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BIOLOGICAL EFFECTS OF ANTI-PEPTIDE ANTIBodies AGAINST THE HER-2/NEU RECEPTOR TYROSINE KINASE: IMPLICATIONS FOR THERAPY OF HUMAN BREAST CANCER

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

Donna-Beth Woodbine, B.S.

* * * * *

The Ohio State University
1997

Dissertation Committee:
Professor Pravin T.P. Kaumaya, Advisor
Professor Charles Brooks
Professor Dick Mortensen
Professor Vernon Stevens

Approved By
Adviser
Ohio State Biochemistry Program
ABSTRACT

Cancer is the second leading cause of death in the United States and of breast cancers, about one quarter of which express the erbB-2 oncogene. This gene encodes a 185kD growth factor receptor, HER-2/neu and its overexpression correlates with a poor prognosis. Thus, the ability to control the expression of this protein could favorably reduce the number of cancer related deaths. HER-2 is composed of four domains: an extracellular cysteine rich domain, a transmembrane sequence, tyrosine kinase domain and the C-terminal tyrosine autophosphorylation sites. The extracellular accessibility of HER-2 makes it an ideal candidate for immunotherapy. The ability of tumor inhibitory antibodies to recognize linear epitopes of the receptor prompted us to test the efficacy of peptide antibodies raised to p185. Previous work in our lab has demonstrated the ability of high titered antibodies from an outbred population to recognize native protein.

No ligand has yet been found for HER-2. Studies have shown that its action is mediated by heterodimerization with the other three members of the epidermal growth factor receptor family: EGFR, HER-3 and HER-4, recruiting diverse molecules to the membrane and increasing the number of signaling pathways accessible to the receptor complex. Dimerization engages HER-2's tyrosine kinase (TK) activity signaling a cascade of intracellular events which result in aberrant growth. Monoclonal antibodies can modulate this TK activity by causing receptor internalization, tumor retardation and differentiation to...
the mature state. Our studies involve generating antibodies to conformational epitopes of Her-2/neu by peptide immunization in an attempt to generate antibodies of defined specificity.

Peptide candidate antigens from HER-2/neu were studied by computer-aided analysis. B cell epitopes were synthesized collinearly with a T cell epitope, MVF 288-302, capable of binding to a broad range of MHC haplotypes. We have raised a panel of peptide antibodies capable of affecting the biology of HER-2: DW1MVF (376-395), MVFDW4 (628-647), DW5MVF (115-136), DW6MVF (410-429). The MVF sequences were engineered into chimeric constructs with HER-2 B cell epitopes and elicited high titered antibodies. These antibodies are able to specifically recognize HER-2 and selectively inhibit tumor cell proliferation in vitro. The anti-peptide antibodies also retarded tumor growth in a nude mouse model. These findings suggest that antibodies raised to a peptide vaccine in humans may offer an effective, selective and less toxic system of HER-2 positive tumor management than currently available methods. Synthetic vaccines would also avoid the dangers involved in using attenuated strains of viruses or infectious biological material as carriers and provide a cost-effective method of treatment. Further studies to induce a CTL response will explore the efficacy of CTL involvement in this form of therapy.
Dedicated to my family.
ACKNOWLEDGMENTS

I wish to thank my advisor Dr. Pravin Kaumaya for guidance, resources and expertise in my training. I extend my appreciation to Dr. Pierre Triozzi, one of the collaborators on this project, for providing laboratory space, use of instrumentation for the Western blotting and immunoprecipitation studies, critical reading of my dissertation and manuscripts and for his insight into the clinical aspects of breast cancer.

I thank Dr. Vernon Stevens for his collaboration and expertise in vaccine design and implementation and critical reading of the manuscripts. I would also like to acknowledge the members of my committee, Dr. Charles Brooks and Dr. Dick Mortensen.

My sincerest thanks goes to Dr. John Lowbridge for poignant commentary and critical review of the solid phase peptide synthesis section of the manuscript. I would like to recognize Dr. Herbert Bressler for review and analysis of the manuscript and insight on experimental procedures.
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American Chemical Society

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PUBLICATIONS


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INTRODUCTION

The statement of the problem

A safe, effective treatment for breast cancer has been fervently studied for several decades but none has been realized. Experimental immunological based therapies have used irradiated whole tumor cells, tumor cell lysates, adoptive transfer of LAK cells or TILs, recombinant constructs and antiidiotypic Monoclonal antibodies. Results have been variable and short lived. This is partly due to the inability to (1) define tumor antigens (2) target the necessary arms of the immune system. Peptide vaccines offer an attractive alternative to present therapies. Antitumor activity is produced entirely within the host eliminating toxicities that inevitably arise by introduction of foreign biological material. Preparations are readily characterized and purity can be carefully controlled.

HER-2/neu is an ideal candidate for immunodiagnosis and therapy. It is a 185kd transmembrane glycoprotein whose oncogenic activity is conferred by over expression. HER-2 can be found in about 25 - 30% of human breast cancers and gene amplification has been shown to correlate with poor clinical prognosis. Monoclonal antibodies to HER-2 have been shown to reduce tumor progression in vivo. This project studied the ability of anti-peptide antibodies to retard tumor growth in vitro and in a nude mouse model. As such, it presents a unique method of cancer therapy.
The specific aims of the project were

1. *To identify B-cell epitopes in p185 HER-2/neu.* Antigenic sites were predicted by computer algorithms along with secondary structural characteristics.

