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REVIEW

Yum L. Yip · Robyn L. Ward

Anti-ErbB-2 monoclonal antibodies and ErbB-2-directed vaccines

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Abstract The tumour antigen ErbB-2 belongs to the epidermal growth factor receptor family. Numerous studies have shown that ErbB-2 is overexpressed in many cancers and it is prognostically important in a subset of malignancies. It is well recognised that this receptor has many characteristics that make it an excellent target for tumour-specific immunotherapy. One anti-ErbB-2 monoclonal antibody, Herceptin or TrastuzuMab, has already shown clinical efficacy for the treatment of metastatic breast cancer. However, despite this success, it is still currently unclear how monoclonal antibodies inhibit tumour growth in vivo. This review will summarise the biological activities of a range of anti-ErbB-2 Mabs, as well as their possible mechanisms of action. In addition, as an active mode of immunotherapy, the current vaccine strategies for inducing or enhancing ErbB-2-specific immunity will also be discussed. It is anticipated that a better understanding of the activities of anti-ErbB-2 Mabs will aid in the development of both passive and active immunotherapies against this important receptor.

Keywords ErbB-2 · Anti-ErbB-2 monoclonal antibodies · Immune responses · Vaccine

The Mab 4D5 is the murine counterpart of the humanised Mab Herceptin or TrastuzuMab. In order to remain faithful to the original studies, the name 4D5 will be used whenever the experiments were conducted with the murine Mab.

Y.L. Yip
School of Medicine,
University of New South Wales,
Sydney, NSW 2052, Australia

R.L. Ward (✉)
Department of Medical Oncology,
St. Vincent's Hospital, Darlinghurst,
NSW 2010, Australia
E-mail: r.ward@garvan.unsw.edu.au
Tel.: +61-2-92958412
Fax: +61-2-83823386

Introduction

The ErbB-2 receptor belongs to the epidermal growth factor receptor (EGFR) family. It consists of a glycosylated extracellular domain (ECD), a hydrophobic transmembrane domain, and an intracellular tyrosine kinase domain [22]. Although a direct ligand for ErbB-2 has not yet been found, ErbB-2 plays a major coordinatory role in the signalling network initiated by the EGFR family. In particular, ErbB-2 functions as a preferential heterodimerisation signalling partner with EGFR, ErbB-3 and ErbB-4 [119]. The resultant ErbB-2-containing heterodimers are characterised by their higher affinity for EGF-like ligands and diversified signalling capability [83]. ErbB-2 is overexpressed in a number of cancers [52]. While the mechanism by which ErbB-2 alters the growth of normal or malignant cells is still not entirely clear, it is possible that ErbB-2 overexpression provides tumours with an enhanced signalling activity via several pathways (for example, the MAPK pathway), and thus leads to uncontrolled growth.

For a decade, ErbB-2 has been considered as an attractive target of cancer immunotherapy. This is not only because ErbB-2 overexpression occurs in many human cancers, but also because overexpression is associated with a poor prognosis in some malignancies (for example, breast cancer). Moreover, expression appears to be stable and homogeneous in primary tumours as well as their metastases [19, 77], and ErbB-2 is only expressed at low levels in normal adult tissues. On the basis of these findings, it has been proposed that immunotherapy against ErbB-2 is likely to have an effective tumour-specific effect.

As a passive immunotherapy module, numerous anti-ErbB-2 ECD monoclonal antibodies (Mabs) have been isolated and some of them are able to inhibit tumour growth. One such antibody, Herceptin (or TrastuzuMab), is now being used in clinics with favourable results against metastatic breast cancer with ErbB-2 overexpression [106]. The molecular mechanisms

underlying these growth inhibitory effects are not well understood. It is, however, becoming clear that besides acting as activators of immune and inflammatory responses, the anti-ErbB-2 Mabs mainly exert their effects by directly altering the physiological activity of the receptor. In accordance with this point of view, it has been reported that besides the well-known tumour-inhibitory effect, anti-ErbB-2 antibodies can be tumour-stimulatory [44].

In this review, we will summarise the effects of a range of anti-ErbB-2 Mabs discovered to date and discuss their possible mechanism of action. Such a review is necessary for the optimal exploitation of anti-ErbB-2 Mabs for therapeutic purposes, as well as for the understanding of the physiological activities of the ErbB-2 receptor. In addition, recent advances in anti-ErbB-2 vaccine development will also be discussed in the context of our understanding of anti-ErbB-2 Mabs.

Approaches based on anti-ErbB-2 monoclonal antibodies

Effects of anti-ErbB-2 Mabs in vitro, in animal models and in clinical trials

The potential role of ErbB-2-specific antibodies as tumour inhibitors was originally demonstrated in neutransformed cells, where an anti-rat neu antibody, 7.16.4, induced the reversal of the transformed phenotype [30]. Subsequently, numerous anti-human ErbB-2 Mabs have been generated by immunising mice with either cancer cells expressing high levels of ErbB-2 or cell lines transfected with ErbB-2. The list of the best-characterised antibodies isolated to date is shown in Table 1.

The initial in vitro characterisation of these Mabs showed the following. (1) Anti-ErbB-2 antibodies can mediate diverse effects on cell growth, either inhibitory, stimulatory, or no effect. (2) There is a relationship between the level of ErbB-2 expression and sensitivity to the growth inhibitory effect of Mabs in breast cancer cell lines [65, 108]. Such a relationship is not necessarily observed in other ErbB-2 overexpressing tumour cell lines, such as the MNK7 gastric cells [61, 65]. (3) The intrinsic ability of anti-ErbB-2 Mabs to alter receptor function accounts for most of their differential effects on cell growth, and antibodies that share the same epitope appear to have the same spectrum of activity [6, 32, 48, 65, 114, 127].

The effect of some anti-ErbB-2 Mabs has also been evaluated in mouse models (Table 1). The panel of anti-ErbB-2 Mabs isolated by Stancovski et al. was injected intraperitoneally (i.p) or intravenously (i.v) into athymic mice bearing tumours [111]. This group showed that while most of the Mabs had significant anti-tumour effects, one of the Mabs, N28, significantly accelerated the in vivo growth of murine fibroblasts transfected with the *c-erb*B-2 gene [111] or the N87 gastric tumour xenograft [44]. Similarly, Harwerth found that Mabs could either inhibit or have no effect on tumour cell growth in nude mice [40]. Although different in vivo effects of the Mabs were observed, no simple correlation was found between the in vitro growth assays (either anchorage dependent or independent) and in vivo effects. For instance, a Mab that showed a growth stimulatory effect in vitro (e.g. N10 or FSP77) might have no or an inhibitory effect in vivo. It is clear that the in vivo effect of a Mab might not be mediated solely by a direct receptor-related function, but also by cell killing in concert with the host immune system (such as antibody-dependant cell-mediated cytotoxicity). The tumour micro- and macro-environment in vivo can further contribute to the lack of concordance between effects observed in vivo and in vitro. It is interesting to note that the various in vivo effects of the Mabs were not related to affinity or isotype, but to their different epitopes [40, 111].

Strong evidence that a single anti-ErbB-2 Mab could reduce the growth of human tumours in mice and prolong their survival was provided by Ohnishi et al. [80]. In their study using severe combined immunodeficiency (SCID) mice bearing an ErbB-2 overexpressing gastric cancer cell line, the Mab 4D5 inhibited tumour growth, reduced the mass of established tumour xenografts, and significantly prolonged mouse survival [80]. The humanised form of 4D5, termed Herceptin or TrastuzuMab [16], contains the complementarity-determining regions (CDRs) of the murine Mab together with human framework regions and IgG_1 constant domains [16]. It was found that Herceptin had similar in vitro and in vivo effects as its murine counterpart [117]. Further experiments in MCF-7-HER2 and BT-474 xenograft mouse models treated with a total dose of 3, 10, 30, or 100 mg/kg Herceptin showed the dose-dependent antitumour activity of this Mab [109].

