NCL-CB11, A NEW MONOCLONAL ANTIBODY RECOGNIZING THE INTERNAL DOMAIN OF THE c-erbB-2 ONCOGENE PROTEIN EFFECTIVE FOR USE ON FORMALIN-FIXED, PARAFFIN-EMBEDDED TISSUE

IAN P. CORBETT, JAMES A. HENRY, BRIAN ANGUS, CAROLINE J. WATCHORN, LINDA WILKINSON, COLM HENNESSY*, WILLIAM J. GULICK†, NADIA L. TUZIT, FELICITY E. B. MAY, BRUCE R. WESTLEY AND CHARLES H. W. HORNE

Departments of Pathology and *Clinical Surgery, University of Newcastle upon Tyne and †ICRF Oncology Group, Hammersmith Hospital, U.K.

Received 14 December 1989
Accepted 30 January 1990

SUMMARY

The c-erbB-2 proto-oncogene encodes a 190 kDa protein representing a putative growth factor receptor with considerable homology to the EGF receptor. Gene amplification and overexpression of the oncogene protein have been demonstrated in a variety of tumours including breast cancer, where it is associated with a poor prognosis.

In this study we have produced and characterized a mouse monoclonal antibody, designated NCL-CB11, to the c-erbB-2 protein. NCL-CB11 was generated to a synthetic peptide sequence corresponding to a site of predicted antigenicity near the C-terminus of the internal domain of the protein. NCL-CB11 produces intense membrane-associated immunohistochemical staining in a proportion of human cancer cells. The specificity of the antibody is supported by Western blotting and immunoprecipitation studies. Reactivity with an internal site of the protein is confirmed by the necessity of cell permeabilization for reactivity in fluorescence-activated cell sorter (FACS) analysis. A high degree of correlation between immunohistochemical staining using NCL-CB11, and c-erbB-2 gene amplification has been observed.

NCL-CB11 should prove to be a valuable reagent for investigations into the pathological significance of c-erbB-2 protein expression.

KEY WORDS—c-erbB-2, oncogene, monoclonal antibody, immunohistochemistry, breast cancer.

INTRODUCTION

The c-erbB-2 proto-oncogene, located on chromosome 17, band 21,1 encodes a 190 kDa (p190) protein, which has been identified in a variety of tumours including breast cancer. There is evidence to suggest that the c-erbB-2 protein is a growth factor receptor closely related to EGFR.2,3 Evidence for a central role in tumorigenesis is suggested by the finding that overexpression of c-erbB-2 in NIH 3T3 cells is transforming and that transgenic mice bearing the c-erbB-2 oncogene driven by a mouse mammary tumour virus promoter develop mammary carcinomas.4,5

A number of studies have indicated that gene amplification and enhanced c-erbB-2 expression are associated with poor prognosis in breast cancer.6-10 Indeed Wright et al.7 found that oncoprotein expression determined immunohistochemically ranked second only to lymph node status as a prognostic indicator. Furthermore, preliminary data suggest that c-erbB-2 oncoprotein expression can predict response to endocrine therapy.11 It seems likely, therefore, that c-erbB-2 oncoprotein determination will find a place in determining patient allocation to different treatment regimes in human breast cancer.
The potential for immunotherapy using antibodies directed against c-erbB-2 oncoprotein has been demonstrated both in vitro and in vivo.\textsuperscript{12} Growth of a neu-transfected cell line derived from a rat neuroblastoma (B104.1.1) can be inhibited in vitro by monoclonal antibody 7.16.4 directed against the external domain of c-erbB-2 oncoprotein.\textsuperscript{13} In vivo, growth of tumours derived from the B104.1.1 cell line implanted in nude mice can also be inhibited by this antibody.\textsuperscript{14} Recently, Hudziak et al. have demonstrated a similar inhibition effect with the human breast tumour cell line SK-BR-3 using an external domain antibody 4D5.\textsuperscript{15} The use of anti c-erbB-2 antibodies provides a possible approach to immunolocalization and therapy in human breast cancer. Identification of the ligand for c-erbB-2 may permit development of antagonists, possibly synthetic polypeptide analogues.