2. *To evaluate predicted B-cell epitopes for their immunogenicity.* Peptides were synthesized incorporating a 'promiscuous' helper T cell epitopes. Outbred animals were immunized and antibody titers followed over a minimum of 12 weeks.

3. *To determine the specificity of peptide antibodies for HER-2/neu.* Specificity was determined by immunoprecipitation of the native protein from human breast tumor cells.

4. *To identify the biological effects of antipeptide antibodies.* The ability of antipeptide antibodies to modulate tumor growth was studied *in vitro* by $^3$H thymidine proliferation assay. A nude mouse tumor model was used to determine tumor inhibiting effects *in vivo.*

5. *Identify Cytotoxic T cell (CTL) epitopes.* Computerized prediction determined the CTL epitopes in the extracellular domain of HER-2. One epitope which overlapped with a B-cell epitope was studied.

Long range goals included

1. Engineering a construct that contains B, T\textsubscript{h} and T\textsubscript{c} epitopes.

2. Testing the engineered construct in an outbred population that develops HER-2 tumors.

3. Optimizing peptide delivery protocols for microsphere utilization

    The results of this study may lead to a new method of HER-2 positive tumor
management by vaccination. This method is selective and might eliminate the toxicities and side effects of current treatments. It could also be used as a preventive strategy for individuals genetically susceptible to breast cancer.

Breast cancer is a significant public health concern in the United States and throughout the globe. Because of the emotional significance of the subject many women delay medical intervention when they develop symptoms. In the United States there is increasing awareness of breast cancer issues due to the media and governmental efforts to increase mammography screening. Breast cancer is the leading female cancer exceeding respiratory and digestive system cancers.

There will be 180,000 new cases of breast cancer diagnosed in 1997 making cancer the leading cause of death for women ages 35-74. The U.S. has the highest incidence of breast cancer in the world. Incidence rates have continued to rise by about 3% per year since 1980. This could be attributed to greater use of screening programs and an aging population (CA 1997 47:14-17). Cancer death rates since 1930 have decreased for colon, uterus and stomach cancers. Rates that continue to rise are pancreatic and ovarian cancer. The age adjusted breast cancer mortality rate, after rising slightly between 1962 to 1989, has decreased slightly but overall remained unchanged.

**Mechanisms of carcinogenesis**

The biology of human tumors has come to be understood in large part through the advances in modern genetics and biochemistry. These methods have enabled us to look at the differences between normal and cancer cells at the level of DNA. Although the machinery for copying DNA is quite accurate, *mutations* can arise leading to cancer. Some
mutations may go unnoticed as in 'silent' mutations where a DNA base substitution renders the same protein, while others can cause the synthesis of abnormal proteins. Outside forces such as chemicals, viruses and radiation can cause deletion, translocation mutation or gene amplification. The visible effects of many mutations are imbalances in normal cellular activity, cells growing without apparent control. This is the salient feature of cancer.

An altered regulatory gene is called an oncogene while the normal gene is called a proto-oncogene. In some individuals malignancy is the result of DNA deletions. Deletion of the gene at 13q14 results in retinoblastoma (Iversen p146, Abeloff p. 83,84). The RB gene product (pRB) is responsible for inhibiting cell proliferation and its activity is regulated by phosphorylation. As the cell cycles, pRB is phosphorylated in G1 phase blocking progression into S phase. Some tumor causing viruses bind pRB allowing S-phase entry. In Burkitt's lymphoma the c-myc gene on chromosome 8 is translocated to one of the immunoglobin loci on chromosomes 2, 14 or 22. This causes a B-cell malignancy found mainly in children (Vile p.79). Finally, proto-oncogene activation may cause proliferation in cells that should not normally be proliferating. In humans HER-2/neu activation is through gene amplification, there are many DNA copies of the gene instead of the normal two. In rats a multistep process is initiated by the chemical carcinogen ethylnitrosourea.

The multistep theory of cancer proposes that in the initiation stage DNA is altered by a carcinogen. The gene then (1) undergoes repair - no cancer (2) permanently changes - without cancer (3) transforms and produces cancer. In the second stage, promotion, cocarcinogens damage the proliferating mechanism giving cell transformation. In step three, progression, morphologic changes in the cell give differing grades of malignancy.

Methods of control at each stage are 1) avoidance/prevention 2) chemoprevention and early
detection 3) therapy. Once carcinogenesis has begun the problem becomes developing accurate, efficient detection methods and therapies with minimal toxicity.

**Breast Cancer Causation**

Women are more likely than men to have breast cancer. BRCA1, a major breast cancer susceptibility gene, was recently mapped to chromosome 17q and is linked in most breast-ovarian cancer families and in about half the families with hereditary site specific breast cancer (Marcus 1996). Clinical features indicating possible hereditary breast cancer are: Earlier age at diagnosis, bilateral breast cancer, predisposition to cancers at other sites, genetic transfer through an autosomal dominant gene. Breast cancer risk is 2 - 3 times greater if a woman's first degree relative such as mother, sister, grandmother or daughter had breast cancer. It is even greater if the relative developed disease under age 50 (Baum 1994, Ladig 1994).

Obesity and dietary fat have been linked to increased breast cancer risk for 2 reasons: (1) excess adipose tissue contains an abundance of the enzyme which converts substrate to the hormones estrone or estradiol. (2) decreased sex-hormone-binding globulin (SHBG) which binds estradiol. These are important since increased estrogen is associated with cancer risk.