Following the promising results in vivo, the Mab Herceptin was evaluated in clinical trials and was approved by the American Food and Drug Administration (FDA) on September 1998 for clinical use [103]. Herceptin is the first biological agent that slows the progression of breast cancer when used as a monotherapy and in combination with chemotherapy (reviewed in [103, 106]). Clinical trials showed that Herceptin inhibits ErbB-2 overexpressing cells, but does not effect normal cells or ErbB-2-negative breast cancer cells. The most impressive therapeutic efficacy was achieved by utilising paclitaxel in combination with the Mab. Herceptin was found to be well tolerated; however, the combination of Herceptin with anthracyclines resulted in a high rate of cardiac side effects [103, 106].

Mechanisms of action of anti-ErbB-2 Mabs

Anti-ErbB-2 Mabs can affect tumour growth by both indirect and direct mechanisms. The indirect mechanism involves the classical pathways in which Mabs kill tumour cells by mediating antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent

Table 1 Growth effects of the best characterised anti-ErbB-2 Mabs (*IM* immunogen *N/A* not assessed, *U* Unknown, *CHO* carbohydrate, *HER2 cells* cells transfected with ErbB-2, *s.c.* subcutaneous)

Mab	IM[a]	In vitro[b]	Assay used (cell lines)	In vivo (mouse model)	Epitope[c]	Reference
4D5	1	Inhibition	Cell proliferation (breast cancer cell lines)	Inhibition (SCID mouse with gastric carcinoma xenograft)	CHO 529–627	[43, 65, 117, 109]
2C4	1	Inhibition	Cell proliferation (breast cancer cell lines)	N/A	U	[43, 32]
7F3	1	Inhibition	Cell proliferation (breast cancer cell lines)	N/A	Same as 2C4	[43, 32]
BD5	1	Inhibition (70%) Inhibition (60%)	Cell proliferation (SK-BR-3) Soft agar (SK-BR-3)	N/A	U	[72, 127]
RC1	1	No effect Inhibition (20%)	Cell proliferation (SK-BR-3) Soft agar (SK-BR-3)	N/A	U	[72, 127]
TA1	1	Inhibition (90%) No effect	Cell proliferation (SK-BR-3) Soft agar (SK-BR-3)	N/A	U	[72, 127]
NB3	1	No effect Inhibition (20%)	Cell proliferation (SK-BR-3) Soft agar (SK-BR-3)	N/A	78–242	[72, 127, 10]
PB3	1	Inhibition (83%) Inhibition (20%)	Cell proliferation (SK-BR-3) Soft agar (SK-BR-3)	N/A	78–242	[72, 127], [10]
ID5	1	Inhibition (65%) Inhibition (30%)	Cell proliferation (SK-BR-3) Soft agar (SK-BR-3)	N/A	U	[72, 127]
OD3	N/A	No effect	Cell proliferation (SK-BR-3) Soft agar (SK-BR-3)	N/A	U	[127]
N10	2	Stimulation (247%)	Cell proliferation (SK-BR-3)	Inhibition: 54% (s.c. injection of HER2 cells)	U	[111]
N12	2	Inhibition (63%)	Cell proliferation (SK-BR-3)	Inhibition: 2% (s.c. injection of HER2 or N87)	Class I 531–586	[44, 53, 130]
N24	2	Stimulation (196%)	Cell proliferation (SK-BR-3)	Inhibition: 84% (s.c. injection of HER2 cells)	U	[111]
N28	2	No effect (107%)	Cell proliferation (SK-BR-3)	Stimulation: 41% (s.c. injection of HER2 or N87)	Class VIII 216–235	[44, 53, 130]
N29	2	Inhibition (72%)	Cell proliferation (SK-BR-3)	Inhibition: 0.3% (s.c. injection of HER2 cells)	Class IX	[111, 44, 53]
L87	3	N/A	N/A	No effect (s.c. injection of N87 cells)	Class III 220–235	[53, 130]
L26	3	N/A	N/A	Inhibition: 15% (s.c. injection of N87 cells)	Class II	[53]
L140	3	N/A	N/A	Inhibition: 39% (s.c. injection of N87 cells)	Class IV	[53]
L431	3	N/A	N/A	Inhibition: 18% (s.c. injection of N87 cells)	Class I	[53]
MRG2	4	No effect	Cell proliferation (Calu-3)	N/A	U	[114, 110]
MRG3	4	Inhibition	Cell proliferation (Calu-3)	N/A	U	[114, 110]
FRP5	2	No effect Stimulation	Cell proliferation (SK-BR-3) Soft agar (NIH3T3#3.7)	No effect (s.c. injection of NIH3T3#3.7 or SKOV3 cells)	U	[39, 40]
FSP16	2	No effect Stimulation	Cell proliferation (SK-BR-3) Soft agar (NIH3T3#3.7 cells)	No effect (s.c. injection of NIH3T3#3.7 or SKOV3 cells)	Same as FRP5	[39, 40]
FWP51	2	No effect Inhibition	Cell proliferation (SK-BR-3 cells) Soft agar (NIH3T3#3.7)	Inhibition (s.c. injection of NIH3T3#3.7 or SKOV3 cells)	U	[39, 40]

Table 1 Continued

Mab	IM[a]	In vitro[b]	Assay used (cell lines)	In vivo (mouse model)	Epitope[c]	Reference
FSP77	2	Inhibition (10%)	Cell proliferation (SK-BR-3)	Inhibition (s.c. injection of NIH3T3#3.7 or SKOV3 cells)	U	[39, 40]
		Stimulation	Soft agar (NIH3T3#3.7)			
TAb250	1	Inhibition (52%)	Soft-agar (SK-BR-3)	N/A	U	[107, 62]
		Inhibition	Cell proliferation (SKOV-3)			
TAb255	1	Inhibition (59%)	Soft-agar (SK-BR-3)	N/A	U	[107, 62]
		Inhibition	Cell proliferation (SKOV-3)			
TAb257	1	Inhibition (45%)	Soft-agar (SK-BR-3)	N/A	U	[107, 62]
		Inhibition	Cell proliferation (SKOV-3)			
TAb260	1	Inhibition (44%)	Soft-agar (SK-BR-3)	N/A	U	[107, 62]
		No effect	Cell proliferation (SKOV-3)			
TAb263	1	No effect	Soft-agar (SK-BR-3)	N/A	U	[107, 62]
			Cell proliferation (SKOV-3)			
E401/CH401	5	Inhibit	Cell counting (SV22, SK-BR-3)	N/A	292–370	[47, 102]

[a] *1* NIH-3T3 cells transfected with ErbB-2; *2* SK-BR-3 cells; *3* purified ErbB-2 ECD; *4* Calu-3 cells; *5* SV22 cells transfected with ErbB-2
[b] Percentage figures in parentheses indicate extent of growth compared to the no-treatment control
[c] Classes of epitopes as defined by Klapper et al. [53]

cytotoxicity (CDC). The direct mechanism involves the binding of Mabs to ErbB-2 to directly alter the receptor's signalling properties. Although the latter mechanism is considered to be the principle mechanism by which anti-ErbB-2 Mabs alter tumour cell growth [65, 109], the precise mechanisms of action are still poorly understood. Several mechanisms have been proposed [6, 44, 51, 53, 74], and these are outlined below, as well as in Fig. 1 and Table 2.

Internalisation and/or down-regulation of ErbB-2

One of the first mechanisms of action attributed to inhibitory Mabs was the removal of ErbB-2 from the cell surface. This process has been variably termed receptor internalisation, endocytosis, receptor down-regulation, or receptor turnover. In this review, the term ErbB-2 receptor internalisation or endocytosis will refer to the process by which ErbB-2 is drawn into the cytoplasm. This normally coincides with antibody internalisation. The term receptor down-regulation or receptor turnover will be used to describe a long-term decrease in the number of receptors as a net result of internalisation and degradation. Receptor turnover is usually determined by ^{35}S-methionine labelling of the cells, followed by antibody treatment and immunoprecipitation, or by specific labelling of surface ErbB-2. In defining these terms, it is clear that internalisation of the receptor and/or antibody can occur without a significant effect on receptor turnover, since the receptor can be recycled to the surface after internalisation.

The inhibitory effect of the first isolated anti-neu Mab, 7.16.4, was demonstrated to be related to down-regulation of neu from the surface [12, 30, 48]. One of the first effects induced by 4D5 treatment was also reported to be the down-regulation of ErbB-2 [43, 101]. In addition to 4D5, other inhibitory Mabs, such as N24, N29, FSP5, Tab-250, have been reported to cause ErbB-2 down-regulation or increase the rate of ErbB-2 turnover [39, 107, 111].

