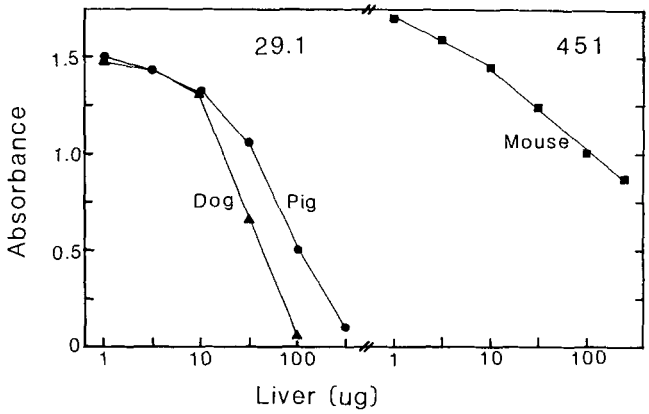

Fig. 2. Standard curves for human EGFR using antisera 451 and 29.1. Solubilized A-431 membranes (50 μl) in 5% Tween-20 were added to a polystyrene dish coated with 20 μg/ml A-431 membranes. Fifty microliters of a 1:40,000 dilution of 451 antiserum or a 1:50,000 dilution of 29.1 antibody was then added and mixed. This mixture was incubated for 1 h on a rocker at RT. A 1:3,000 dilution of aR/HRP or aM/HRP was added as appropriate and incubated as described in Materials and Methods. The enzyme substrate was allowed to incubate in the wells for 1 h. The absorbance was read at 410 nm. The x-axis indicates the number of EGFR added in the form of solubilized A-431 membranes.

slope indicates that the antibody has the same affinity towards the placental EGFR as for the A-431 EGFR [24].

The ability of EGFR from different species to compete in the ELISA assay was tested in the experiments shown in Figure 2. Standard curves were produced using dilutions of mouse, dog, and pig liver membranes to compete against A-431 membranes bound to PS plates. Antiserum 451 was used for mouse membranes and antiserum 29.1 was used for dog and pig membranes. These antisera were selected for each species on the basis of the data shown in Table II. Classical sigmoidal curves were obtained for all three species, and a minimum of 10 μg of membranes in all cases competed significantly (Fig. 2).

Compare the slopes in Figure 1 to those in Figure 2. The slopes in Figure 2 vary widely, indicating that each antiserum has a different affinity towards EGFR from different species. This finding emphasizes the importance of using EGFR from the same species as the tissue being measured in the standard curve. We did not obtain sufficient sensitivity when we tried to use liver membranes bound to the PS dishes as a source of EGFR (data not shown).

Calibration curves for human EGFR are also shown for antiserum 451 and monoclonal antibody 29.1 (Fig. 3). The graphs indicate that both of these antibodies are also suitable for competition assays.

The sensitivity of the assay can be increased approximately threefold by a variation of the procedure (Fig. 4). Antibody 989 was preincubated in the presence of the competing substrate in a separate plate. Thus the competing substrate had a temporal advantage to bind to the antibodies [24]. This procedure requires an extra step of transferring the preincubated mixture to the EGFR-coated plate.

Differential Expression of EGFR in Different Human Tissues

The number of EGFR in different human tissues was examined in the experiment shown in Table III. Placenta and epidermis are tissues known to have high levels of