Up to 20% of total cancer incidence in humans is virally associated (Vile p137). (See Table 1 Ras signal transduction cascade). In 1911, Peyton Rous showed that an agent could pass tumors between chickens. This agent was later found to be a retrovirus containing the v-src gene which caused the tumors. These viruses copy their RNA into DNA which is then integrated into host DNA. Thus it evades detection and produces viral proteins. Later
studies showed that normal cells possessed homologous genes. This gene may be virally controlled or sustain mutations that alter the protein's function (Vile p8-11). HER-2/neu is homologous to a viral gene and was identified in transformed mouse fibroblasts by hybridization with the retrovirus associated v-erbB gene probe (Bargmann 1986).

The major physical carcinogen in our environment is solar ultraviolet (UV) radiation giving rise to a proportion of skin cancers. Other types of radiation such as ionizing radiation including nuclear fallout, has been shown to cause leukemias, breast, lung, esophagus, thyroid, colon, bladder and ovarian cancer. X-rays have been associated with breast cancer after repeated exposure.
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<th>Associated Tumors</th>
<th>Risk Factors</th>
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<td>Human Papilloma virus (HPV)</td>
<td>Genital, laryngeal, skin warts</td>
<td>Sunlight, Genetic</td>
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<td>In situ and invasive cancers of the vulva and uterine cervix</td>
<td>Genetic disorders</td>
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<tr>
<td>Herpesvirus</td>
<td>Epstein-Barr virus (EBV)</td>
<td>Burkitt's lymphoma</td>
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<td>Immune deficiency</td>
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<td>herpes simplex type 2 (HSV-2)</td>
<td>cancer of uterine cervix</td>
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<td>Human T-cell leukemia virus-1 (HTLV-1)</td>
<td>Adult T-cell leukemia/lymphoma</td>
<td>Histocompatibility</td>
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Table 1: Oncogenic viruses

Avoidance of habitual and environmental risk factors can do much to reduce cancer incidence. Table 2 provides a list of agents and processes considered carcinogenic in humans (Botstein p152). Two cancers whose risk can be almost eliminated are lung cancer and skin cancer. Behavioral changes such as the use of protective clothing, sunscreen, natural shade and avoidance of tanning parlors reduces exposure to carcinogen.
<table>
<thead>
<tr>
<th>Agent</th>
<th>Type of exposure</th>
<th>Site of cancer</th>
</tr>
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<tbody>
<tr>
<td>alcoholic beverages</td>
<td>drinking</td>
<td>mouth, pharynx, esophagus, larynx, liver</td>
</tr>
<tr>
<td>arsenic</td>
<td>mining and smelting of certain ores,</td>
<td>lung, skin, liver (angiosarcoma)</td>
</tr>
<tr>
<td>asbestos</td>
<td>pesticide manufacture</td>
<td></td>
</tr>
<tr>
<td>benzene</td>
<td>manufacturing and use</td>
<td>lung, pleura, peritoneum</td>
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<tr>
<td>chlornaphazine</td>
<td>leather, petroleum</td>
<td>leukemia</td>
</tr>
<tr>
<td>estrogens</td>
<td>medication</td>
<td>bladder</td>
</tr>
<tr>
<td>immunosuppressants</td>
<td>medication</td>
<td>cervix, vagina</td>
</tr>
<tr>
<td>(cyclosporin)</td>
<td></td>
<td>Non-Hodgkin’s lymphoma, skin, soft tissue tumors</td>
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<tr>
<td>mustard gas</td>
<td>manufacturing</td>
<td>lung, nasal sinuses</td>
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<td>infection</td>
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<td>bladder (squamous)</td>
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<tr>
<td><em>Clonorchis sinesis</em></td>
<td></td>
<td>liver (cholangiocarcinoma)</td>
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<tr>
<td>tobacco smoke</td>
<td>smoking, especially cigarettes</td>
<td>lung, pharynx, mouth, esophagus, bladder, pancreas, kidney</td>
</tr>
<tr>
<td>UV radiation</td>
<td>sunlight</td>
<td>skin, lip</td>
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<tr>
<td>X-rays</td>
<td>diagnosis</td>
<td>breast</td>
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Table 2: Environmental agents and processes considered carcinogenic in humans.
**Tumor morphology and biochemistry**

Several characteristics of cancer cells that separate them from the norm are apparent: appearance, differences in mortality, density dependent growth, cell surface expression and sensitivity to growth factors.

*Microscopic properties.* Unlike normal cells, the bundles of microfilaments and microtubules in transformed cells do not associate into the organized polymers necessary for cell shape. Therefore transformed cells are of heterogeneous size and shape (pleomorphism). Some exhibit membrane ruffling which can be attributed to reorganization of actin by oncogenes $v$-$src$ and $v$-$ras$. The terminal phosphorylated protein in the ras signal cascade (see Figure 1 Ras signal transduction cascade) is mitogen associated protein kinase (MAP-K). Growth factors stimulate MAP-K to phosphorylate microtubule associated proteins for mitosis. Incorrect phosphorylation can affect assembly, organization and thus tumor course. The chromatin of transformed cells often stains darker (hyperchromatism), there may be unequal segregation of chromosomes during mitosis or an increased nuclear-to-cytoplasm ratio.