Inhibition of tumour growth has also been associated with an intrinsic ability of anti-ErbB-2 antibodies to induce endocytosis, without a significant effect on receptor down-regulation [44, 53, 71, 114]. ErbB-2 endocytosis is usually monitored by measuring the internalisation of Mab [44, 71, 114] or ErbB-2 itself [53]. In the former case, the assumption is made that the anti-ErbB-2 antibodies are always coupled to ErbB-2 even when internalised. Maier et al., by using gold-labelled Mab TA1 and electron microscopy, reported that after 30 min, the TA1-gold was mainly found on the cell surface or endosomes, with no significant routing to lysosomes [71]. Similarly, it has been reported that internalised receptor was apparently replaced within 1 h after TA1 or ID5 treatment, as down-regulation of the receptor was not detected [127].

Whilst tumour inhibitory effects can be related to the ability of the Mab to cause receptor internalisation

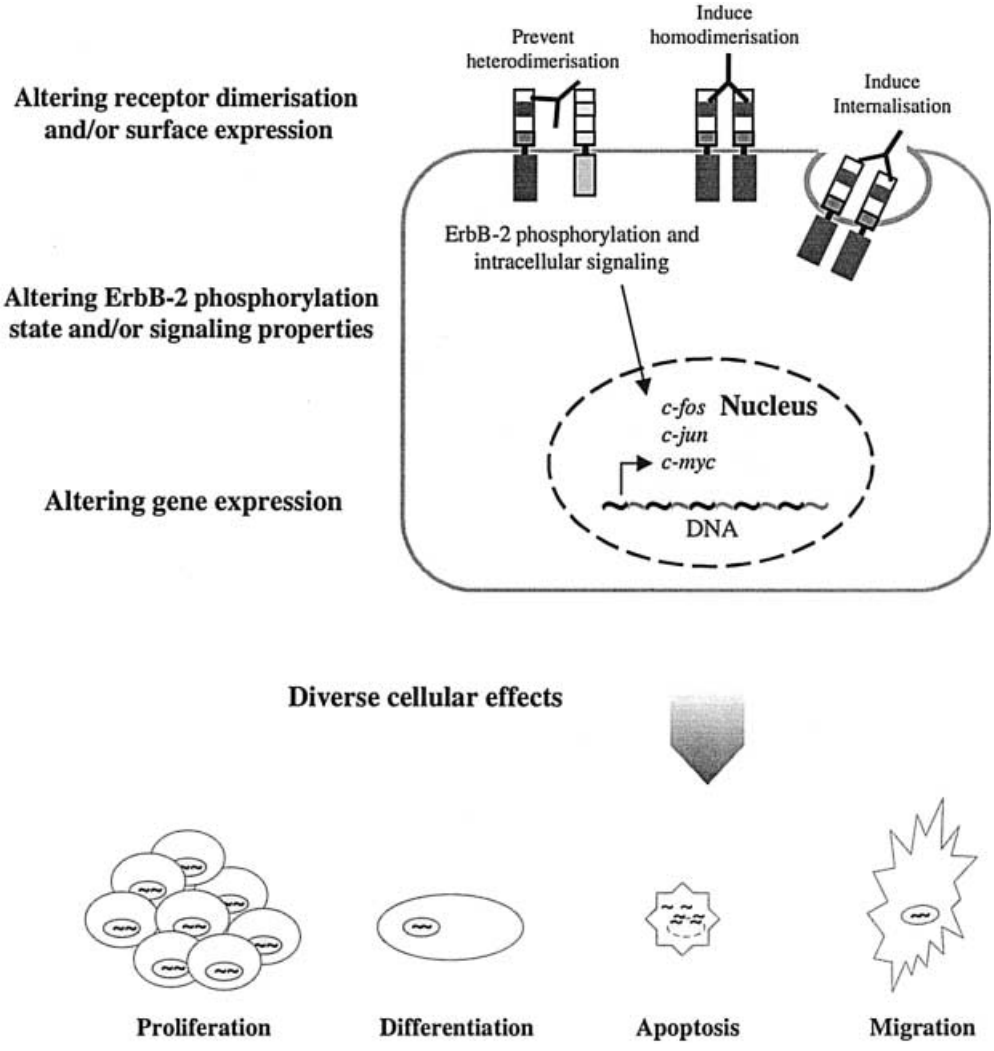

Fig. 1 Proposed mechanisms of action of anti-ErbB-2 Mabs

and/or degradation, studies that compared the internalisation rate and clonogenic growth of tumour cells in the presence of effective and ineffective antibodies showed that growth inhibition did not always correlate with internalisation of ErbB-2 receptors [111, 127]. Also, while the Mabs FWP51 and FSP77 could both inhibit tumour growth, only FSP77 effectively down-regulated ErbB-2 [40]. Shawver et al. further argued that increased receptor degradation was cell-line-dependent and was not required for growth inhibition [107]. Moreover, it should be noted that the stimulatory Mab N28 appeared to cause ErbB-2 down-regulation as well [111].

Clearly, it may be argued that down-regulation of ErbB-2 could induce growth inhibitory effects on its own by decreasing the signalling efficiency of the entire signalling network. In accordance with this is the established fact that the growth inhibitory effect of anti-ErbB-2 Mabs is dependent on the extent of ErbB-2 overexpression [65, 114]. The down-regulation of ErbB-2 could result in the disruption of ErbB-2 homodimer or heterodimer formation and consequently decreased receptor phosphorylation and catalytic activity. In a recent study, Klapper et al. showed that after treatment with an inhibitory Mab, L26, enhanced ErbB-2 degradation was observed and this was preceded by the recruitment of the c-Cbl ubiquitin ligase to ErbB-2 and subsequent ErbB-2 poly-ubiquitination, which directed ErbB-2 to a proteolytic pathway [54]. However, the same group demonstrated that the tumour stimulatory antibody N28 could also recruit c-Cbl [64]. Further studies are surely required to clarify these nuances. Finally, it should be noted that while ErbB-2 internalisation and down-regulation by Mab is relatively slow and inefficient [7, 71], most of the experiments performed to date use a relative short time-course to measure these events (about 1 h). A longer time course may be required in order to take into account the possible recycling event.

Blocking receptor heterodimerisation

An alternative mechanism of Mab-induced tumour inhibition may involve the prevention of heterodimerisa-

Table 2 Mechanisms of action of the best characterised Mabs isolated to date (*N/A* not assessed)

Mab	Effects on cell growth	Internalisation	ErbB-2 down-regulation	Phosphorylation of ErbB-2	Effects on cell cycle, differentiation and apoptosis	Ligand blocking	Reference
4D5	Inhibition	Yes	Degradation	Controversial	Cell cycle arrest at G_1 No effect on apoptosis	Yes, HRG	[32, 68] [109]
2C4	Inhibition	N/A	N/A	N/A	N/A	Yes, HRG	[32, 66]
7F3	Inhibition	N/A	N/A	N/A	N/A	Yes, HRG	[32, 66]
BD5	Inhibition	N/A	N/A	N/A	N/A	Partial, HRG	[127]
ID5	Inhibition	N/A	N/A	Increased	N/A	Partial, HRG	[126, 127]
TA1	No effect	Yes (Mab)	No effect	No effect	Differentiation	N/A	[5, 71, 126]
N10	Inhibition (in vivo)	N/A	No effect	No effect	N/A	No	[111]
N12	Inhibition (in vivo)	Yes (Mab)	No effect	Slightly increased	Differentiation	No	[6, 111, 44]
N24	Inhibition (in vivo)	Yes (Mab)	Degradation	Slightly increased	Differentiation	No	[6, 111, 44]
N28	Stimulation (in vivo)	No	Controversial	Strongly increased	Decrease cells in G_0/G_1	No	[6, 111, 44]
N29	Inhibition (in vivo)	Yes (Mab)	Degradation	No effect	Differentiation. Cell cycle arrest at late S, early G_2	No	[6, 111, 44]
L87	No effect (in vivo)	No	No effect	No effect	N/A	No	[53]
L26	Inhibition (in vivo)	Yes (ErbB-2)	Degradation	Increased	N/A	Yes, EGF and HRG	[53, 54]
L140	Inhibition (in vivo)	Yes (ErbB-2)	Moderate	Increased	N/A	No	[53]
L431	Inhibition (in vivo)	Yes (ErbB-2)	Degradation	Increased	N/A	No	[53]
MRG2	No effect	No	N/A	No effect Slightly increased	N/A	N/A	[110, 114]
MRG3	Inhibition	Yes (Mab)	N/A	Increased	N/A	N/A	[110, 114]
FRP5	No effect (in vivo)	N/A	Degradation	Increased	N/A	N/A	[39, 40]
FSP16	No effect (in vivo)	N/A	Degradation	Increased	N/A	N/A	[39, 40]
FWP51	Inhibition (in vivo)	N/A	Degradation	Increased	N/A	N/A	[39, 40]
FSP77	Inhibition (in vivo)	N/A	Little effect	Slightly increased	N/A	N/A	[39, 40]
TAb250	Inhibition	Cell line dependent	Cell line dependent	Increased	N/A	N/A	[107]
TAb255	Inhibition	N/A	N/A	Increased	N/A	N/A	[107]
TAb257	Inhibition	N/A	N/A	Increased	N/A	N/A	[107]
TAb260	Inhibition	N/A	N/A	Increased	N/A	N/A	[107]
TAb263	No effect	N/A	N/A	No effect	N/A	N/A	[107]
Mab74	Inhibition	N/A	N/A	Increased	Apoptosis	N/A	[51]
E401/CH401	Inhibition	Yes (Mab)	Down-regulation	Increased	Cell cycle arrest at G_1 Apoptosis.	No	[47, 102]

