The human EGF receptor as a target for cancer therapy: six new rat mAbs against the receptor on the breast carcinoma MDA-MB 468

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Summary Using the breast carcinoma cell line MDA-MB 468 as immunogen, we have produced six new rat monoclonal antibodies (mAbs) against the human EGF receptor (EGFR) and are investigating their use for diagnostic and therapeutic applications in cancer patients whose tumours overexpress these receptors. The mAbs (three IgG2b and one each of IgG2a, IgG1 and IgA) were selected on the basis that they bound to the extracellular domain of the EGFR and blocked growth factor-receptor interaction. Competitive assays showed that, with the exception of antibody ICR85, the mAbs bound to one of two distinct epitopes on the external domain of the EGFR. ICR85, ICR65, & ICR55 immunoprecipitated the 170 kDa glycoprotein from cells expressing the EGFR but not the 185 kDa product of the related c-erbB-2 proto-oncogene. Unlike EGF and TGFα none of the mAbs stimulated the growth of quiescent human foreskin fibroblasts but they inhibited the EGF and TGFα induced growth stimulation of these cells in vitro. When tested for their effect on tumour cells the mAbs were found to inhibit the growth in vitro of a number of human tumours that overexpressed the EGFR (e.g. HN5, HN6, HN15, A431, MDA-MB 468) but they were without effect on tumour cell lines expressing low or undetectable amounts of the receptor. Our initial results indicate that this new generation of antibodies which bind with high affinity to the EGFR, block growth factor-receptor interaction and inhibit the growth of human squamous carcinoma cell lines overexpressing the receptor have potential for clinical application.

Data from a number of laboratories indicate that overexpression of the receptor (EGFR) to which EGF, TGFα and other polypeptide hormones bind, plays an important role in the development of certain types of human malignancies (Santon et al., 1986; Ozanne et al., 1986; Velu et al., 1987; Difore et al., 1987; Harris et al., 1990). In addition, some of these tumours produce ligands for the receptor and it has been suggested that an autocrine mechanism is involved in progression of cancers of this type (Sporl & Roberts 1985; Imanishi et al., 1988; Nistare, et al., 1988; Dernycyk 1990; Tateishi et al., 1990; Yoshida et al., 1990; Morishige et al., 1991). Over-expression of the EGFR by tumour cells, compared to their normal counterparts, has been reported for a number of squamous cell carcinomas (e.g. Cowley et al., 1984; Hendler et al., 1984; Sainsbury et al., 1985; Gullick et al., 1986) and this in turn was also correlated to poor prognosis in patients with some of these carcinomas (reviewed by Gullick, 1991).

The high level of expression of the EGFR on squamous cell carcinomas and the important role of the receptor (a 170 kD transmembrane tyrosine kinase) in signal transduction (Ullrich & Schlessinger, 1990) make it potentially an excellent target for antibody directed therapy (Mendelsohn, 1989). Ideally, the antibody of choice would have the ability first to inhibit cell growth by blocking growth factor-receptor interaction and second to activate complement and recruit host effecter cells to bring about tumour cell destruction. Although a number of mouse monoclonal antibodies (mAbs) have been developed in the last decade using the A431 cell receptor as immunogen (e.g. Schreiber et al., 1981; Waterfield et al., 1982; Sato et al., 1983; Liveh et al., 1986; Murthy et al., 1987; Hendly et al., 1990; Pellegrini et al., 1991) only a few have either of the desired properties. Mouse antibodies are less effective in activating human effector functions than are rat antibodies of the IgG2b isotype (Hale et al., 1985; Dyer et al., 1989). Furthermore, the effectiveness of monoclonal antibodies in harnessing host effector functions depends not only on the isotype of the antibody but also, importantly, on the particular epitope bound on the target antigen. For this reason it is necessary to search for an antibody that maximises the biological and immunological functions.