*Kinetic properties* 1) Immortality- During normal cell division telomeres, the protecting cap at the ends of DNA, shorten a bit with each cell division thus determining the number of times a cell can divide. In transformed cells, the telomerase enzyme replaces snipped caps allowing indefinite replication. 2) Density dependent growth- Cells growing in culture will continue to divide in the presence of sufficient nutrients until a blanket of cells reaches the edges of the container. At this point they maintain their density in that cell growth is equal
to cell loss. This is termed density dependent growth or contact inhibition. Cancer cells seem to ignore these restriction signals. 3) Attachment independence- Cancer cells continue to grow on top of one another forming irregular clumps of cells and they no longer require a surface on which to grow. They are able to grow in a bed of soft agar.

The extracellular matrix (ECM) secreted by normal cells and upon which they rely is made up of protein and carbohydrate constituents such as collagen and fibronectin. Neoplastic cells usually a) produce less ECM b) have a lower membrane glycoprotein content including fibronectin which along with collagen, elastin, etc. makes up the ECM c) secrete greater amounts of proteases such as cathepsin D, stromelysin-3 and collagenase that may dissolve the ECM d) express new cell surface antigens that may or may not be associated with the cause of the disease e) produce new transport proteins that alter membrane permeability and transport f) produce plasminogen activator which lessens cell adhesion to the ECM.

**Hormonal modulation** Breast cancer and gynecologic cancers in general have been shown to be modulated hormonally. Hormones mediate their activity in target cells through interaction with receptors that transduce a signal internally and initiate a cascade of events that leads to appropriate physiological response. Hormone-related cancers make up about 40% of new cancer cases in the U.S. (Henderson 1993). The changes that cause a cell to be cancerous involve increasing expression of proteins linked to cell proliferation, which raising the possibility of metastasis and decreasing production of proteins that slow or halt cell growth.
The impact of hormones on cancer is dramatically demonstrated when ovarian and endometrial cancer incidence is plotted against age at cancer diagnosis. The slope continues to rise steadily until menopause, about 50 years of age, when it plateaus significantly. Women who have taken combination oral contraceptives for 5 years have a decreased cancer incidence rate (Henderson 1993). If we think of the profound cell proliferation that takes place during every hormonal cycle and keep in mind the proliferation rate of cancer cells, we could come to the conclusion that every ovulation, every cycle, increases the likelihood of an aberrant event in mitosis- an initiating event of a cancer. It is this line of reasoning, ‘the ovulation hypothesis’ that sheds light on the reason why oral contraceptives seem to reduce cancer risk (Marshall 1993).

Hormones are molecules secreted into the bloodstream that initiate a regulatory function on their target organs at the cellular and molecular level. They are chemically classified as amines such as epinephrine, polypeptides or glycoproteins like insulin, and steroids such as estrogen. Hormones control ovulation and the proliferative cycles of the uterus. The primary event occurs in the hypothalamus as the release of gonadotropin releasing hormone (GnRH). GnRH stimulates the pituitary to secrete two gonadotropic hormones- follicle-stimulating hormone (FSH) and luteinizing hormone (LH) that controls the structure and function of the ovaries. The ovaries, by secretion of estrogen and progesterone, modulate the growth of endometrial cells.

Hormones may be natural or exogenous. Exogenous hormone includes the contraceptive pill or hormone replacement therapy. Use of exogenous hormones in the contraceptive pill inhibits ovulation. Combination oral contraceptives (COCs) contain both estrogen and progestogen. They are safer than historical (sequential) oral contraceptives that
contained estrogen without progesterone- "unopposed estrogen"- which were taken off the market in 1978. The other source of exogenous hormone is from hormone replacement therapy given to menopausal women. The most common being conjugated equine estrogens and medroxyprogesterone acetate (Hulka 1995). Short term use relieves some of the symptoms associated with this stage such as hot flashes, bone loss, etc. The use of contraceptive pills decreases the number of proliferative events and reduces the chances of mutation.

The effects of hormones on breast cancer is a bit more complicated. While progesterone inhibits the effects of estrogen and decreases ovarian and epithelial cancer risks, the combination of estrogen plus progesterone causes mitogenic activity in breast tissue. The two main epithelial phenotypes in the mammary gland are the luminal or secretory cells and the basal or myoepithelial cells. The pattern of antigen expression can be used to differentiate between the two. A majority of breast cancers arise in the cells of the terminal duct lobular unit (TDLU) of luminal cells. Their proliferation is highest in the luteal phase when estrogen and progesterone are at work (reviewed in Hulka 1995, Taylor-Papadimitriou 1993).

*Altered antigen/receptor expression*- Cell surface proteins also provide such things as attachment and acquisition of nutrients. Recent studies have looked at the link between receptor status and cancer recurrence or mortality. Estrogen receptor is found in luminal cells while greater expression of EGFR is found in basal cells (Taylor-Papadimitriou 1993). About 50-80% of breast cancers have estrogen receptors. Patients with both estrogen and progesterone receptor cancers more favorably respond to endocrine treatments such as
tamoxifen or surgery. Presence of progesterone receptor in primary tumors correlates with a lower chance of metastasis (Baulieu 1990). In some cancers Her-2 receptors are upregulated. This increased expression is linked to poor clinical outcome.

The idea that tumor cells might express certain specific antigens is illustrated by an interesting case in which a gastric cancer patient received an incompatible blood transfusion. The patient developed an antibody response against an antigen which was expressed on the tumor. The tumor regressed and the patient remained tumor free for 25 years (Botstein p.100). Later studies have found antibodies to cancer cell lines in patient sera (Disis 1994). However, these tumor associated antigens that induce an immune response have been elusive.