tion of ErbB-2 with other ErbB proteins, thereby effectively blocking growth-factor-mediated signal transduction. For example, antibodies that bind within or near the postulated low-affinity ligand binding site(s) might block ligand binding by direct competition or steric hindrance. Indeed, a group of anti-ErbB-2 Mabs (Class II) have been shown to inhibit the binding of HRG and EGF to their direct receptors [53]. These Mabs were effective as Fab fragments and they mediated their effect by accelerating ligand dissociation and blocking cross-talk between the receptors [53]. In previous studies, the Mabs BD5 and ID5 [127], as well as 2C4 and 7F3 [66], were also reported to inhibit HRG binding.

Phosphorylation of ErbB-2

The question of whether ErbB-2 phosphorylation is necessary for Mab-induced inhibition has been the subject of constant debate. Both a decrease and an increase of receptor phosphorylation have been associated with the tumour-inhibitory effects of these Mabs. Shawver et al. reported that receptor phosphorylation is not necessary for the inhibitory effect of the Mabs [107]. In contrast, several groups reported that anti-ErbB-2 Mabs exert their growth-inhibitory effects by having a partial agonist effect, leading to the stimulation of ErbB-2 tyrosine autophosphorylation and the induction of receptor internalisation [43, 71]. In a study where the phosphorylation of ErbB-2 and different intracellular substrates was analysed after 10 min of treatment with various Mabs, a correlation was found between antibodies that inhibit cell growth and the intensity of tyrosine phosphorylation [125]. This group further showed that at later time intervals serine phosphorylation of at least three intracellular substrates was increased preferentially by growth-inhibitory antibodies [125]. In addition, antibodies had no effect on cells transfected with ICD-truncated ErbB-2 or with kinase-region-deleted ErbB-2, thus demonstrating that tyrosine kinase activity is required for growth inhibition mediated by anti-ErbB-2 antibodies [125]. However, it should be noted that the tumour stimulatory antibody N28 was also reported to strongly increase phosphorylation of ErbB-2 [111].

For the most studied Mab, 4D5 (Herceptin), conflicting results have been reported. Some studies have shown that 4D5 stimulates ErbB-2 tyrosine kinase activity and mediates the phosphorylation of intracellular substrates [101, 104]. Kern et al. further reported that inhibition of tyrosine kinase function with Genistein reversed the 4D5-induced growth inhibitory effect, thus supporting the hypothesis that 4D5 has agonistic properties [50]. In contrast, Kumar et al. reported the inhibition of serum-stimulated ErbB-2 phosphorylation in SK-BR-3 breast cancer cells with 4D5 [59]. This study showed that 4D5 inhibited ErbB-2 tyrosine phosphorylation in the long term, and this effect could not be completely accounted for by ErbB-2 down-regulation [59]. A 4D5-mediated blockade of signal transduction is also supported by Lane et al., who showed that treatment of 4D5 for 1 h decreased the phosphorylation level of ErbB-2, as well as other intracellular proteins [61].

Several factors may contribute to these conflicting results. The cell line used may be important. For example, the breast cancer cell lines SK-BR-3 and BT-474 had different responses in terms of receptor phosphorylation and turnover after Tab-250 treatment [107]. The duration and condition of treatment can be another factor. Recently it has also been proposed that the composition of the lysis buffer used to prepare the cell lysates may affect the results [109]. Herceptin or 4D5 results in ErbB-2 tyrosine phosphorylation only when lysates are prepared in non-denaturing conditions (non-ionic detergent, Triton X-100), but not in denaturing conditions (sodium dodecyl sulphate). Under non-denaturing conditions, the observed phosphorylation may not be physiological [109].

Interference with gene expression

Although the downstream events that occur following the interaction between ErbB-2 and anti-ErbB-2 antibodies are uncharacterised, some evidence suggests that the antibodies can interfere with specific programmes of gene expression [85, 104].

Treatment of confluent BT-474 cells with 4D5 caused a transient and early (1 h) 5- to10-fold increase in *c-fos* mRNA levels after the stimulation of intracellular substrates [104]. Park et al. also reported that the attenuation of neu receptor signalling observed after anti-neu Mab 7.16.4 treatment was associated with activation or recovery of a functional tumour suppressor-like gene TBP-1 [85].

Modulation of cell cycle progression

Cell cycle progression is modulated by a range of gene products, such as cyclins and cyclin-dependent kinases (CDKs). Increasing evidence suggests that downstream gene targets link signalling pathways to cell cycle control molecules, such as cyclin D1 and c-Myc (reviewed in [57]). Several anti-ErbB-2 Mabs that effect cell growth have been reported to alter normal cell cycle progression.

Bacus et al. first reported that the tumour inhibitory Mab N29 could specifically induce growth arrest at late S or early G_2 phase of the cell cycle in AU-565 breast cancer cells [6]. Recently, several reports demonstrated the effects of inhibitory Mabs on the G_1 phase of the cell cycle. The Mab ID5 caused G_1 arrest by a mechanism that was independent of p53 [63]. Similarly, 4D5 treatment also resulted in a block of the G_1/S transition in ErbB-2 overexpressing cells, such as BT-474, SK-BR-3 breast cancer cells and OVCA420 ovarian cancer cells [61, 109, 129]. In both cases, an increased amount of the $p27^{kip1}$ CDK inhibitor was found in cells treated with the anti-ErbB-2 Mabs [61, 63, 129].

In contrast, incubation with the stimulatory N28 Mab was reported to reduce the proportion of AU-565 breast cancer cells in the G_0-G_1 phase, and slightly increase the proportion of cells in other phases of the cycle [6].

Taken together, these data provide evidence that anti-ErbB-2 Mabs can modulate cell growth by affecting the progression of cells through the cell cycle.

Induction of cellular differentiation

The induction of cellular differentiation by Mabs has been proposed as an additional mechanism by which they exert their tumour-inhibitory effects [6].

Differentiation of AU-565 breast cancer cells was achieved by treating the cells with the Mabs TA1 or N29 [5, 6]. After Mab treatment, AU-565 cultures exhibited morphological maturation, characterised by synthesis and secretion of milk components (casein and lipids), and the induction of mature cell morphology (large lacy nuclei surrounded by flat cytoplasm). Differentiation in the AU-565 cells was also associated with the translocation of ErbB-2 from the membrane to a perinuclear site and cell cycle arrest at the G_2 phase [5, 6].

Apoptosis

The mechanism of tumour growth inhibition by anti-ErbB-2 Mabs was previously considered to be purely cytostatic [48]. Several studies have now shown that certain anti-HER2 antibodies can exhibit cytotoxic activity [51, 67, 102, 127].