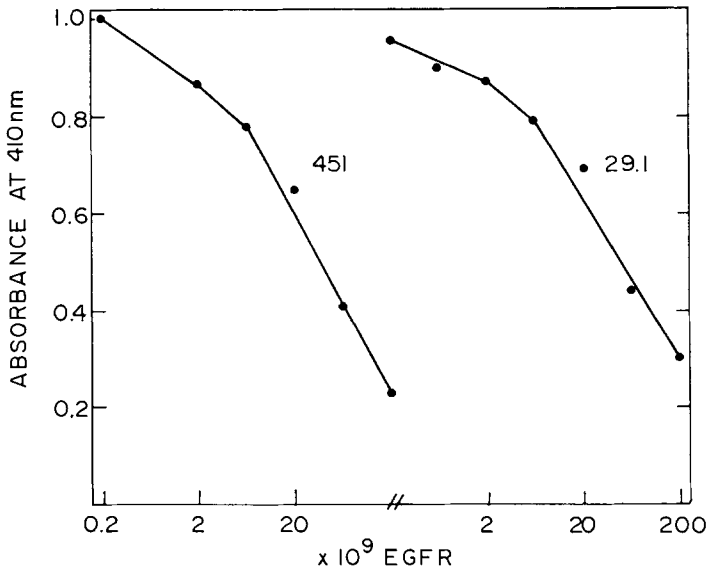

Fig. 3. Standard curve for human EGFR using an extra sensitive method. Dilutions of A-431 cells (55 μl) and 55 μl 1/50,000 986 were added to a Limbro 96-well Disposo tray (New Haven, CT) and incubated for 2 h at RT. One hundred microliters was transferred to a plate coated with 20 μg/ml A-431 membranes. The plate was incubated for 1 h on a rocker at RT followed by washes. A 1:3,000 dilution of aR/HRP was added and incubated for 1 h at RT. After washing, the TMB reagent was incubated in the wells for 30 min and the reaction was stopped with 1 M phosphoric acid. The absorbance was read at 450 nm.

EGFR, and skeletal muscle is known to have low levels of EGFR [23,25,26]. The ELISA reflects these known differences and for the first time provides a direct quantitative comparison for the number of receptors in these different tissues. The antiserum used does not recognize rabbit EGFR, so rabbit tissues can provide a control for possible interfering constituents of various tissues.

An experiment was performed to determine whether EGFR number in human epidermis as determined by this ELISA assay reflected that determined in a previous study. Using morphometric analysis of colloidal gold-labeled antibodies to the EGFR as well as autoradiography of radioactive EGF binding, we previously determined that the stratum corneum has approximately seven times fewer EGFR than the stratum basalis of skin [27]. Using the ELISA described above, we were able to determine the number of EGFR in 50 μg of callus (stratum corneum) and thin epidermis (approximately 30% stratum basalis). In callus we found 75 million EGFR/μg protein, and in thin skin there were 400 million EGFR/μg protein. This is a 5.33-fold difference and agrees well with the sevenfold difference previously determined. The small difference between these two figures can be accounted for by the fact that thin epidermis is not pure stratum basalis and thus would be expected to have fewer EGFR.

Assay Variability

Intraassay and interassay variability was examined in the experiments described in Table IV. Each experiment was performed on a different day. The same placenta homogenate was used for each of these experiments. The experiments show that the coefficient of variance (CV) can vary widely from experiment to experiment, but it did not exceed 18% in any case. The average intraassay CV was 11%. The interassay CV

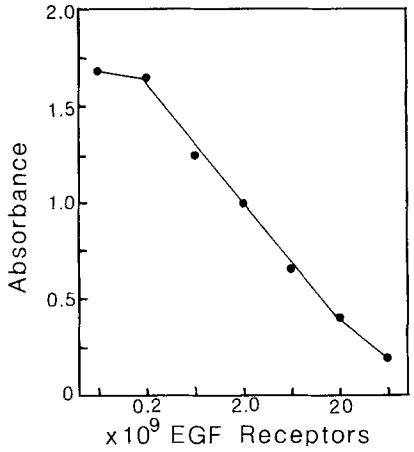

Fig. 4. Standard curves using liver from different species. Solubilized extracts (50 μl) were added to a polystyrene dish coated with 20 μg/ml A-431 membranes. Fifty microliters of a 1:50,000 dilution of 29.1 antibody was added to pig and dog liver extracts or 1:40,000 dilution of 451 antiserum was added to the mouse liver extract. This mixture was incubated for 2 h on a rocker at RT followed by washes. A 1:10,000 dilution of a M/HRP or aR/HRP was added as appropriate and incubated for 1 h at RT. After washing, the TMB reagent was incubated in the wells for 2 h and the absorbance was read at 630 nm. Each sample was performed in duplicate.

was 15% for 10 μg placenta and 8% for 20 μg placenta. The lower CV in the latter case may be due to the fact that the amount of EGFR in the 20 μg amount of placenta is closer to the center of the standard curve.