We have reported (Modjtahedi, et al., 1992) the preparation and properties of ten rat mAbs that bind to three distinct epitopes (A,B,C) on the external domain of the EGFR using as immunogen the human squamous cell carcinoma HN5. While all of the mAbs against epitopes B and C blocked the binding of EGF and TGFα to the receptor on a number of squamous carcinoma cell lines the antibodies against epitope C were an order of magnitude better than the others at inhibiting cell growth. The best antibody, ICR16, inhibited completely the growth of HN5 cells in vitro at antibody concentrations above 1 nM. However, none of the antibodies was of the IgG2b isotype and therefore would not be expected to interact efficiently with the host immune effector functions in rat, mouse or man. Since our intention is to test the effectiveness of antibodies in the clinic we report here the preparation of a second series of antibodies using as immunogen the EGFR over-expressing breast carcinoma MDA-MB 468 (Filmus et al., 1985) searching particularly for IgG2b antibodies with growth inhibitory properties.

Materials and methods

Cell lines

The following cell lines were kindly provided by Dr M.J. O'Hare: (a) carcinomas with >10⁶ receptors/cell, LICR-LON-HN5, LICR-LON-HN6 and LICR-LON-HN15 (head and neck), A431 (vulval), MDA-MB 468 (breast); (b) cells with <10⁶ receptors/cell, EJ (bladder), SKOV3 (ovarian) and SKBR3 (breast). SKOV3 and SKBR3 also overexpresses the c-erbB2 product at a level some tenfold greater than the EGFR. The EGFR expressing A172 (glioblastoma) and A549 (lung) cell lines were obtained from ECACC, Porton Down. MDA-MB 435, a human breast adenocarcinoma which does not express detectable levels of the EGFR and the mouse EGFR expressing C66 cells were provided by Dr S.A. Eccles. Cells were cultured routinely in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and the antibiotics penicillin, streptomycin and neomycin.

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Production and testing of anti-EGFR monoclonal antibodies

Two immunisation protocols were performed with cells harvested from confluent flasks by trypsinisation. In the first, CBH/cbi rats were immunised via their Peyer’s patches with 4 × 10⁷ MDA-MB 468 cells contained in 100 μl of phosphate buffered saline, pH 7.2. The rats were rechallenged via the same route and with the same number of cells two weeks later and again 4 weeks after the primary immunisation. Alternatively, the rats were immunised at five sites (4 × s.c. and 1 × i.p.) with a total of 10⁴ MDA-MB 468 cells contained in PBS then rechallenged two weeks and six weeks later via the same route with 3 × 10⁶ cells. Lymphocytes were prepared for fusion from either mesenteric nodes or spleens three days after the last immunisation.

Hybridomas were prepared (Dean et al., 1984) by fusing 10⁶ lymphocytes with 5 × 10⁵ Y3 myeloma cells. Between eight to twelve days later the wells were screened for the presence of antibodies that inhibited the binding of ¹²⁵I-EGF (10⁶ c.p.m./well) to MDA-MB 468 or EJ cells that had been grown to confluence in 96-well plates. Colonies from positive wells were picked individually, grown up and cloned twice by limiting dilution. The isotype of each mAb was determined as described previously (Styles et al., 1990).

Control antibodies used in this investigation

mAb ALN/11/53 is directed against a membrane antigen on the rat sarcoma HSN (Dean et al., 1984). ICR12 (Styles et al., 1990) and ICR55 are directed against distinct epitopes on the extracellular domain of the c-erbB-2 p185. Antibodies ICR10 and ICR16, prepared against the receptor on HN5 cells (Modjtahedi et al., 1992), were used as positive controls.

Purification of monoclonal antibody

Antibodies were precipitated from culture supernatant or ascites (prepared in athymic rats) with (NH₄)₂SO₄ to 45% of saturation. mAbs of the IgG2a and IgG2b isotype were purified by ion-exchange chromatography on DE52 cellulose (Whatman Ltd., Maidstone, Kent). IgA and IgG1 antibodies were purified by affinity chromatography on MARK1 (Bazin et al., 1984) covalently coupled to Sepharose 4B. All preparations were dialysed extensively against PBS, filter sterilised and stored frozen.

Radioiodination of proteins

Monoclonal antibodies, mouse or human EGF (Collaborative research, Waltham, Mass.) or human recombinant TGFα (Boehringer Mannheim, Germany) were labelled, using the Iodogen procedure (Fraker and Speck, 1978), with Iodine-125 (Na¹²⁵I, Amersham International) to a specific activity of 10 pCi μg⁻¹.