Kita et al. first showed that cytotoxicity mediated by Mab74 was due to the induction of apoptosis, as judged by morphological assessment and the TUNEL apoptosis assay [51]. Later, another group also reported that tumour growth inhibition could be induced by apoptosis [60, 102]. The Mab CH401 caused cell cycle arrest and apoptosis in an ErbB-2-transfected cell line SV22 [102]. Similarly, Kunisue et al. showed Herceptin (4D5) induced both apoptosis and cell cycle blockade in an ErbB-2-transfected MCF-7 cell line, ML-20 [60]. The cytotoxic mechanism of CH401 was independent of down-regulation of ErbB-2 receptors or of internalisation of the Mabs [102]. Interestingly, both Mab74 and CH401 appeared to stimulate tyrosine phosphorylation of the ErbB-2 protein, thus suggesting that ErbB-2 signalling activity might be required to induce apoptosis [51, 102].

It is also worth noting that the use of multimeric antibodies directed against ErbB-2 has also been shown to activate apoptotic cell death pathways in tumour cells that overexpress ErbB-2 protein [35].

Synergistic effects of Mabs with chemotherapy drugs

Clinical trials clearly showed that Herceptin and chemotherapy treatment was superior to chemotherapy alone (see above). Combination studies with cisplatin revealed that 4D5, as well as other anti-ErbB-2 Mabs potentiate cytotoxicity by decreasing DNA repair following cisplatin-induced DNA damage [4, 90]. Additionally, 4D5 enhanced the inhibitory effects of cytokines [58] and anti-oestrogens such as tamoxifen [60, 124]. A recent report further showed that combination therapy with external radiotherapy and 4D5 yielded potent synergistic anti-tumour effects in a breast cancer xenograft model [91].

Although the mechanisms of these additive or synergistic responses are only just beginning to be understood, they seem to involve the Mab effects on cell cycle control. Pietras et al. evaluated the mechanism for the synergy between Mab and cisplatin by measuring DNA synthesis as well as the formation and repair of cisplatin-induced DNA adducts in cancer cells [90]. They found that treatment of ErbB-2-overexpressing cells with cisplatin led to a dose-dependent increase in DNA synthesis, which was significantly reduced by combined treatment with 4D5. Therapy with Mab also led to a 35–40% inhibition of DNA repair after cisplatin exposure and, as a result, promoted drug-induced killing in target cells [90]. In another study, Pietras et al. showed that anti-ErbB-2 Mabs caused irradiated cells to exit cell cycle arrest more rapidly than normal, even before their DNA was repaired [91]. Consequently, cells were forced to divide with damaged DNA and, as a result, triggered pathways that eventually lead to apoptosis. These effects appear to be caused by anti-ErbB-2-stimulated changes in the key cell-cycle regulator protein $p21^{WAF1}$, which is responsible for the cell cycle arrest induced by irradiation [91].

Effects on invasion and metastasis

Apart from the ability to effect cell growth, recent studies showed that anti-ErbB-2 may regulate the processes involved in metastasis.

Treatment of SKBR-3 human breast cancer cells in vitro with 4D5 resulted in a dose-dependent reduction in the expression of potent angiogenesis growth factors VEGF/VPF [89]. In contrast, the Mab ID5 appeared to induce tyrosine phosphorylation of ErbB-2, increase invasiveness, and increase expression of the adhesion molecule CD44 (HCAM) [126].

Summary

As summarised above, the proposed mechanisms of action of the anti-ErbB-2 Mabs are diverse. It should be noted that some of the effects induced by the Mabs reminisce those induced by the range of EGF-related ligands [113], thus further supporting the fact that Mabs act through actively altering the signalling activity of ErbB-2. At this stage, however, too few antibodies have been compared to know which of the functional effects

are linked and which are independent. It is also not yet clear which functional effects are necessary or sufficient for efficacy in vivo.

It is currently unclear whether ErbB-2 activation is necessary for receptor internalisation. On one hand, it has been shown that tyrosine kinase activity was required for down-modulation of oncogenic neu [12]. In fact, the rate of internalisation and degradation of the oncogenic neu protein were remarkably higher than wild-type neu [112, 128]. Mutation of human ErbB-2 to the activated form also facilitated down-regulation by Mabs [40, 120]. In addition, non-activated neu-transfected cells were not sensitive to anti-receptor antibody treatment, and the neu receptor on these cells could not be down-regulated [84]. On the other hand, experiments that made use of various mutants of ErbB-2 led to the conclusion that neither autophosphorylation sites nor tyrosine kinase activity were required for antibody-mediated endocytosis of ErbB-2 [71].

The relationship between internalisation and differentiation [5, 6], or between cell cycle progression, differentiation and apoptosis is also unclear. Whilst Bacus et al. reported that the anti-ErbB-2 antibodies with tumour inhibitory effects could specifically induce cell growth arrest at late S or early G_2 phase, as well as cellular differentiation [6], Mab ID5 was shown to induce G_1 arrest without causing differentiation or apoptosis [63].

Conceivably, several independent mechanisms may be responsible for the tumour growth inhibitory effects of anti-ErbB-2 Mab. The difficulties in interpretation also stem from the fact that the function of ErbB-2 itself is not well identified. Therefore, the question as to which in vitro assays best reflect the in vivo tumour inhibitory properties of a Mab is still not clear. Several factors, however, clearly determine the efficacy of anti-ErbB-2 Mabs.

Regarding the antibody itself, factors such as bivalency and its epitope can influence the efficacy of the Mab. Whilst some groups showed that intact antibodies were not required for growth inhibition and Fab fragments from Mabs could still inhibit the anchorage-independent growth tumour cells [127], others found that the bivalency of the Mab was required for tumour inhibitory effects [107, 110]. For example, the bivalent $F(ab')_2$, but not Fab, stimulated receptor autophosphorylation and inhibited tumour cell growth [107]. Entire Mabs were reported to induce internalisation in cancer cells, whereas their Fab portions were not internalised [110]. Clearly, as classified by Klapper et al., two types of Mabs are present; one type that can function as a Fab and another type that cannot [53]. The important determinant of this differential property is the epitope of the Mabs. We have recently provided direct evidence to the fact that the epitope location of an anti-ErbB-2 Mab does determine its biological activity [130]. It can be anticipated that with more epitopes of anti-ErbB-2 Mabs being mapped, it could be possible to derive an epitope-function relation of these Mabs and provide a better understanding of the whole range of biological effects of anti-ErbB-2 Mabs.

The measurement of the efficacy of anti-ErbB-2 Mabs can also be influenced by the set of experiments chosen to evaluate their effects, as well as experimental conditions. Although the cell proliferation assay is one of the most commonly used methods to determine the effects of Mabs on tumour cell growth, our experience has shown that it is necessary to perform this assay using a range of cell lines in order to obtain a more representative result. Furthermore, as variations between assays are observed, where possible this assay should not be used as the sole method of determining the effect of a Mab on cell growth. Biochemical studies, such as the determination of tyrosine phosphorylation level of ErbB-2, may not be conclusive and could lead to confusing results. For example, in the case of 4D5, some studies have shown an agonist effect with increased tyrosine phosphorylation of ErbB-2 [50, 101, 104], while others have shown decreased phosphorylation [59, 61]. Some reports have also variably related other Mab-induced inhibition to phosphorylation [125]. Clearly, experimental conditions may lead to these conflicting results. While short-term treatment of Mab can increase phosphorylation (e.g. 10 min) [125], long-term treatment (>1 h) may result in dephosphorylation [61, 101]. Moreover, the results can be cell-line-dependent. It appears that cellular assays, such as cell cycle analysis and apoptosis, correlate better with the ultimate biological effect of a Mab. It should, however, be noted that the use of transfected cell lines may not be ideal, as they do not represent true malignant cells and may be deficient in certain downstream components important in mediating the effects of the Mabs.

Anti-ErbB-2 immune responses

Treatment of cancer patients with an anti-ErbB-2 Mab requires repeated intravenous treatments with large doses of antibodies. The generation of an active immune response to ErbB-2 in patients represents an attractive alternative to passive immunotherapy. Moreover, an appropriately activated tumour-specific immune response can be further amplified, thereby inducing a long-lasting protection.