DISCUSSION

An ELISA for the EGF receptor was developed using three different antisera, one of which is commercially available. Using one of the antisera (986), the assay could detect as few as 667 $\times 10^6$ receptors. This is equal to 1.11 fmol. Thus the assay can detect the number of EGFR in 330 A-431 cells or approximately 271 ng of whole cellular protein (assuming that A-431 cells have 0.82 ng protein per cell). Assuming that most normal cells have 50-fold fewer EGFR [15], 16,500 normal cells, or 13.5 μg protein, would be required for the assay. A variation of the ELISA requiring an additional preincubation step increases the sensitivity an additional threefold.

TABLE III. Quantitation of EGFR in Different Tissues*

	$\times 10^7$ EGFR/μg protein	
Tissue	Human	Rabbit
Placenta	78.4	ND[a]
Epidermis	26.5	ND
Muscle	2.42	ND

*The experiment was performed as described for Table IV except that each data point was determined in duplicate. Fifty microgram samples of each tissue were examined.

[a]Rabbit liver was used as a negative control because rabbit placenta was not available.

TABLE IV. Reproducibility of the EGFR ELISA*

Experiment	Placenta protein(μg)	$\times 10^9$ EGFR	Standard deviation	Coefficient of variance (%)
1	10	12.7	1.6	13
	20	30.6	5.6	18
2	10	18.3	2.6	14
	20	36.6	5.6	15
3	10	15.6	0.7	4
	20	37.0	2.3	3
Average	10	15.5	2.3	15
	20	34.8	2.3	8

*The solubilized extracts (50 μl) were added to a polystyrene dish coated with 20 μg/ml A-431 membranes. The same placenta and A-431 extracts were used for all three experiments. Fifty microliters of a 1:50,000 dilution of 986 antiserum was then added and mixed. This mixture was incubated for 2 h on a rocker at RT followed by washes. At 1:10,000 dilution of aR/HRP was added and incubated for 1 h TRT. After washing, the TMB reagent was incubated in the wells for 30 min and the reaction was stopped with 1 M phosphoric acid. The absorbance was read at 450 nm. Each data point is the averge of four samples except for the standards, which were determined in duplicate. Each experiment was performed on a different day.

The normal cell estimate is presented only as a rough guideline. The number of EGFR of normal cells can vary to a great extent due to a number of parameters such as tissue source, exposure to growth factors (including EGF), and metabolic state. The ELISA format described in this paper should be applicable to any specific antiserum to the EGFR. The only modification would be to optimize the dilution of antibody used.

An advantage of the format described above is that high amounts of protein of low specific activity can be used in the assay. ELISA plates have a limited capacity to bind protein, so the sensitivity of an assay requiring binding of cell extracts directly to the plastic is limited by the proportion of EGFR protein in it. In most cells, this percentage is very small; for example, in fibroblasts, EGFR represents 0.0035% of the total cellular protein [7]. This format also uses low amounts of specific antibody compared with the type of ELISA using an antibody bound to the ELISA dish in order to capture the antigen.

A disadvantage of the competitive ELISA is that proteases must be carefully inhibited. Active proteases can degrade EGF receptors bound to the dish and in solution as well as degrading the antibodies. EDTA, iodoacetic acid, and leupeptin are general protease inhibitors and were added to all incubation solutions. Since the EGFR is associated with the plasma membrane, proteases as well as other interfering cellular components can be removed by centrifugation (the EGFR would be in the pellet fraction). To determine whether proteases are present, the wells can be preincubated with the test tissue extract (compared with buffer alone) and washed out before adding the antiserum. A resulting decrease in the final absorbance indicates that the tissue extract degraded EGF receptors bound to the plate.