A novel approach to anti c-erbB-2 therapy has recently been described by Sternberg and Gullick.\textsuperscript{16} They propose that neu activation depends on receptor dimerization via the transmembrane domain. They present data to show how this can occur in the absence of ligand in association with a single amino acid substitution caused by mutation, and suggest that dimerization might be inhibited by an analogue comprising only the transmembrane portion. Such an approach could possibly be applied to the human situation where it appears that c-erbB-2 amplification rather than mutation causes increased receptor activity.\textsuperscript{17}

Thus, both from a prognostic and potentially from a therapeutic point of view, it appears that determination of c-erbB-2 status will prove to be of value in human breast cancer. Immunohistochemistry provides a simple and rapid method of determining antigen expression. Polyclonal antisera used in such determinations have the disadvantage of increased background staining and inter-batch variation.\textsuperscript{18} In this study, we have produced a monoclonal antibody, NCL-CB11, raised against a synthetic peptide of predicted antigenicity from the c-erbB-2 molecule, which will circumvent these disadvantages. NCL-CB11 is highly effective in formalin-fixed, paraffin-embedded material.

**MATERIALS AND METHODS**

*Production of monoclonal antibodies*

Synthetic peptides corresponding to regions of the c-erbB-2 oncoprotein sequence were used as immunogens, selected on the basis of computer-predicted antigenicity. The peptides were prepared on a semi-automatic peptide synthesizer (Cambridge Research Biochemicals Pepsynthesiser II) using Fmoc chemistry.\textsuperscript{19} Peptides were conjugated to keyhole limpet haemocyanin (KLH) and bovine serum albumin (BSA) for immunization and immunoassay procedures, respectively.

Immunization of Balb/c mice was by administration of a sequence of four peptide–KLH conjugate injections, each of approximately 30 µg, at 14-day intervals. Initial injections were in Freund’s complete adjuvant, followed by Freund’s incomplete adjuvant, both administered subcutaneously, and finally two inoculations in phosphate-buffered saline (PBS) administered by the peritoneal route.

Four days after the final immunization the spleen was removed and a cell suspension produced. The spleen cells were fused with P3-NS1/1-Ag-1 cells using polyethylene glycol, suspended in selective growth medium, and plated out onto 96 well plates. The cultures were incubated for approximately 10 days, or until colony growth became apparent. Supernatant was removed from each well and assayed by an enzyme-linked immunosorbent assay (ELISA) against peptide–BSA conjugate. Wells exhibiting antipeptide activity were then microscopically examined for growth, and where possible individual colonies were transferred to separate wells in 24 well growth plates. After a further incubation period the growth plate wells underwent a second ELISA, and wells exhibiting no antipeptide activity were discarded. Supernatant was removed from all remaining wells and tested immunohistochemically, using an indirect immunoperoxidase technique, on formalin-fixed, paraffin-embedded tissue sections (see below). Known positive cases for the assay were determined using polyclonal antibody 21N.\textsuperscript{20} Colonies demonstrating any positive immunohistochemical staining then underwent a minimum of three cloning procedures using the doubling dilution technique. The immunoglobulin sub-type of cloned monoclonal antibodies was determined by immunodiffusion using a commercial kit (ICN Immunobiologicals, U.S.A.).

*Cell culture*

MCF-7 cells were cultured at 37°C and 5 per cent CO₂ in a humidified incubator, in RPMI 1640 containing glutamine, antibiotics, sodium hydrogen carbonate, and 10 per cent fetal calf serum (FCS). SK-BR-3 cells (a gift from Dr N. Hynes, Friedrich Miesner Institute) were maintained using
Dulbecco’s modified Eagle’s medium (DMEM), containing antibiotics and 10 per cent FCS (all tissue culture reagents, Northumbria Biologicals, U.K., and Gibco, U.K.).