As a potential vaccine target, ErbB-2 has a number of advantages. Firstly, some patients with ErbB-2-positive cancers develop an immune response to the protein, thus indicating that tolerance to this self-protein can be overcome (see below). Secondly, the high expression of ErbB-2 on cancer cells in comparison to normal tissues suggests that such a response should be specific for the tumour with no or minimal toxicity to normal tissues. Finally, as ErbB-2 has an important role as a growth factor receptor, an immune response (especially an appropriate antibody immune response) can induce cancer cell death by multiple mechanisms. Numerous vaccine strategies against ErbB-2 have been described and these will be discussed below.

The endogenous anti-ErbB-2 immune responses in cancer patients

Incidence

The existence of serum antibodies and T cells reactive to ErbB-2 was first reported in patients with breast cancer whose tumours overexpressed ErbB-2 [25, 95]. Since then, a number of other studies have documented the existence of this endogenous immune response, both humoral and cellular, against ErbB-2.

In a study of 127 breast cancer patients, Disis et al. detected serum anti-ErbB-2 antibody titres of greater than 1:100 in 14 of 127 (11%) patients versus none of 200 normal women. In particular, five patients with early-stage disease had a substantial humoral response, with antibody titres greater than 1:5000 [29]. Anti-ErbB-2 antibodies were also found in colorectal cancer patients [122] and in advanced metastatic breast cancer patients with circulating ErbB-2 ECD [121]. A recent study has shown that antibody responses to all the ErbB receptors were present in patients with different types of epithelial malignancies [8]. The presence of higher titres of anti-ErbB-2 antibodies correlated with ErbB-2 overexpression in the primary cancer of those individuals with early-stage disease [29, 122]. This correlation was not observed in advanced-stage breast cancer patients, and the incidence of antibodies in these patients was also less than that observed in early stage disease [29].

Helper T cell immunity to ErbB-2 has been detected concurrently with anti-ErbB-2 antibodies [25]. In fact, as the serum antibodies against ErbB-2 are mainly IgG or IgA [29, 121, 122], and the switch from IgM to an IgG or IgA response requires T cell help, it follows that ErbB-2 contains helper T cell epitopes. Indeed, several groups have successfully identified helper T cell epitopes on ErbB-2 [25, 28, 34, 55, 118]. In all of these studies, potential helper T cell epitopes were first chosen by computer algorithms. Synthetic peptides that corresponded to these potential epitopes were then synthesised and assessed for their ability to produce lymphokines or to stimulate the proliferation of peripheral blood mononuclear cells (PBMC) from healthy or cancer patients [25, 34, 118]. In a recent study, Kobayashi et al. have identified several helper T cell epitopes with promiscuous HLA-DR binding characteristics using this strategy. One of the peptides was able to induce ErbB-2-specific helper T cell responses restricted to a range of HLA-DR in vitro. Moreover, the peptide-reactive helper T cells were able to recognise naturally processed ErbB2 protein presented by antigen-presenting cells (APC) [55].

The presence of ErbB-2 reactive cytotoxic T lymphocytes (CTLs) has also been described in patients with ErbB-2 overexpressing tumours [46, 87, 132]. These CTLs were isolated and expanded by in vitro stimulation of tumour-associated lymphocytes with autologous tumour cells [46, 56, 131]. As the induction of ErbB-2-specific CTLs would require the processing and presentation of peptides, several HLA-type-restricted ErbB-2 CTL peptide epitopes have been identified, based on consensus motifs for binding to specific HLA types. These potential CTL epitopes were then used to pulse dendritic cells, which were tested for their ability to generate CTLs capable of lysing either peptide-sensitised target, ErbB-2-transfected or autologous cancer cells [11, 69, 99, 105]. Table 3 shows the list of the identified ErbB-2 CTL and helper T cell epitopes.

The ability of certain generated ErbB-2-specific CTLs to lyse autologous tumour cells indicates that multiple ErbB-2-derived epitopes can be naturally processed and presented on the surface of tumour cells. Indeed, the existence of ErbB-2 antigenic peptides presented on the tumour cell surface by HLA class I has been directly demonstrated by acid elution of tumour cells [87, 88]. Several tumours, such as breast [87], ovarian [87, 99], non-small cell lung [88], pancreatic [86], renal cell [11, 105] and colon [11], were found to express ErbB-2 CTL epitopes. Moreover, it has been shown that some ErbB-2 CTL epitopes are shared between several distinct types of epithelial tumours [11, 87].

Taken together, these studies suggest that ErbB-2 is not only presented by tumour cells, but also can elicit both humoral and cellular immune responses in cancer patients.

The role of existent immunity in immune surveillance and cancer progression

Clearly, one of the important questions raised by the finding of endogenous immune responses in patients relates to the anti-tumour effects of such a response. Several studies have suggested a possible beneficial role of these endogenous immune responses.

In a study of more than 1,000 breast cancer patients, ErbB-2 overexpression was associated with a favourable prognosis in stage I patients with a lymphoplasmacytic infiltration (LPI) in their primary tumour [98]. A recent study further showed that whilst ErbB-2 overexpression correlated with poor prognosis in node-negative patients without LPI, overexpression correlated with a good prognosis in node-negative LPI-positive patients [115]. These findings may suggest that the immune response can decrease tumour progression during its early stages of development. Consistent with this hypothesis is the fact that the presence of higher anti-ErbB-2 antibody titres correlated with the presence of ErbB-2 expression by the primary cancer only in early-stage disease [29, 95]. It has also been shown that there is a significant link between the presence of HLA-A2-positive, ErbB-2-negative tumours and intratumoral T cells in primary breast tumours. In these HLA-A2-positive breast cancer patients, it has been suggested that the host immune response contributed to the elimination of ErbB-2 overexpressing tumour cells [78, 79].

Despite these promising speculations, the evidence to date only reflects part of the immune repertoire in a subset of patients. It is not yet possible to know whether

endogenous T cells, and especially antibody responses, are truly beneficial and can cause immune destruction of cancer. The demonstration of a beneficial effect of the anti-ErbB-2 immune responses will require large epidemiological studies and many years of follow-up. Such studies have not yet been conducted.

The induction of an active immune response to ErbB-2

While it is not clear whether an immune response to HER-2/neu results in improved survival, the existence of such a response does predict that vaccines will be able to induce and boost immunity to HER-2/neu. Over the years, several animal models have validated the fact that immunity to ErbB-2 can be elicited by vaccination. Evaluation of anti-ErbB-2 vaccines in animals must address safety, immunogenicity and efficacy.

Factors to consider in the design of vaccines against ErbB-2

The major goals of any cancer vaccine are to induce a specific immune response against the tumour antigen that can mediate tumour rejection, and to establish a long-term immunological memory. Factors such as the form of immunogen used and the mode of tumour challenge need to be considered when constructing a vaccine and designing an immunisation schedule. In the case of anti-ErbB-2 vaccines, the problem of tolerance and autoimmune toxicity should also be taken into consideration, as ErbB-2 is a self-protein. Animal models using transgenic mice allow an examination of the effects of immunological tolerance to ErbB-2. Two types of rat neu transgenic mice have been developed. In one type of transgenic mice, the wild-type rat *neu* cDNA was under the control of a murine mammary tumour virus (MMTV) 3' long terminal repeat promoter [38]. Female mice develop spontaneous focal mammary adenocarcinomas as a consequence of overexpression of rat neu. These tumours are histologically similar to human breast cancer, and thus provide a good model for identifying vaccine approaches potent enough to overcome mechanisms of immune tolerance. In another type of transgenic mice, the activated form of neu containing a point mutation was used [37, 75]. These mice developed spontaneous mammary tumours as a result of oncogenic activation of neu. In both type of mice,

Table 3 List of identified helper T-cell and cytotoxic T-cell epitopes on ErbB-2 (numbers represent the amino acid number of the ErbB-2 protein)