*Altered signal transduction*- Oncoproteins may include growth factors, growth factor receptors, signal transducers and transcription factors (Langdon, 1995). Growth factors can be made in either an autocrine (made by the cell that requires it) or paracrine fashion (synthesized nearby and transported to the target cell usually by the bloodstream). The involvement of growth factors and receptors in oncogenesis has received widespread attention in the past decade (Aaronson, 1991). In normal cell signaling pathways, binding of a ligand to its inactive receptor initiates receptor oligomerization and in the case of receptor tyrosine kinases (RTKs), activation of intrinsic protein kinase activity and autophosphorylation. Autophosphorylation regions of the protein provide docking sites for Src-homology-2 (SH2) domains of signaling molecules such as phospholipase C, phosphotidylinositol 3 kinase and p21 rasGTPase-activating protein (GAP) (Langdon, 1995). These proteins in turn interact with other kinases ultimately switching on genes
associated with cell proliferation, cell migration and shape. Thus, the external signal can begin a series of activation/deactivation events in the cytoplasmic compartment that eventually reaches the nucleus turning on nuclear transcription factors and initiating protein synthesis. In normal cells, growth factor stimulation presents a transient and reversible signal. Protein phosphorylation and dephosphorylation are rapid reversible mechanisms carried out by protein kinases and phosphatases, respectively. Amino acids which can be modified by phosphorylation include serine, threonine, tyrosine and histidine. The family of tyrosine kinase receptors includes epidermal growth factor receptor (EGF), HER-2/3/4, Fibroblast growth factor receptor (FGFR) and platelet derived growth factor receptor (PDGFR).

Transformed cells are able to produce their own growth factors. Thus they can survive under conditions in which normal cells would die (Dean 1994). Adjacent cells can also nourish a tumor. Activated platelets contain large amounts of growth factors including transforming growth factor α (TGF-α), platelet derived growth factor (PDGF) and transforming growth factor β (TGF-β). Both primary and secondary tumors require a vasculature in order to grow larger than a few millimeters. This process of blood vessel growth is called angiogenesis. Promoters of angiogenesis include endothelial mitogens fibroblast growth factor (FGF-2), vascular endothelial growth factor (VEGF) and TGF-α. Factors that enable migration but not proliferation are PDGF, TGF-β and angiotropin (Marx 1993, Macdonald 1993). Thus the receptors for these hormones represent a critical component of tumor biology by promoting growth.

The estrogen receptor (ER) is an intracellular transcription factor receptor that has DNA binding ability. Ligand binding induces interaction with gene sequences called
hormone-response elements (HREs). It possesses four domains (1) a region with transactivation function (2) the Zinc-finger DNA binding/dimerization domain (3) heat shock protein interaction domain and (4) hormone binding and nuclear localization domain (Findlay ed. 1994). ER concentration is increased by estrogen itself but progesterone inhibits this induction (Baulieu and Kelley 1990). Estrogen stimulates TGF-α synthesis and secretion (Gullick 1990). Binding of estrogen to its receptor causes dimerization, association with HRE’s and activation of target genes.

Progesterone receptors (PR) become activated by ligand-dependent phosphorylation. Its structure is similar to that of the estrogen receptor. Although PR DNA does not contain a typical estrogen response element, estrogen can induce transcription of PR in the rabbit and the same is thought to be true of the human system (Findlay 1994). In the presence of estrogen, progesterone receptor concentration increases. Progesterone however inhibits its own receptor numbers (Baulieu and Kelly 1990).

Tyrosine kinase (TK) receptors are composed of four domains: extracellular cysteine rich ligand binding domain, transmembrane domain, tyrosine kinase domain and carboxyl terminal tail. Ligand binding promotes receptor dimerization and activation of the tyrosine kinase activity. Epidermal growth factor (EGF) receptor is the best studied example. Other family members may be disulfide linked i.e. insulin-like growth factor receptor (IGF-1) or possesses immunoglobulin-like domains (PDGFR). Both EGF and TGF-α bind to the EGFR (Findlay 1994). Increased levels of EGFR are found in several epithelial carcinomas (Modjtahedi 1994, Dean 1994). Over expression of both molecules has been shown to be necessary for cell transformation (Gullick 1990).

The HER-2/neu receptor exerts its transforming ability through gene amplification
and over expression. It is able to dimerize with the EGFR. Heterodimerization of receptors plays an important role in increasing the repertoire of signals within a cell. A different pair of heterodimers would induce a different set of signaling molecules and cellular events. 

HER-2 over expression is linked to pathology of the breast, ovary, colon and lung.

HER-2/neu oncogene

The discovery of neu dates back to the late 1970's. A group studying chemical carcinogens found that when pregnant BDIX rats were fed ethylnitrosourea (ENU) at 15 weeks gestation, the offspring developed neuro/glioblastomas (Shih 1979). To try to discern the responsible gene, DNA from these tumors was introduced into the standard mouse fibroblast cell line NIH/3T3 (Shih, 1981). This DNA was able to cause transformation of NIH/3T3 cells. Cells grew as foci of densely packed cells in soft agar and exhibited tumorigenicity in nude mice (Schecter 1984). The oncogene was called neu for neuroblastoma.