Tissue and fixation

Mastectomy and lumpectomy specimens were received fresh in the Department of Histopathology. Tumours were dissected and block size pieces placed in neutral buffered formalin overnight. Following primary fixation, the blocks were further trimmed and fixed with mercuric chloride (saturated aqueous mercuric chloride and 37 per cent formaldehyde, 9:1) for approximately 3 h. Blocks were then routinely paraffin-embedded.

Immunohistochemistry

Three μm sections of tissue were prepared, dewaxed, and rehydrated. The sections were then treated with 0.5 per cent hydrogen peroxide in methanol for 10 min, washed in a running water tray, and placed in Tris-buffered saline (TBS) for 5 min. The sections were covered with normal rabbit serum for 10 min, after which excess rabbit serum was removed and replaced by NCL-CB11, at a dilution of 1:40. Sections were incubated in a humid chamber at 4°C overnight. After washing in two changes of TBS for 10 min, the sections were covered with rabbit anti-mouse peroxidase conjugated secondary antibody (Dakopatts, Denmark) and incubated for 30 min at room temperature. Sections were then washed in two changes of TBS for 10 min, developed with diaminobenzidine tetrahydrochloride substrate (Sigma, U.K.), counterstained with haematoxylin, dehydrated, cleared, and mounted.

Immunohistochemical staining of tumour cell membranes was scored according to both intensity and the proportion of cells staining. Intensity was scored on a four-point scale: no staining, score 0; equivocal weak staining, score ±; unequivocal staining, score +; intense staining, score ++. The proportion of tumour cells staining was scored on a five-point scale: no cells staining, score 0; 1–24 per cent cells staining, score 1; 25–49 per cent staining, score 2, 50–74 per cent staining, score 3; 75–100 per cent staining, score 4.

Cytospins were prepared from cultured SK-BR-3 and MCF-7 cells, fixed in acetone for 10 min, and stained as above.

FACS analysis

The human breast cancer cell lines SK-BR-3 and MCF-7 were aliquoted into 10^6 cells per sample. Prior to analysis, samples were treated with 0.1 per cent saponin and then incubated with 7.5 μl of the mouse monoclonal antibody NCL-CB11 for 30 min, centrifuged, and washed in 1 per cent BSA. Fluorescinated goat anti-mouse IgG (GAM-FITC) (Becton Dickinson) was used as the secondary antibody, at 1 μg per sample. The controls used were (a) cells incubated with both the primary and the secondary antibodies, but without saponin; and (b) cells treated with saponin and incubated with GAM-FITC, but without the primary antibody. Cytometry was performed using a Becton-Dickinson flow cytometer FACS 420 with log amplifier on green fluorescence, thus allowing the median channel of fluorescence to be recorded for each sample. The stored data were analysed by B-D Consort 30 software.