Helper T-cell	Cytotoxic T-cell	Compatible HLA class	Tumours on which CTL epitope is displayed	Reference
42–56		N/A	–	[25]
	48–56	HLA-A2.1	N/A	[99]
	63–72	HLA-A24	Ovarian	[81]
	106–114	HLA-A2.1	Gastric	[56]
	369–377	HLA-A2.1	Ovarian	[33]
			A range of tumours	[69]
			Gastric	[56]
			Renal cell and colon	[11]
			Ovarian and melanoma	[99]
			Renal cell	[105]
396–406			–	[34]
	435–443	HLA-A2.1	Ovarian and melanoma	[99]
			Renal cell	[105]
474–487			–	[34]
	654–662	HLA-A2.1	Breast and ovarian	[87]
			Ovarian	[131]
			Pancreatic cancer	[86]
			Renal cell and colon	[11]
	665–673	HLA-A2.1	Ovarian	[99]
	689–697	HLA-A2.1	Gastric	[56]
			Ovarian and melanoma	[99]
			Renal cell	[105]
	754–762	HLA-A3	–	[49]
	767–775	HLA-A2.1	N/A	[99]
	773–781	HLA-A2.1	A range of tumours	[69]
777–789		HLA-DR4	–	[34, 118]
	780–788	HLA-A2402	N/A	[45]
783–797		N/A	–	[25]
	785–793	HLA-A2.1	N/A	[99]
883–892		HLA-DR1, HLA-DR4, HLA-DR52, HLA-DR53	–	[55]
884–899			–	[34]
	952–960	HLA-A2.1	Ovarian	[99]
	971–980		Ovarian	[46, 33]

vaccine efficacy can be evaluated by challenging the animals with syngeneic tumour cells expressing the transgene.

Current strategies of anti-ErbB-2 vaccination

Numerous vaccination strategies, including the use of whole ErbB-2 or ECD protein, synthetic peptides and naked DNA, have been described and these will be discussed (see Table 4).

Vaccines using tumour cells, whole ErbB-2 or ECD

Bernards et al. first reported the use of vaccination to protect mice against tumour challenge with a rat neuroblastoma cell line expressing mutated rat neu protein. This study used as the vaccine a vaccinia virus recombinant expressing the ECD of rat neu. However, the same protocol did not protect rats from tumour challenge with the rat cell line, reflecting the fact that rats were tolerant to rat neu [9]. Similarly, Disis et al. also showed that it was impossible to induce an anti-rat neu immune response in rats immunised with whole neu protein [28].

To overcome tolerance, one group has tried to immunise rats with recombinant human ErbB-2 ECD, which showed high homology with rat neu. They reasoned that a cross-reactive immune response to rat neu might be generated and would protect animals against subsequent rat neu challenge. Indeed, the rats developed a response to ErbB-2, which was also cross-reactive to rat neu. However, the induced rat neu cross-reactive antibody response was too weak to protect the mice against subcutaneous rat-neu transfected tumour cell challenge [116].

Recently, several surprising results were obtained in studies using transgenic animals. Esserman et al. reported successful induction of an anti-self immune response by immunisation with neu ECD protein [31]. In this study, a N202 transgenic mouse line that overexpressed wild-type rat neu was used. Immunisation with the neu ECD induced anti-neu humoral and cellular immunity and prevented tumour development in 50% of immunised animals [31]. Using a similar transgenic mouse model, Reilly et al. further showed that, despite significant tolerance to the transgene, neu-specific immune responses similar to those observed in breast cancer patients could be demonstrated in mice prior to vaccination. More importantly, both cellular and humoral neu-specific responses in transgenic mice could be boosted with neu-specific vaccination using irradiated whole-cell and recombinant vaccinia virus. Mice were not only protected from a neu-expressing tumour challenge, but the onset of spontaneous tumour formation was delayed [97]. In another study, an allogeneic cell-based vaccine was used in an activated rat neu transgenic mouse model [17]. While a neu-specific anti-tumour response could be induced and mice were protected from spontaneous or transplanted tumour formation, established spontaneous tumours themselves were never affected [17]. These results suggest significant differences in the immunological requirements for the rejection of established versus transplanted tumours [17].

Taken together, the data discussed above appear to suggest that despite tolerance to ErbB-2, it may be possible to induce or augment an ErbB-2-specific immune response via immunisation. Whether this truly means that tolerance can be overcome is uncertain, as it is possible that tolerance was not completely existent in certain transgenic mice. Moreover, in terms of whole ErbB-2 or cell-based vaccines, the difficulties in production and storage, and the possible existence of contaminating oncogenic materials may hamper their potential use in the clinic.

Vaccines using synthetic peptides

The use of synthetic peptides offers an alternative vaccination strategy, and one with several advantages. Peptides are simple, cheap to produce and can induce a very specific response. In addition, some groups have suggested that synthetic peptides may help circumvent the problem of tolerance [28]. Several peptide vaccination strategies are currently being evaluated.

The form in which the peptide antigen is delivered can be important in determining its immunogenicity. Several methods have been employed, and these include direct peptide vaccination with an adjuvant [24], polysaccharide-peptide conjugates [36], or peptide loading onto splenocytes or dendritic cells [76]. As an example of the second approach, Gu et al. constructed a hydrophobised polysaccharide/oncoprotein complex vaccine in order to facilitate the delivery of a 147-amino-acid long fragment of the ErbB-2 protein, which contains one or several T cell epitopes, to the MHC class I pathway [36].

The identification of defined peptide epitopes recognised by either helper T cells or CTLs (see Table 3) is of considerable interest for anti-ErbB-2 vaccine design. Disis et al. have shown that although whole rat neu protein is not immunogenic, immunisation of rats with multiple T-helper peptides derived from the rat neu elicited strong humoral and helper T cell responses [28]. Recently, these investigators further showed that immunisation of breast and ovarian cancer patients with multiple human ErbB-2 helper T cell peptides could elicit both peptide- and ErbB-2-specific T-helper cell responses [27]. This is the first demonstration that an anti-ErbB-2 immune response can be induced by vaccination in humans.

The fact that there are shared CTL epitopes between various cancers further increases the population of patients that can be targeted by peptide vaccines based on CTL epitopes (Table 3). Nagata et al. showed that this

Table 4 Summary of animal models of anti-ErbB-2 vaccination strategies. The immune responses to ErbB-2 following vaccination in a range of animal models (*N/A* not assessed, *s.c.* subcutaneous, *HTL* helper T cells, *TM* transmembrane domain)

Immunogen	Mouse model	Tumour challenge (route)	Antibody response	T cell response	Effects and remarks	Autoimmunity	Reference
Tumour cells, whole ErbB-2, ECD							
Neu-transfected allogenic mouse fibroblasts	Transgenic FVB/N mice with wild-type rat neu	Tumour implantation	Yes	Yes	Prevent formation of transplanted and spontaneous tumours. Established tumours not affected. Protection mediated by T cells	N/A	[17]
ErbB-2 transfected mouse fibrosarcoma	Balb/c mice	ErbB-2 transfected murine cells (s.c.)	N/A	Yes, CTL	The growth of tumour cells in mice was suppressed.	No	[76]
Whole 3T3-neu cells or recombinant vaccinia virus	Transgenic FVB/N mice with wild-type rat neu	NT cells derived from spontaneous mammary tumours in mice (s.c.)	Yes	Yes	Protect mice from tumour challenge. The protection is T-cell-dependent. Delay onset of spontaneous tumour	N/A	[97]
Human ErbB-2	BDIX rat	Rat B104 tumour cells expressing mutated neu (s.c.)	Yes (cross-reactive to rat neu)	Yes	Response too weak to affect growth of tumour	N/A	[116]
Whole rat neu	Rat	No	No	No	N/A		[28]
Neu ECD	N202 mice transgenic with wild-type rat neu	Spontaneous tumour	Yes	Yes	Prevent tumour development in 50% of mice	N/A	[31]
Synthetic peptides							
Polysaccharide/ErbB-2 complex	Balb/c mice	ErbB-2 transfected cells (s.c.)	Yes	Yes, CTL	Protection. CTL are the major effector cells	N/A	[36]
Rat neu synthetic helper-T cell epitopes	Rat	No	Yes	Yes, HTL	N/A	No	[28]
Spleen cells pulsed with murine ErbB-2 CTL epitope	Balb/c mice	ErbB-2 transfected murine cells (s.c.)	N/A	Yes, CTL	Protection from tumour cells challenge.	No	[76]
Dendritic cells pulsed with murine ErbB-2 CTL epitope	Balb/c mice	Pretransplanted ErbB-2 expressing tumour CMS7ME (s.c.)	N/A	Yes, CTL	Growth of CMS7ME in mice was suppressed.	N/A	[45]
Synthetic B cell epitopes	Rabbit or N202 mice transgenic with wild-type rat neu	No	Yes	N/A	Inhibit ErbB-2-overexpressing tumour cell growth in vitro. Prevent formation of spontaneous tumours in transgenic mice.	N/A	[23]
B cell epitopes	Balb/c mice	No	Yes	N/A	Epitope recognised by tumour-inhibitory effect can induce a specific tumour-inhibitory response	N/A	[130]
DNA							
Neu DNA	Balb/c mice	No	Yes (cross-reactive to murine ErbB-2)	N/A	Antibodies can inhibit tumour cell growth in vitro	No	[20]