Determination of the responsible protein was carried out by injecting mice with the transformed cells. Their sera was used to immunoprecipitate transformed cell lysates and a band corresponding with 185kD was absent in untransformed cell lysates (Padhy 1982). Since the only difference between normal and abnormal rats was the carcinogen, a 'vector' such as a virus could be ruled out. ENU probably induced expression of the protein. Drebin et al. (1984) raised monoclonal antibodies which were able to bind to both the transformed cells and the tumor.

Unfortunately the exact mechanism by which ENU initiates neu tumors is unknown. Other chemical carcinogens act by inducing mistakes in a cell's DNA ie. truncation, mutation
or substitution, or creating chromosomal instability (Vile 1992, p12). For example, proflavin binds tightly to DNA inserting itself between the base pairs. This pushes the base pairs apart lengthening the DNA molecule. Distortion then leads to either addition of an extra base (insertion mutation) or a base is omitted (deletion mutation) (Frieseider 1987, p.370).

Rat neu contains 1260 amino acids while the human homolog 1255 (Yamamoto 1986, Bargmann 1986). The human homolog of *neu* was found using v-erbB, which is the chicken EGFR in avian erythroblastosis virus, as a screening probe (Coussens 1984, Gullick 1990). Both rat and human forms possess extensive homology to other members of the family EGFR, HER-3/erbB3 (Yarden 1990) and HER-4/erbB4 (Plowman 1993). HER-3 was identified by reduced stringency hybridization of human genomic DNA using v-erbB as a probe. A 2.6kb mRNA was identified in human epithelial cells. The full length glycosylated protein is 160kd. The 1323 amino acid erbB3 was mapped to chromosome 12q13 (Kraus 1989). HER-3 binds heregulin with low affinity but when co-expressed with HER-2, a higher affinity receptor is generated which is capable of a tyrosine phosphorylation signal (Sliwkowski 1994). Primers used to isolate murine genomic EGFR, erbB2 and erbB3 were used to identify HER-4/erbB4. A distinct region from this clone was then used to identify the human HER-4 from a breast cancer cell line (Plowman 1993). The 1284 amino acid protein was found to transform NIH3T3 cells when expressed with either EGFR or HER-2 (Zhang 1996).

HER-2 contains a cysteine-rich extracellular ligand binding domain with several potential glycosylation sites. It also has an amphipathic transmembrane domain, a highly conserved kinase domain and an autophosphorylation site domain which differs significantly from EGFR (Gullick 1990). Unlike the rat protein which contains the substitution of glu 664
for val in the transmembrane region, Her-2 contains no mutation. Oncogenicity is conferred by gene amplification and over expression of the normal protein or over expression with EGFR (Venter, 1987; DiFiore, 1987; Ben-Baruch, 1994).

Although the crystal structure of EGFR family members is not yet available, crystalized serine/threonine and tyrosine kinases show they possess not only sequence homology but similar topology. Homology molecular modeling in which the kinase domains of cAMP dependent protein kinase (cAPK), HER-2 and EGFR are aligned shows three distinct kinase domains: ATP binding, activation loop, catalytic domain. ATP binding residues are conserved throughout the family (Murali 1996). The most flexible region is the activation loop where the autophosphorylation tyrosines are located. The overall kinase topology is bilobal, with a smaller amino terminal (N) lobe for ATP binding and large C-terminal lobe. Docking substrates bind in the cleft between the two lobes. The N lobe is mainly twisted antiparallel β sheet and the C-lobe is primarily α-helical (McDonald 1995).

Activation occurs through point mutation (rat neu) or over expression (human c-erbB2). The Val 664 to Glu mutation in rat neu confers no significant change in the conformation of the transmembrane domain. This laid to rest the idea that the mutation mimicked a conformational change caused by ligand binding and receptor activation (Gullick 1992). The transmembrane region is necessary for proper cell surface expression since deletion mutants are retained in the ER (Hudziak 1991). Since the receptors are free to move about the plane of the membrane, the substitution may stabilize hydrogen bonds formed when receptors encounter each other (Burke 1997). In protooncogenic neu, enzymatic cleavage of the 110kd extracellular domain (ECD) gives constitutive activation (Leitzel 1992, Pupa 1993). Inability to arrest the phosphorylation and growth results in tumor
formation. The ECD is important in the oncogenesis of neu in transgenic mice. Mutations in the conserved cysteine rich domain cause constitutive disulfide bond induced dimerization of receptors causing cellular transformation (Siegel 1996).

EGF binds the EGFR causing receptor dimerization and concomitant activation of the tyrosine kinase. Autophosphorylation allows cellular substrates to bind to the activated receptor ultimately causing cell growth and differentiation. High levels of EGFR expression in NIH/3T3 cells has been shown to cause transformation (reviewed in Dougall 1993). Studies report that HER-2 is indirectly activated by EGF (Connelly 1990) resulting in increased tyrosine phosphorylation. Cell lines created that simultaneously over express HER-2 and EGFR were transformed while those overexpressing HER-2 only did not exhibit transformation (Kokai 1989). Both HER-2 and HER-4 lack transforming ability when expressed alone (Zhang 1996). This illustrates the close regulation of one receptor by the other and how different signaling pathways can be affected.