Immunoprecipitation studies

SK-BR-3 cells were metabolically labelled as follows: 9 cm diameter petri dishes (Nunc, Gibco, U.K.) of SK-BR-3 cells were grown to approximately 90 per cent confluence. Cells were washed twice with DMEM without methionine and labelled with 0.5 mCi[^35S]methionine (Amersham, U.K.) in 10 ml of DMEM consisting of nine parts of methionine free medium and one part of normal DMEM and 5 per cent FCS for 18 h at 37°C[^35S]Methionine-labelled SK-BR-3 cells were then washed twice with PBS containing 2 mM ethyleneglycol-bis (β-aminoethyl ether) tetraaetate acid (EGTA) and lysed in 1 ml of ice-cold 50 mM Tris–HCl buffer, pH 7.4, containing 1 per cent Triton X-100, 5 mM EGTA, 150 mM NaCl, 25 mM benzamidine, and 3 mM phenylmethylsulphonyl fluoride (PMSF) (lysis buffer). The lysate was clarified by centrifugation at 16,000 g for 5 min at 4°C. 100 μl of NCL-CB11 supernatant or 5 μg of affinity purified 21N antibody was absorbed onto 20 μl of a 1:1 slurry of protein A Sepharose (Sigma, U.K.) for 20 min at room temperature and then washed with 1 ml of PBS. Competing peptide (10 μg) or 20 μl of 1:100 dilution of normal mouse serum (NMS) was employed as controls. 170 μl of clarified cell lysate was added and the total volume made up to 500 μl with lysis buffer. The tubes were then tumbled for 2 h at 4°C. The immune complex was washed by brief centrifugation once with 1 ml of PBS containing 0.2 per cent (v/v) Triton X-100 and twice with...
1 ml of PBS supplemented with NaCl to a final concentration of 0.5 M and 0.2 per cent (v/v) Triton X-100. Bound proteins were eluted with 40 μl of sample buffer containing β-mercaptoethanol and run on a 5 per cent polyacrylamide gel containing sodium dodecyl sulphate (SDS). The gels were stained with Coomassie blue and destained using standard procedures. The gels were then dried and autoradiographed for 65 h at −80°C using Kodak XAR5 film.

**Autophosphorylation of c-erbB-2**

Immunoprecipitations were performed as above using unlabelled SK-BR-3 cell lysate, but with an additional wash with 1 ml of 20 mM Hepes buffer, pH 7.4, containing 0.1 per cent Triton X-100 and 3 mM MnCl₂ (phosphorylation buffer). Carrier-free [³²P]ATP (Amersham, U.K.) (5 μCi) was added in 30 μl of phosphorylation buffer to each immunoprecipitate and incubated for 10 min at room temperature. The reaction was stopped by adding 40 μl of 5× sample buffer containing β-mercaptoethanol and boiling for 3 min. The samples were separated on 5 per cent PAGE gels as above, but autoradiographed for 8 days.

**Western blotting analysis**

2, 5, 10, and 20 μg of SK-BR-3 cell lysate prepared as described above (protein concentration estimated using the method of Bradford) and rainbow molecular weight markers (Amersham, U.K.) were run on a 5 per cent polyacrylamide gel containing SDS. Electrophoretic transfer of proteins to nitrocellulose membranes (Schleicher and Schuell, F.R.G.) was performed at 180 V for 3 h in 25 mM Tris base, 192 mM glycine buffer containing 20 per cent methanol and 0.01 per cent SDS. The nitrocellulose blot was then block with 10 mM Tris-HCl buffer, pH 8-0, containing 150 mM NaCl, 0.05 per cent Tween 20 (TBST buffer), and 1 per cent BSA for 30 min. The nitrocellulose blot was then incubated with NCL-CB11 antibody (1:20 dilution in TBST) for 40 min at room temperature, rinsed three times for 5 min each with TBST buffer, and the first antibody revealed using the Protoblot Western Blot AP System (Promega, U.S.A.).