Epitope determination by competitive radioimmunoassay

Triplicate samples (50μl) of doubling dilutions of each purified mAb (50 μg ml⁻¹ initial concentration) in DMEM-3%FCS or neat culture supernatant (ICR65), were mixed with an equal volume containing 2.5–3.0 × 10⁶ cpm/well of the ¹²⁵I-labelled mAb, ¹²⁵I-EGF or ¹²⁵I-TGFα. Controls containing medium alone or ALN/11/53 were set up in the same way. Aliquots of 90 μl of each mixture were transferred to monolayers of MDA-MB 468 or EJ cells grown to confluence in 96-well plates. After incubation for one hour on ice the cells were washed three times with diluent, then lysed in 1 M NaOH containing 1% sarkosyl and the bound radioactivity determined in a Hydragramma spectrometer (Oakfield Instruments Ltd, Oxford). Antibodies were considered to be in the same epitope cluster if they inhibited the binding of each other to the cells by more than 80%.

Dissociation of antibody affinity

Dissociation constants were determined from Scatchard plots of binding of ¹²⁵I-labelled antibodies to the EGFR receptor isolated from HN5 cells as described by Modjtahedi et al. (1992). Immunoreactivity, i.e. the proportion of radiolabelled antibody that would bind antigen at infinite antigen excess, was determined using a protocol similar to that described by Lindmo et al. (1984).

Immunoprecipitation of ¹²⁵S-methionine-labelled proteins

The proteins of HN5, SKOV3 or A431 cells were radio-labelled by incubation for 16–20 h with ¹²⁵S-methionine (200 μCi/80 ml⁻¹ flash containing 5 ml methionine-free DMEM), and cell lysates were prepared using Triton-X 100 as described previously (Styles et al., 1990). Immunoprecipitates were made by incubating 1 ml aliquots of the radio-labelled cell extracts with 50 μl of Sepharose-linked rabbit/rat Fab (5 mg ml⁻¹) gel that had been incubated previously with one of the specific rat/EGFR mAbs, ICR12 or ALN/11/53 (50 μg mAb/50 μl anti-Fab beads). ¹²⁵S-methionine-labelled truncated EGFR secreted into the culture supernatant by A431 cells was immunoprecipitated in the same way. After rotation overnight at 4°C the samples of gel were washed four times in lysis buffer then analysed by SDS-PAGE on 7.5% reducing gels.

Effect of antibody and/or TGFα on quiescent human fibroblasts

DE532 cells (Flow laboratories) were seeded at 4 × 10⁴ cells ml⁻¹ in DMEM containing 10% FCS and grown to confluence in 24 well plates, then the medium was replaced with DMEM containing 1% FCS. After 48 h in this medium, 50 μl aliquots of mAb (25 μg ml⁻¹) and/or TGFα (5 ng ml⁻¹) were added to quadruplicate wells and the cells were incubated overnight at 37°C then pulsed for 6 h with 2 μCi/ well of ³H-thymidine. The acid insoluble radioactivity incorporated into DNA was determined in a liquid scintillation counter.

Effect of antibodies to the EGFR on growth of tumour cells in culture

About 5 × 10⁴ cells in 100 μl of DMEM containing 2% FCS were seeded into each well of a 96-well plate. After incubation for 4 h at 37°C, 100 μl aliquots of dilutions of each mAb (starting at 50 μg ml⁻¹) were added in triplicate to the wells and the cultures were incubated at 37°C. Controls were set up that contained either medium alone or medium containing dilutions of a control antibody (ALN/11/53). The cultures were incubated for a further 3 to 10 days until the controls incubated in medium alone were almost confluent and had increased in number to a maximum of 5 × 10⁴ cells per well. All cells were fixed with 0.25% glutaraldehyde then washed in water, air dried and stained with 0.5% methylene blue (100 μl/well) for 15 min. After washing with tap water and air drying, 200 μl of 0.33N HCl was added to each well and the A₅₄₀ of each supernatant was determined in a Titertek Multiscan. To determine the initial number of cells present at the start of treatment in each experiment, an extra plate was set up and the cells were fixed 4 h after the start of incubation at 37°C. Growth as a percent of control was determined from the formula:-

\[
\%\text{ growth } = \frac{B - A}{C - A} \times 100
\]

Where \( A = A_{540} \) at start of experiment

\( B = A_{540} \) after treatment with antibody

\( C = A_{540} \) after incubation in medium alone

To investigate the effect of endogenous growth factors present in FCS on the activity of the antibodies, experiments were carried out using DMEM containing 2%, 5% or 10% FCS.
Results