Table 4 Continued

Immunogen	Mouse model	Tumour challenge (route)	Antibody response	T cell response	Effects and remarks	Autoimmunity	Reference
Neu DNA	FVB/N mice transgenic with activated rat neu	Spontaneous tumour	Yes	Yes	Delay tumour onset in 10/12 mice, prevent tumour in 2/10 mice, prevent metastasis	No	[3]
Neu DNA. Full length or truncated	FVB/N mice transgenic with activated rat neu	Mouse tumour cells expressing mutated neu (s.c.)	Yes	Yes	Protection. Antibody response not required for protection	N/A	[18]
Human ErbB-2 DNA. Full length or truncated	Balb/c mice	ErbB-2 transfected cells (s.c.)	N/A	N/A	Protection of tumour development in full-length DNA-immunised mice	N/A	[123]
Neu DNA (TM + ECD)	Balb/c mice transgenic with activated rat neu	Syngeneic tumour cells expressing rat neu (s.c.)	Yes	No CTL	Partial protection against tumour challenge, but stop development of spontaneous tumour	N/A	[100]

approach could successfully induce a CTL response capable of mediating tumour rejection in mice without producing any autoimmune toxicity [76]. This group further showed that the same peptides (p63 or p780) could be used to sensitise PBMC from cancer patients or normal individuals in vitro [45, 81]. The induced ErbB-2-specific HLA-A24-restricted CTL could further lyse ErbB-2 overexpressing cancer cells. The peptide p63 is now being evaluated in clinical trials [81].

Most recently, Dakappagari et al. [23] and our group [130] have shown that it is possible to use B-cell epitopes to induce a tumour-inhibitory immune response. In particular, it has been found that peptides located at the membrane proximal region of ErbB-2 ECD most sufficiently induced a tumour-inhibitory antibody response [23, 130]. Together with the fact that the epitope of Herceptin is located within the membrane-proximal region [109], it is tempting to speculate that this region will be effective in mounting a strong protective immune response.

DNA-based vaccines

DNA-based vaccines involve the direct inoculation of expression plasmids into animals. The injected DNA, once taken into the cell, can pass through the nuclear membrane and persist as a non-replicating episomal molecule, thus resulting in a long-lived foreign protein expression. Therefore, DNA vaccination has the potential advantage of inducing long-lasting immune responses against the expressed antigens [73].

Several groups have used this approach to induce anti-ErbB-2 immune responses [3, 18, 123]. Concetti et al. first reported that injections of rat neu full-length DNA into mice could generate cross-reactive anti-murine ErbB-2 autoantibodies [20]. The same group, by using rat neu transgenic tumour-bearing mice, showed that rat neu DNA vaccination could both prevent the outgrowth of mammary neoplasms and reduce their metastatic ability [3]. In another study using a transgenic mouse model, DNA vaccination with plasmid encoding transmembrane domain and ECD of rat neu elicited an anti-neu antibody response. Although the mice were only partially protected against tumour cell challenge, tumour progression was markedly inhibited [100]. This result suggested that the immune mechanisms responsible for the rejection of transplantable tumours might not be the same as those that inhibited the tumour progression [100].

Although promising, the effectiveness of this DNA-based vaccine is still limited [3]. It has been suggested that DNA vaccination might not be able to break CTL tolerance [100]. In addition, the possibility of mutagenic integration and subsequent tumour development with the use of plasmid DNA has not been fully evaluated [70].

Over the last few years, the in vivo evaluation of ErbB-2 vaccines has been performed using complex animal models. Such models show that it is feasible, and probably beneficial, to elicit an anti-ErbB-2 immune

response. With respect to the problem of tolerance, although Disis et al. and several other groups showed that immunisation with ErbB-2 whole protein or ECD would induce tolerance [9, 28], other studies using transgenic mouse models that closely mimic the clinical situation demonstrated that tolerance could be overcome even using a large ErbB-2 molecule [17, 31, 97]. In fact, in cancer patients with endogenous anti-ErbB-2 immune responses, tolerance to ErbB-2 is already overcome or partially overcome [25, 95]. Effective immunisation against ErbB-2 may thus involve the activation of an endogenous natural immunity to a set of self-epitopes that had not maintained tolerance. Cefai et al. further suggested that anti-ErbB-2 vaccines might induce a specific immune response against spontaneous ErbB-2-expressing tumours. In contrast to current belief, this group showed that established tumours might not cause a systematic immune defect that prevented the elicitation of immune responses [17]. In this respect, anti-ErbB-2 vaccines may have both preventative and therapeutic roles.

Clearly, if an anti-ErbB-2 immune response is generated, the potential for autoimmune toxicity should be evaluated. The low expression of ErbB-2 in normal tissues should allow the targeting of the immune response to tumour cells without autoimmune toxicity. It is encouraging that all the studies to date showed that the generation of neu-specific immunity revealed no evidence of autoimmunity in animals [3, 20, 28, 76] (Table 4). However, as ErbB-2 is widely expressed in fetal tissues, the possibility of fetal toxicity should be considered [96].

From the animal experiments it is apparent that whilst vaccination may be sufficient to enable animals to reject transplantable or spontaneous tumours [17, 97], and to slow the progression of hyperplastic lesions [3, 100], it was usually not effective in animals with established carcinomas [100]. This suggests that established tumours have distinctive properties and can develop mechanisms to escape immune rejection. Immune escape mechanisms can be mediated by the tumour microenvironment. For example, defective tumour capillaries may alter tumour cell accessibility [1]. The release of many suppressive factors by tumours could also render the tumours resistant to immune attack [21, 41]. In addition, the overwhelmingly high frequency of HLA-class-I down-regulation in primary breast (up to 88%) [13] and other cancers [14,15] may limit the effectiveness of vaccines that rely solely on the induction of CTL [2, 42, 97]. The occurrence of ErbB-2-negative variants, as well as the observed resistance of ErbB-2-positive cancer cells to natural killer cell and TNF-mediated cytotoxicity, may likewise represent a potential problem (reviewed in [26]).

Conclusion

Effective immune responses often involve combined antibody, helper T-cell, and CTL responses. All arms of the immune system can be directed against HER-2/neu. At present, it is still not known what type of immunity will have a therapeutic anti-tumour effect and how it can be safely generated or augmented. It is also not known what is the ideal form of immunogen. As discussed, antibodies to the ECD can profoundly alter ErbB-2 function, resulting in either stimulation or inhibition of cell growth. Whilst infusion of an inhibitory anti-ErbB-2 Mab shows beneficial results in the clinic, the paucity of data about the nature of the endogenous antibody responses in patients raises the question of whether inducing such responses would be advantageous. In fact, Prehn has suggested that the interaction between an immunogenic tumour and the immune system may be a complex one, such that the tumour may actually be stimulated by cytokines and antibodies resulting from a weak anti-tumour immune response [93, 94]. It is thus essential to have a better understanding of the anti-ErbB-2 antibodies. Such knowledge would not only help in deriving a vaccination strategy that can safely and efficiently augment anti-ErbB-2 immune response, it may also help in the understanding of the physiological function of ErbB-2. Indeed, due to the absence of a defined ErbB-2 ligand, numerous studies have used anti-ErbB-2 Mabs to induce ErbB-2 homodimers and interpret the results as a specific property of the ErbB-2 signalling effect [82, 92]. Clearly, these findings may need to be interpreted with caution as the epitope of the anti-ErbB-2 Mabs could have effects on the signalling outcome.

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