**DNA analysis**

DNA was extracted from dismembrated tumour specimens as described previously. High molecular weight DNA digested with the restriction endonuclease EcoRI was extracted from 79 tumour specimens (comprising 74 primary breast cancers and five deposits of breast carcinoma metastatic to lymph nodes) for which fixed tissue suitable for immunohistochemical staining was available. Samples of tumour DNA (5 μg) were digested with EcoRI present in either 2- or 10-fold excess, separated by gel electrophoresis for 500 Vh in a 0.8 per cent agarose gel, and transferred to nitrocellulose filters using the method of Southern. Following transfer, membranes were washed in 3× standard saline citrate (SSC) and baked under vacuum for 2 h at 80°C. The filters were then hybridized with 3.5×10⁶ cpm/ml of a nick translated [³²P]-labelled 1.6 kb EcoRI fragment of the c-erbB-2 oncogene which spanned the central portion of the cDNA (a gift from T. Yamamoto, Tokyo), subcloned into the commercially available Stratagene Bluescript Vector (obtained from Northumbria Biologicals). Hybridization solutions and conditions were as described previously. Unbound probe was removed from the filters by serial washes in 0.3×SSC, 0.1 per cent SDS at room temperature followed by two washes at 65°C in the same solution. The filters were then exposed to pre-flashed X-ray film at −70°C for 1–17 h. To correct for variation in DNA loading and transfer, and to allow assessment of gene amplification, filters were washed for 30 min in two changes of 0.03 M sodium hydroxide at room temperature and rehybridized with a tubulin probe, labelled by nick translation with [³²P], and hybridized under the same conditions as already described at 3×10⁶ cpm/ml prior to washing and autoradiography. Hybridization of the two probes was determined by scanning densitometry of the autoradiographs, and the ratio of c-erbB-2 to tubulin hybridization was calculated.

**RESULTS**

**Monoclonal antibody production**

A fusion using the spleen from a mouse immunized with a peptide from an internal domain c-erbB-2 site yielded 30 colonies from wells showing initial antipeptide activity. Secondary screening, following subculture, revealed three of the initial colonies to have strong antipeptide activity. An aliquot of supernatant was removed from each of the three colonies for immunohistochemical assay. Each supernatant demonstrated characteristic c-erbB-2 membrane staining at various dilutions.
Fig. 1—Immunohistochemical staining of invasive ductal carcinoma of breast using NCL-CB11 anti c-erbB-2 monoclonal antibody. Note intense staining of tumour cell membranes. (a) Low power; (b) high power (c) shows intensive membrane-associated staining of tumour cells (centre). Note the absence of staining of epithelial cells of the normal lobule (top right) and of the normal duct (lower right).

One colony was selected on the basis of strong cell growth, and the remaining two were cryopreserved for future use. The selected colony was cloned by limiting dilution, cultured, and then the cloning procedure was repeated twice. The resultant clone was then cultured for further investigation and characterization of its anti c-erbB-2 monoclonal antibody, designated NCL-CB11.

NCL-CB11 produced intense membrane staining, with minimal background staining, of up to 20 per cent of breast carcinomas examined (Fig. 1). In some cases, weaker cytoplasmic staining was also observed. The SK-BR-3 breast carcinoma cell line, which contains 4- to 8-fold gene amplification and 128-fold overexpression of c-erbB-2 mRNA, also showed intense membrane-associated staining while control cells without amplification remained unstained (Fig. 2).

Characterization of immunoreactivity

The specific reactivity of monoclonal antibody NCL-CB11 with the proteins of appropriate M, was determined by Western blotting analysis and immunoprecipitation of 35S-labelled cell lysate or by immunoprecipitation followed by autophosphorylation. Intense reactivity at 190 k was shown for each determination (Fig. 3). In the Western blot (Fig. 3C), two additional bands at 150 k and 130 k M, were observed when large amounts of cell lysate were applied. Reactivity with an internal site was supported by fluorescence-activated cell sorter (FACS) analysis. Strongly positive fluorescence, due to NCL-CB11, was seen on SK-BR-3 cells only in those samples treated with the detergent Saponin (Fig. 4), indicating that the antibody is detecting an internal portion of the oncoprotein. No significant increase in fluorescence was observed in the MCF-7 control cells, which express only very low levels of c-erbB-2 protein.

DNA analysis

The c-erbB-2 probe hybridized to two discrete bands of approximately 7 and 5-2 kb (Fig. 5). The ratio of c-erbB-2 to tubulin hybridization ranged from 0-13 to 24 in the 79 tumour DNA samples. The ratio lay between 0-13 and 0-53 for the majority of samples (50, 63 per cent) and for these samples the mean ratio was 0-34 (standard deviation = 0-1). It
Fig. 2—Immunohistochemical staining of cytospin preparations of cultured cells using NCL-CB11 anti c-erbB-2 monoclonal antibody. (a) SK-BR-3 cells. Note intense membrane staining. (b) MCF-7 cells. Note the absence of membrane staining; nuclei are demonstrated by a light haematoxylin counterstain.