Production of hybridomas

Five hybridomas (ICR60–64) producing mAbs that specifically inhibited the binding of $^{125}$I-EGF to its receptor were obtained from two fusions using mesenteric node cells taken from rats immunised via the Peyer's patches with MDA-MB 468 cells. Recently, a further antibody (ICR65) has been obtained using spleen cells from a third immune rat and all the results reported here with this antibody have been obtained using culture supernatant. The effect of these mAbs on the binding of $^{125}$I-EGF to the bladder carcinoma cell line EJ, is presented in Figure 1. Three of these antibodies (ICR61, ICR62 and ICR65) were of the IgG2b isotype (Table I). Competitive assays showed (Table II) that antibodies ICR60–64 bound to two distinct epitopes on the external domain of the EGF receptor one of which (ICR62 and ICR63) was the same (epitope C) as that recognised by antibodies ICR11 and ICR16 produced previously against the receptor on HN5 cells (Modjtahedi et al., 1992). ICR60, ICR61 and ICR64 did not compete with any of the antibodies raised against HN5 cells and were considered to bind to an independent epitope (D) on the EGFR (Table I). ICR65, however, competed with all the new antibodies (Figure 2) for binding to the receptor suggesting that epitopes C and D were adjacent. The immunoreactivity of the antibodies tested varied from 31% (ICR61) to 74% (ICR64, see Table I). The new antibodies were of high

![Figure 1](image)

**Table I** Rat monoclonal antibodies to the human EGF receptor

| Immunogen/antibody | Isotype | Epitope cluster | Affinities (nm) | Immuno-reactivity | Reference |
|---------------------|---------|-----------------|-----------------|-------------------|-----------|
| (a) HN5             |         |                 |                 |                   |           |
| ICR9                | IgG2a   | +               | 3.5             | 53%               | Modjtahedi et al (1992) |
| ICR10               | IgG2a   | +               | 6.7             | 69%               |           |
| ICR11               | IgG2a   | +               | 5.00 ; 1.2      | 68%               |           |
| ICR14               | IgG2a   | +               | 2.90 ; 70.0     | 73%               |           |
| ICR15               | IgG2a   | +               | 5.50 ; 68.0     | 33%               |           |
| ICR16               | IgG2a   | +               | 0.37 ; 3.49     | 49%               |           |
| ICR28               | IgA     | +               | N.D             | N.D               |           |
| ICR29               | IgA     | +               | N.D             | N.D               |           |
| (b) MDA-MB468       |         |                 |                 |                   | this work |
| ICR60               | IgA     | +               | N.D             | N.D               |           |
| ICR61               | IgG2b   | +               | 0.25 ; 1.40     | 31%               |           |
| ICR62               | IgG2b   | +               | 7.50            | 69%               |           |
| ICR63               | IgG2a   | +               | 0.15            | 54%               |           |
| ICR64               | IgG1    | +               | 0.61 ; 1.80     | 74%               |           |
| ICR65               | IgG2b   | +               | N.D             | N.D               |           |

N.D = Not determined.

**Table II** Effect of rat mAbs to the Human EGFR on the binding of $^{125}$I EGF (A) or $^{125}$I TGFr (B) to human carcinoma cells

**A**

| mAb (78ns) | Head and neck (%) | $^{125}$I EGF bound to: | Lung (A549) | Brain (A127) | Bladder (EJ) |
|------------|--------------------|--------------------------|-------------|-------------|-------------|
| ICR60      | 48.7               | 27.4                     | 8.5         | 22.0        | 30.0        |
| ICR61      | 33.7               | 11.5                     | 19.0        | 10.5        | 11.2        |
| ICR62      | 23.4               | 9.5                      | 18.6        | 16.7        | 12.9        |
| ICR63      | 22.8               | 4.2                      | 10.0        | 9.3         | 7.7         |
| ICR64      | 24.2               | 10.1                     | 4.9         | 9.2         | 5.4         |
| ICR65      | 49.5               | 24.3                     | ND          | 22.6        | 8.5         |