Soluble polypeptide growth factors such as those for EGFR are unable to directly cross the cell's phospholipid bilayer. They must transduce their signal by binding a transmembrane receptor. The neu differentiation factor (NDF) family of ligands are alternatively spliced hydrophilic proteins produced by cleavage of the membrane bound protein. More than 10 isoforms exist which are able to bind HER-3 and HER-4. No ligand has been found which directly activates HER-2.
<table>
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<td>*</td>
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* heregulin isoform

Table 3: Indirect activators of Her-2

Located at chromosome 8p11-22, the EGF ligands include EGF, β-cellulin, amphiregulin, TGF-α and heparin-binding EGF (Findlay 1994). They possess a conserved
EGF-like motif consisting of 3 cysteine loops linking cysteines 1 & 3, 2 & 4, 5 & 6. Indirect activators of HER-2 (Table 3) do so by binding their cognate receptor and transphosphorylating HER-2. These activators also possess an EGF-like domain that is sufficient to activate their receptor (Barbacci 1995). The studies that have isolated potential HER-2 ligands indicate that some are stimulatory and others inhibitory (Davis, 1991; Dobashi, 1991; Lupu, 1990/2; Peles, 1992/3). Several of these ligands are alternatively spliced neuronal and mesenchymal factors (Ben-Baruch, 1994).

Two models exist for ligand-induced receptor dimerization/activation (Samanta 1994). In the intramolecular process, binding induces a conformational change in the receptor which is propagated through the transmembrane segment to give the active conformation of the kinase. This is the case with the insulin receptor. It exists as a disulfide bonded dimer at the cell membrane. Ligand binding causes trans-activation where the kinase domains may either undergo a 'scissors' motion to bring them closer or a relative rotation (McDonald 1995). In the intermolecular model, binding shifts the equilibrium from inactive monomers to active dimers without large conformational changes. The human growth hormone (hGH) induces dimerization of the extracellular domain of its receptor in a sequential manner. hGH contains two binding sites. A monomer of hGHbp binds hGH at the first site. The complex then diffuses to an unloaded receptor, the second binding event takes place and tyrosine kinase activity is initiated. Interestingly, sites 1 and 2 of hGH are completely different yet they bind identical sites on the hGHbp (Wells 1994). Thus the binding of ligand to a receptor can induce receptor dimerization invoking changes in
conformation and the activation of signaling pathways which affect cell growth, proliferation and differentiation. In much the same way, Ab raised to a receptor can mimic the actions of the ligand.

There has been no evidence for a conformational change in activated neu or dimeric HER-2. Over expression increases the likelihood of contact with another receptor by passive diffusion. One receptor acts as a substrate for the enzymatic activity of its partner. The effect is that an increase in the fraction of active receptors permits excessive signal transduction.

Tyrosine plays an integral part in the functions of EGFR family members. In the case of Her-2, the γ phosphate of adenosine triphosphate (ATP) covalently modifies a tyrosine side chain and is neutralized by charged residues such as Lys or Arg. Lys 757 is necessary for the oncogenic activity of transformed neu implicating tyrosine kinase activity in oncogenesis (Qian 1995, Qian 1994). Tyrosine kinase negative or cytoplasmic deletion mutants give defective complexes. Also, tumorigenesis in nude mice is reversible by conditional expression of the tyrosine kinase (Baasner 1996). Once the phosphate is covalently added to the receptor, cytoplasmic proteins with phosphotyrosine docking regions called Src homology 2 (SH2) or SH3 domains bind. SH2 domains are short sequences of about 100 amino acids that possess high affinity to phosphorylated tyrosine residues within certain motifs. They are present in enzymes, transcriptional regulators and adaptor proteins such as Grb-2 and Shc. SH3 domains can be found in signal transducers and recognize cellular proteins with proline-rich sequences (Ricci 1995). Hence ligand binding results in the assembly of multiprotein complexes at the cell membrane (Sun 1994).

Heterodimerization maximizes the assortment of proteins that can be recruited to the
membrane. This allows for connection of the receptor to several signaling pathways and broadens the cellular effects of one ligand. Oncogenic neu is more phosphorylated than the proto-oncogenic form. This may either increase recruitment of cytoplasmic proteins or attract a slightly different complement of docking proteins.

Phosphorylation of HER-2 couples it to the ras/MAP kinase pathway. The unique amino acid environment around each tyrosine determines which signaling molecule docks (Carraway 1995). Several proteins are shown to bind the phosphorylated HER-2. GRB2 and Shc bind tyrosines 1139 and 1196 or 1248 respectively- amino acid numbering of Coussens (Ricci 1995). The SH3 sites of GRB2 are able to activate a molecule named sos (son of sevenless) which in turn activates ras. In the cascade that follows ras activates raf-1 which turns on MEK; MEK activates MAP kinase which is translocated to the nucleus activating the transcription factors myc, jun and fos (Figure 1). Fos and jun interact to form the transcription complex AP-1 (activator protein -1 ) which turns on proliferative genes (Langdon 1995).

Treatment of SKBR3 cells with phosphatase inhibitor allows detection of phospholipase C-γ(PLC-γ) and GTPase-activating protein (Jallal 1992). Activated phospholipase C rapidly hydrolyzes phosphatidyl inositol 4, 5 bis phosphate (PIP₂) to inositol triphosphate, I(1,4,5)P₃. IP₃ in turn can cause the release of sequestered calcium from the smooth endoplasmic reticulum. This stimulates a myriad additional cytoplasmic proteins (Gennis 1989 p.362,363 ).