Fig. 3—(A) Autoradiograph of immunoprecipitated c-erbB-2 from [35S]methionine-labelled SK-BR-3 cells. Track 1: 5 µg AP21N antibody; track 2: 5 µg AP21N antibody including 10 µg competing 21N peptide; track 3: 100 µl NCL-CB11 supernatant; track 4: 20 µl of 1:100 dilution of NMS. (B) Autoradiograph of autophosphorylated c-erbB-2 immunoprecipitated from SK-BR-3 cells as described in Materials and Methods. Track 1: 5 µg AP21N antibody; track 2: 5 µg AP21N antibody including 10 µg competing 21N peptide; track 3: 20 µl of 1:100 dilution of NMS; track 4: 100 µl NCL-CB11 supernatant. (C) Western blot of c-erbB-2 performed as described in Materials and Methods. Track 1: molecular weight markers; tracks 2–5: 2, 5, 10, and 20 µg total protein from lysed SK-BR-3 cells.
Fig. 4—Fluorescence of SK-BR-3 cells measured by flow cytometry, having been (A) incubated with NCL-CB11 and goat anti-mouse FITC, (B) treated with 0.1 per cent saponin and incubated with GAM-FITC, and (C) treated with saponin and incubated with CB-11 and GAM-FITC. Fluorescence of MCF-7 cells measured by flow cytometry, having been (D) incubated with NCL-CB11 and GAM-FITC, (E) treated with 0.1 per cent saponin and incubated with GAM-FITC, and (F) treated with saponin and incubated with CB-11 and GAM-FITC. Note that only SK-BR-3 cells permeabilized with saponin show reactivity (C)
Fig. 5—The c-erbB-2 probe hybridized to discrete bands of approximately 7 and 5.2 kb in EcoR1 digests of breast tumour DNA. Levels of hybridization are compared with hybridization of a tubulin probe. Four samples show amplification.

has been assumed that in these samples the c-erbB-2 gene was unamplified. Using these criteria the c-erbB-2 gene was amplified 2-fold in a further eight tumours and amplified more than 2-fold in 25 (31 per cent of total). Of these, the gene was amplified by 5-fold or more in 18 tumours (22 per cent). In four of the five instances where paired primary tumours and lymph node metastatic deposits were available for analysis, comparable gene copy numbers were present in the two samples. In one instance, the primary tumour showed slight (3-fold) amplification of the c-erbB-2 gene, yet there was no detectable amplification in the metastatic deposit.

In general, immunohistochemical staining for the c-erbB-2 proteins product correlated well with the presence of an amplified c-erbB-2 gene in the 79 tumours studied. Amplification of the c-erbB-2 gene by greater than 2-fold was considered genuine. In 14 tumours, unequivocal immunohistochemical staining (+, + +) of a portion of the tumour (score > 1) was found in association with genuine amplification and 48 tumours neither stained immunohistochemically nor showed gene amplification. A further 11 tumours had an amplified c-erbB-2 gene yet did not stain immunohistochemically, and six stained immunohistochemically but showed no gene amplification ($\chi^2 = 15.91, P < 0.001$, Table I). The significance of the correlation between amplification and immunohistochemical staining was increased still further if only gene amplification of greater than 4-fold and strong immunohistochemical staining (+, + +) over at least 50 per cent of the tumour section (score > 2) were considered positive. Using these criteria, intense immunohistochemical staining correlated with gene amplification in ten instances and there were no examples of tumours showing strong, extensive immunohistochemical staining with less than 4-fold gene amplification; 57 tumours showed neither strong immunohistochemical staining nor gene amplification and 12 had 4-fold or greater gene amplification without intense immunohistochemical staining ($\chi^2 = 25.75, P < 0.001$, Table II).