**B**

| mAb (156ns) | Head and neck (%) | $^{125}$I TGFr bound (%) | Vulva (A431) | Breast (MDA-MB468) | Bladder (EJ) | Ovarian (SKOV3) |
|-------------|--------------------|---------------------------|-------------|---------------------|--------------|-----------------|
| ICR60       | 41.8               | 31.6                      | 19.9        | 14.0                | 34.9         |                 |
| ICR61       | 31.3               | 25.8                      | 12.9        | 10.4                | 6.9          |                 |
| ICR62       | 24.5               | 24.1                      | 10.3        | 7.2                 | 6.4          |                 |
| ICR63       | 38.3               | 24.1                      | 12.5        | 4.6                 | 20.3         |                 |
| ICR64       | 15.4               | 18.7                      | 4.8         | 6.4                 | 7.4          |                 |
| ICR65       | 33.4               | 25.8                      | 9.4         | 5.6                 | 4.3          |                 |
affinity and two of the Scatchard plots (ICR61, ICR64) showed two affinities for binding (Table I).

All six antibodies inhibited the binding of EGF and TGFα to the receptor present on five human tumour cell lines of different origin (Table II) and as expected maximal inhibition was observed with the cell lines (e.g. EJ and SKOV3) that expressed lower levels of the EGFR. This result suggests that most of the antibodies bind at or near to the ligand binding site on the receptor. However, the antibodies did not bind to the mouse EGFR since none of them inhibited the binding of 125I-EGF to the mouse cell line Ca6 (data not shown).

The antibodies do not crossreact with the product of the c-erbB-2 proto-oncogene

MAbs ICR60–ICR64 and ICR10 (positive control antibody raised against EGFR on HN5 cells) all specifically immunoprecipitated the 170 kDa EGFR from detergent extracts of 35S-methionine-labelled HN5 cells whereas a control antibody (ALN1/153) did not (Figure 3). The mAbs also immunoprecipitated the EGFR from detergent extracts of 35S-methionine-labelled SKOV3 cells but not the 185 kDa overexpressed product of the c-erbB-2 gene. The latter was, however, precipitated from these extracts (Figure 3) by antibody ICR12 (Styles et al., 1990). We conclude that none of the antibodies tested (ICR60–64) crossreacted with the c-erbB-2 gene product.

Effect of antibody and/or TGFα on proliferation of human fibroblasts

Both EGF and TGFα stimulate DNA synthesis in quiescent human fibroblasts. We have investigated the effect of our antibodies used alone or in combination with growth factors on DE532 cells. The results, presented in Table III, show that none of the antibodies alone could stimulate DNA synthesis in these cells. All of the antibodies blocked TGFα stimulated DNA synthesis; completely in the case of ICR61–ICR64, but antibody ICR60 was less effective. We conclude that all the

Table III  Effect of anti-EGFR monoclonal antibodies, TGFα or both on DNA synthesis in quiescent human foreskin fibroblasts (DE532)

| TGFα (ng ml⁻¹) | ICR60 25 µg ml⁻¹ | ICR61 25 µg ml⁻¹ | ICR62 25 µg ml⁻¹ | ICR63 25 µg ml⁻¹ | ICR64 25 µg ml⁻¹ | ALN11/53 25 µg ml⁻¹ | NO Antibody |
|----------------|------------------|------------------|------------------|------------------|------------------|-------------------|-------------|
| 0              | 240              | 201              | 247              | 250              | 199              | 223               | 316         |
| 5              | 770              | 189              | 190              | 308              | 221              | 1556              | 1755        |
antibodies acted as antagonists of TGFα. Similar results were obtained when EGF was used instead of TGFα (data not shown).

The mAbs inhibit the growth of tumour cells that overexpress the EGFR

The growth of HN5 and HN6 cells was inhibited completely when cultures were incubated with the antibodies to the EGFR at a concentration of 25 µg ml⁻¹ or greater (Table IV). When tested at lower concentrations, some of the mAbs were found to be more effective than others (Figure 4) and the order of effectiveness for inhibition of growth of HN5 cells was ICR64 (IgG1) > ICR62 (IgG2b) > ICR63 (IgG2a) > ICR60 (IgA) > ICR61 (IgG2b). Antibodies ICR60–64 were also tested for their effect on the growth of a number of other tumour cell lines including those (A431, HN15, MDA-MB 468) with high levels of expression of the EGFR, or those (EJ, SKOV3, SKBR3) with lower levels of EGFR expression (<1 x 10⁵ receptors/cell) or cells that did not express detectable levels of the EGFR (MDA-MB 435). The results of this comparative study using media containing 2% FCS are presented in Table IV. They show that growth of the cell lines with high level expression of the EGFR was inhibited by all of the antibodies tested. At a concentration of 156 nM, however, the antibodies were without effect on the growth of SKBR3, SKOV3, EJ or MDA-MB 435 cells.