Another method of determining whether tissues have interfering components is to use rabbit tissue as a negative control (for rabbit antisera 986 and 451). This type of control has the advantage that both the EGFR and the antibodies are exposed to tissue factors. This control assumes that nonspecific factors are similar in the homologous rabbit tissue. For antibody 29.1, mouse tissue should be suitable. With liver, epidermis, and muscle, no tissue interference was observed (Table III).

The assay is highly species specific, with the order of sensitivity for the EGF receptor from different species dependent on the antiserum used. The order of reactivity of 986 and 451 was expected since these antisera were produced against human EGF receptors in rabbits. Since rabbits usually produce a polyclonal antiserum, the greatest number of antigenic sites would be present in human EGFR. As the EGFR diverges in its structure in different species, more and more antigenic sites disappear and a smaller subset of antibodies are available to bind to those EGFR. Another factor in play is that the immune system tends to avoid producing antibodies to "self" antigens. Thus the rabbits did not produce antibodies to rabbit EGFR and even the mouse monoclonal antibody was unreactive towards mouse EGFR. The preferential reactivity of 29.1 towards pig and dog liver EGFR over human placental EGFR was unexpected since this mouse monoclonal antibody was produced against human EGFR. One explanation is that, since a monoclonal antibody has a single antigenic site, this particular site was more stable in the presence of detergent in pig and dog than in human. The EGFR from A-431 with its extra glycosylation seems to be more stable, using EGF binding as a criterion, in the presence of detergent than that from placenta [26]. Some of the antisera produced against EGFR from A-431 cells have been shown to be directed against the blood group A antigen [14,28], so the blood type of the placenta was examined using a hemagglutinin assay (Ortho Diagnostics). Since the blood type of the placenta was AB, an antiserum to the blood group A antigen should have reacted. Nonrecognition of placental EGFR by this antibody was previously observed [11].

The lower limits of detection of human placenta and livers from mouse, dog, and pig were tested. Serum 986 had the capacity to detect as little as 667 ng membrane protein of placenta (Fig. 1). Antiserum 451 had the capacity to detect as little as 1 μg membrane protein of mouse liver. Antibody 29.1 had the capacity to detect as little as 10 μg membrane protein of dog and pig liver (Fig. 2).

The sensitivity of the ELISA described above compares favorably in sensitivity to radioimmunoassays previously described [11,14,30]. The ELISA is more convenient because of the stability of the components. Also, one of the radioimmunoassays [14] has the disadvantage that both proteases and phosphatases [16] can potentially interfere with the assay. This is because the signals are radioactive phosphate groups on tyrosyl residues of the EGFR.

Another method of quantitating the EGFR is by immunodetection in a Western blot [29,31-33]. This procedure is far more complex than the method described above. Also, we have found it to be nonquantitative for crude membrane extracts from tissues with low EGFR concentrations, perhaps because other proteins block the access of antibodies to the small number of receptors. The advantage of the Western blot procedure is that a less specific antiserum can be used because any immunodetection of proteins that have a molecular weight different from that of the EGFR can be eliminated.

In conclusion, a method to quantitate EGFR in four different species without the use of radioisotopes has been devised. This method is more sensitive than previously described procedures and allows the use of crude membrane preparations of tissues. The presence of EGFR in cancer tissue is thought to play a role in autocrine stimulation of cell proliferation and metastasis [2]. EGFR may also play a role in other diseases, such as psoriasis and pox virus diseases [3,4]. The rapidity and simplicity of the above-described EGFR ELISA should aid in the analysis of perturbations in the number of

EGFR in different diseases and in the progression of disease in order to elucidate the role this receptor plays.

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