**DISCUSSION**

Our results demonstrate that NCL-CB11 is highly effective for immunohistochemistry using paraffin-embedded material. The specificity of the antibody is supported by Western blotting and immunoprecipitation studies. The identity of the protein was further indicated by its ability to autophosphorylate in an immune kinase complex assay. This activity is characteristic of most receptor tyrosine kinases and of some soluble cytoplasmic proteins involved in growth signal transduction. NCL-CB11 gives intense membrane-associated staining consistent with the cellular location of the oncprotein. Reactivity with the internal domain, in keeping with the synthetic peptide sequence used as immunogen, is supported by the requirement for permeabilization of SK-BR-3 cells in order to give immunostaining for FACS analysis. This latter finding highlights a further potential use for NCL-CB11 in determining tumour c-erbB-2 status in small samples of tumour taken by fine needle aspiration.

Relating immunohistochemical staining of any degree to gene amplification revealed a significant correlation. The significance of correlation increased markedly when stringent criteria were applied, i.e., greater than 4-fold amplification and intense staining of greater than 50 per cent of tumour section (score +, + +, 3 or 4). It is perhaps of relevance that Wright et al. applied similar criteria to their prognostic series using 21N for immunostaining. They found that c-erbB-2 staining was strongly associated with poor prognosis, and that the significance of this was reduced when tumours with any staining were considered c-erbB-2 positive (C. Wright, personal communication).
Table I—Correlation between immunohistochemical staining of c-erbB-2 protein product and c-erbB-2 gene amplification. A significant correlation was present between unequivocal c-erbB-2 immunohistochemical staining and greater than 2-fold gene amplification ($\chi^2 = 15.91, P < 0.001$)

<table>
<thead>
<tr>
<th>Immunohistochemistry negative (−, ±)</th>
<th>Immunohistochemistry positive (+, ++)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplification ≤ 2-fold</td>
<td>48</td>
</tr>
<tr>
<td>Amplification &lt; 2-fold</td>
<td>11</td>
</tr>
</tbody>
</table>

Table II—Correlation between strongly positive immunohistochemical staining of c-erbB-2 protein product and c-erbB-2 gene amplification. A significant correlation was present between the presence or absence of strongly positive c-erbB-2 immunohistochemical staining and greater than 4-fold gene amplification ($\chi^2 = 25.75, P < 0.001$)

<table>
<thead>
<tr>
<th>Immunohistochemistry negative (−, ±, +)</th>
<th>Immunohistochemistry strongly positive (+ +)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplification &lt; 4-fold</td>
<td>57</td>
</tr>
<tr>
<td>Amplification ≥ 4-fold</td>
<td>12</td>
</tr>
</tbody>
</table>

Applying stringent criteria, 13 per cent of tumours were positive by immunohistochemistry, and 22 per cent showed gene amplification. These results are in accord with those of previous investigators. Intense immunohistochemical staining has been shown in between 9 and 16.5 per cent of tumours, although when positivity is defined as any significant staining, up to 30 per cent of tumours have been scored positive. In previous studies, the frequency of gene amplification has ranged from 10 to 30 per cent. Slamon et al. have demonstrated that moderate levels of c-erbB-2 protein overexpression, associated with low levels of gene amplification, may not be recorded as positive by immunohistochemical staining as a result of fixation regimes, and this may account for the slight discordance in incidence.

It is worthwhile noting, however, that in our laboratory it is routine to post-fix tissue in formol sublimate (see Materials and Methods), and this appears to enhance immunohistochemical staining. Investigators in other centres using neutral buffered formalin only appear to score a smaller proportion of tumours positive for the oncoprotein using NCL-CB11 (W. Cowan, personal communication), and experiments are currently under way to investigate the effect of various fixation regimes on staining. Gusterson et al. found methacarn fixed tissue to be superior to formol saline for demonstration of c-erbB-2 using the 21N polyclonal antibody.