To investigate if endogenous growth factors present in FCS affected the activity of the anti-EGFR antibodies, experiments were repeated using HN5 cells incubated in media containing 5% or 10% FCS. Only a small shift in baseline (10%) was observed with these antibodies (cf ICR64, Figure 5) even at the highest concentration of FCS used and we conclude that at the concentration present in FCS such growth factors had little effect on the growth inhibitory activity of the mAbs.

Table IV Effect of mAbs to the EGFR receptor on the growth of tumour cells in medium containing 2% foetal calf serum

| mAbs (156nm) | HN15 | HN5 | HN6 | A431 | MDA-MB 468 |
|--------------|------|-----|-----|------|------------|
| ICR60        | 39.6 | 2.7 | ND  | ND   | ND         |
| ICR61        | 32.4 | -1.0| -6.7| 66.1 | 40.1       |
| ICR62        | 18.3 | 1.1 | -7.1| 65.3 | 32.8       |
| ICR63        | -0.1 | -1.4| 3.4 | 71.8 | 67.0       |
| ICR64        | 22.8 | 0.1 | 0.0 | 0.0  | 39.7       |

No effect on SKBR3, SKOV3 and EJ cells. ND = Not determined.

While the head and neck carcinomas HN5, HN6 and HN15 were the most sensitive to antibody induced inhibition of growth, A431 and MDA-MB 468 were less susceptible. A431 cells have been reported to secrete large amounts of a truncated form of the receptor (Weber et al., 1984) and this may have influenced the interaction of the rat antibodies with the transmembrane receptor.

The mAbs bind to the truncated EGFR secreted by A431 cells

When ¹²⁵I-labelled mAbs (2.2–2.5 x 10⁵) were mixed with culture supernatant obtained from A431 cells the binding of antibodies ICR60–64 to EJ cells was abolished (Table V). Also, the 100 kD truncated receptor was precipitated when cell free supernatants from ³⁵S-methionine labelled A431 cultures were immunoprecipitated with mAbs ICR16 and ICR64 (Figure 6) but not when the control antibodies (ALN/11/53, IRC12, IRC55) were used. Clearly, with tumours like A431, the presence of large amounts of truncated receptor may decrease the effective dose of antibody reaching the tumour cells.

Discussion

In this paper we describe the production and some of the properties of six new rat monoclonal antibodies directed against the human EGF receptor. Our results show that these antibodies have the following properties, they: (a) bind with high affinity to two distinct epitopes on the extracellular domain of the human EGFR, (b) do not cross-react with the related product of the c-erbB-2 proto-oncogene, (c) prevent the binding of exogenous growth factors to the receptor, (d) inhibit the growth stimulatory effects of EGF and TGFα on human fibroblasts and (e) inhibit the growth of human tumour cell lines overexpressing the EGFR. Three of these antibodies are of the IgG2b isotype (ICR62, epitope C; ICR61, epitope D and ICR65, epitopes C and D). While all of the antibodies stained frozen sections of cells overexpressing the EGFR, none was found to bind the fully reduced

Table V Effect of supernatant from human epidermoid carcinoma cell line (A431) on the binding of ¹²⁵I anti-EGF receptor mAb to the EGF receptor on EJ cells. Each value is the mean of triplicate samples.

| Treatment              | ICR61 Antibody Bound (cpm) |
|------------------------|----------------------------|
| A431 supernatant       | 88                         |
| Control medium         | 1200                       |
|                        | 700                        |
|                        | 1289                       |
|                        | 2567                       |
Figure 6 Autoradiographs of immunoprecipitates of [35S]-methionine labelled EGF and truncated EGF secreted by A431 cells run on 7.5% reducing gels. Track A: ICR16; track B ICR64; track C ICR12; track D ICR55; track E ALN/11/53; track F medium control.

receptor in Western blots nor did they bind to formol-saline fixed paraffin embedded tissues (data not shown). We conclude that mAbs ICR60–64 all recognise conformational determinants on the receptor.

During the last decade a number of laboratories have generated antibodies against the human EGF R with a view to their use in structural studies on the receptor or for clinical applications in oncology. In most cases the antibodies were raised in mice against the EGF R and the vulval carcinoma cell line A431. Because the A431 receptor also contains the blood group A antigen, many of the antibodies produced crossreact with this normal antigen limiting their use for diagnostic or therapeutic purposes (see review by Sato et al., 1987).

As in A431 cells, the receptor for the EGF R is overexpressed on the surface of MDA-MB 468 cells but, unlike A431 cells, the gene for the EGF R is not rearranged (Filmus et al., 1989). Our aim has been to produce rat monoclonal antibodies that bind to different epitopes on the external domain of the human receptor for EGF so that we can identify the best epitope to target for growth inhibition as well as the best antibody for harnessing host immune effector functions. For this reason we have used rats as our source of antibodies rather than mice because some rat antibodies of the IgG2 isoform interact very efficiently with the effector arm of the human immune system (Dyer et al., 1989).

We have reported (Modjtahedi et al., 1992) that rat mAbs raised against the head and neck carcinoma cell line LICR-LON-HN5 bound with high affinity to one of three distinct epitopes (A,B,C) on the EGF R but none were of the IgG2b isoform. Outstanging, in terms of inhibition of growth of HN5 cells, were antibodies ICR11 and ICR16 both of which were directed against epitope C. Here we report that two out of a further six mAbs raised against the EGF R on MDA-MB 468 cells also bound to epitope C and one of these was an IgG2b (ICR62) with good growth inhibitory properties in vitro. Interestingly, three of the four other mAbs recognised a new epitope (D) on the EGF R and one of these mAbs (ICR61) was an IgG2b. Lastly, antibody ICR65, which is of recent production, is also an IgG2b and this mAb crossreacted with both epitopes C and D. The other properties of this antibody are currently being determined.

The isolation of three antibodies against a novel epitope on the EGF R was surprising since no antibodies of this type were obtained in nine separate fusions when HN5 cells had been used a immunogen. Clearly, this resulted from the use of a different source of EGF R as immunogen (MDA-MB468) which in turn led also to the isolation of three mAbs of the IgG2b isotype. These findings reinforce our view that it is essential to use different source of immunogen to obtain as diverse a population of antibodies (epitope/isotype) from which the best antibody for clinical use can be selected.

In terms of biological activity, antibody ICR61 was the least effective of the new antibodies and complete inhibition of growth of HN5 cells was only obtained at a concentration of 20 nM which was an order of magnitude higher than that required with ICR62 or ICR64, which inhibited completely the growth of these cells at a concentration of 2.4 nM. Indeed ICR64 was one of the best of all the rat mAbs tested and it equalled ICR16 (Modjtahedi et al., 1992), in terms of its capacity to block the binding of EGF R to its receptor and to inhibit the growth of HN5 cells in vitro. We conclude that both epitopes C and D constitute suitable targets for antibody directed therapy.

The antibodies very effectively inhibited the growth in vitro of three different head and neck carcinoma cell lines. They were less effective, however, when tested on A431 or MDA-MB 468 cells. The A431 cell line is aberrant in that it secretes large amounts of a truncated form of the EGF R (Weber et al., 1984) to which all of the rat antibodies bind. This truncated EGF R may therefore reduce the effectiveness of the mAbs at inhibiting growth of this cell line. Furthermore, we have shown that, in addition to overexpression of the EGF R, both A431 and MDA-MB 468 cells produce TGFα constitutively (Derynick et al., 1987; Ennis et al., 1989). High level secretion of this ligand by these cells may also reduce the effectiveness of the antibodies in vitro by competing with them for binding to the receptors and thereby enhancing their internalisation.

Currently, we are testing some of the antibodies for their activity in vivo against xenografted human tumours growing in athymic mice and the results obtained are very encouraging. These experiments are described in the accompanying paper and show that ICR62 and ICR64 were effective at eliminating the tumours when given at the time of tumour cell inoculation. ICR62 was also effective in inducing regression of established tumours.

We believe that this new generation of rat mAbs which bind with high affinity to the EGF R, block ligand-receptor interaction and inhibit the growth of squamous cell carcinomas overexpressing the receptor have potential for clinical application.

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