The NCL-CB11 antibody described is an excellent reagent for immunohistochemistry. However, to explore prospects for immunolocalization and immunotherapy it will be necessary to produce antibodies of high affinity recognizing sites on the external domain. Such a reagent would allow determination of concordance of external and internal parts of the c-erbB-2 molecule in individual tumours; the possibility of loss of part of or all of the external part of the oncoprotein with constitutive activation of the internal part has not yet been addressed, although one report of an apparently
truncated c-erbB-2 gene in a gastric adenocarcinoma has appeared. Antibodies to the external domain would be ideal for use in the fluorescence-activated cell sorter (FACS), obviating the need for cell permeabilization. Although mouse monoclonal antibodies have been used in immunolocalization and immunotherapy, a human or humanized (chimeric mouse Fab/human Fc) antibody would be ideal. If a highly antigenic site can be determined, then the prospects for a true human monoclonal antibody would be greatly increased, and we are currently assessing a number of synthetic peptides of predicted antigenicity in this regard.

Of direct relevance to the possibilities for therapy in human adenocarcinoma is the question of the ubiquity and extent of c-erbB-2 expression. If the gene is widely expressed, then the issue specificity of anti c-erbB-2 reagents may be reduced. Colling et al. have recently described immunostaining for c-erbB-2 in a great variety of normal human tissues, but polyclonal antibodies were used at high concentration and the possibility of cross-reactivity with other molecules remains. Cohen et al. have also described immunohistochemical reactivity in a variety of epithelial tissues, again using polyclonal antisera. Much of the staining observed in these studies was cytoplasmic, which is difficult to explain if the molecule is considered to be a membrane receptor, although, interestingly, it has been reported that twice as much EGF receptor is found inside normal human hepatocytes than is expressed on the cell surface. In accord with these authors, cytoplasmic staining has been observed in some cases stained with NCL-CB11. De Potter et al. found that membrane-associated staining for c-erbB-2 was specific for malignancy and presented evidence that cytoplasmic staining, observed with antibodies 3B5 and 9G6, was accounted for by reactivity with a cytoplasmic protein of 155 kDa. Antibodies NCL-CB11 and 21N also demonstrate reactivity with a 150 kDa band as demonstrated by Western blotting. These may represent precursor forms of the molecule. Studies on non-neoplastic tissues using NCL-CB11 will be of interest in this respect: Preliminary data from immunohistochemistry using NCL-CB11 suggest that the c-erbB-2 oncogene protein may indeed be demonstrated in normal human tissues (P. Quirke and W. J. Gullick, unpublished results), and a comprehensive study using a wide variety of both fixed and frozen tissue will be carried out in our laboratory.

Polyclonal antisera often produce high levels of background staining and because of inter-batch variation, render comparison of studies performed in different centres problematical. The NCL-CB11 monoclonal antibody should enable uniform results to be obtained in different centres, a point of particular importance if c-erbB-2 oncoprotein expression is used to determine therapy, for example, in clinical trials. Efficacy in fixed paraffin-embedded tissue permits convenient tissue handling and will enable retrospective studies to be carried out.

In summary, NCL-CB11 should prove of value as a diagnostic reagent, in determining prognosis and therapy in breast and perhaps other cancers, and in understanding the physiologic and pathological roles of the c-erbB-2 oncoprotein.

ACKNOWLEDGEMENTS

I. P. Corbett would like to acknowledge a grant from the Medical Research Council, C. J. Watchorn the North of England Cancer Research Campaign, J. A. Henry a Wellcome Trust research training fellowship, and F. E. B. May a Royal Society 1983 University Research Fellowship. We would like to thank the ICRF Oncology Group for their cooperation, and Miss B. Kennedy for her patience in the preparation of this manuscript.

REFERENCES