Phosphorylated neu interacts with the cytoskeleton and undergoes internalization. Activated neu is associated with p58, a protein implicated in stabilizing microfilament-membrane interactions (Juang 1996) and the coated pit adaptin AP-2 (Nesterov 1995, Gilboa
The tyrosine residues responsible for internalization are located in NPXY internalization motifs (Gilboa 1995, Chang 1993, Lamaze 1995). Her-2 contains at least 3 such regions. (Figure 2 EGFR and Her-2 internalization motifs)

An association between HER-2 and cancer began in the mid 1980's when HER-2 was found to be overexpressed in a number of adenocarcinomas. Slamon et al. (1989) found that amplification of the c-erbB2 gene correlated with poor clinical outcome. A possible method of therapy was introduced when it was discovered that antibodies could cause receptor internalization and degradation resulting in differentiation and tumor cell growth retardation (Drebin, 1985 & 1986; Harwerth, 1993). This has been linked to downregulation of tyrosine kinase activity. Multiple antibodies can provide a synergistic antitumor effect (reviewed in Dougall 1993). This has prompted the study of antibodies for therapy of HER-2 positive tumors in breast cancer.

Tumor Detection

Breast cancer survival increases with earlier detection. The American Cancer Society has suggested three methods of early detection 1) breast self-examination (BSE)- performed monthly by all women beginning at 20 yrs of age 2) clinical breast examination (CBE)- performed by a health care professional every 3 years from age 20-40 and annually thereafter 3) mammography- beginning at age 40, annually.

Mammography is the only proven means of detecting breast cancer before it can be detected by physical examination or BSE. About 85-90% of breast cancers are detectable by this method (Ladig 1994). The test involves two views of each breast lateral or oblique.
and craniocaudal (from top to bottom) by film-screen imaging. Its accuracy and predictive value is strengthened by the number of growths detected and their correlation with disease presence.

Breast self-examination (BSE) is free, effective, private, simple and has no side effects. Women who practice BSE regularly usually discover smaller lumps with less lymph node involvement than those who discover tumor accidentally (Ladig, 1995). Reasons for not utilizing this detection method include fear of finding tumor, inadequate knowledge, forgetting, disbelief in its effectiveness or discomfort with touching breasts. The exam involves testing for visible and tactile changes in the breast and usually takes about 20 minutes. Visual inspection involves looking for puckering, dimpling, skin retraction, nipple inversion or vein prominence. Proper training increases the ability to detect tumor early. Clinical breast examination may involve demonstration of the BSE with opportunity for practice, questions, answers and discussion of risk factors.

The specificity of monoclonal antibodies has been exploited in the detection of certain cancers (Rosen 1995). Radiolabeled antibody can be administered by intravenous (i.v.) injection and located by radiography (Old 1996) or enzyme linked immunosorbent assay (ELISA) can be used to quantify antigen in biological fluids (Sias 1990, Iwase 1997). Optimal tumor imaging depends on the radionuclide used and the form of the antibody. Common radionuclides used are Iodine-131 ($^{131}$I), iodine-123 ($^{123}$I), indium-111 ($^{111}$In) and technetium-99m ($^{99m}$Tc). $^{99m}$Tc is the least expensive and possesses high-intensity energy though it has a short half-life of about 6 hours (Abeloff 1995). Antibody fragments are more advantageous than whole molecule because they clear more rapidly generating less background noise.
Factors affecting prognosis  Once a suspicious lesion is detected it is tested for malignancy at the cytologic (cells) or histologic (tissue) level. Palpable lesions can be sampled by fine needle aspiration (FNA) biopsy and mounted for review. Nonpalpable masses are treated as above with radiographic guidance using minimal local anesthesia. Tissue sampling employs core needle biopsy for dominant growths. This is usually performed just prior to surgery. The last two biopsies are incisional, removing only a fraction of the tumor or excisional, removing the entire mass.

The most important predictor of recurrence and survival is involvement of axillary lymph node in disease. This is termed staging (Ladig 1995). Staging of breast cancer uses the tumor size (T), lymph node involvement (N), distant spread of metastasis (M) system as follows:

Stage 0: carcinoma in situ
Stage I: tumor 0-2cm with + nodes T0N1, T1N1
   2-5cm with +/- nodes T2N0, T2N1
   >5 cm with - nodes T3N0, all M0
Stage III: lymph node involvement or any size with direct extension to skin or chest wall
Stage IV: any tumor size with distant metastasis

Identifying tumors at later stages correlates with a decrease in survival. DNA ploidy and the percent of cells in S phase (S-phase fraction) are additional indicators of malignancy. High EGFR expression or HER-2/neu over expression has been correlated with poor prognosis.
Current therapies

The most common forms of cancer therapy are chemical intervention, radiotherapy and/or surgery. Each is prescribed dependent on the stage of disease and age of the patient. In the past surgery was the method of choice for physicians. Even today the words 'breast cancer' conjure up images of radical mastectomy and hair loss. However, more conservative strategies are currently being used. Lumpectomy is used when a small solitary tumor is found in one breast with no evidence of spread to lymph nodes, skin, muscle or elsewhere. When infiltration of surrounding tissue is realized, the safest procedure is some form of a mastectomy (simple/total, modified radical (Patey) or classical (Halsted) radical mastectomy). The first two types retain the pectoralis major muscle and the shape it provides. The last procedure which removes this muscle, is no longer in routine use. The side effects of surgery include cosmetic flaws, bruising, wound infection, swelling, stiffness and in radical mastectomy, instability of the shoulder blades. Prostheses or breast reconstruction are available to minimize cosmetic concerns but the other effects sometimes persist.

Radiation treatment is given after surgery to try to destroy any cancer cells in the local area that evade surgery. The side effects include skin sensitivity or itchiness, interference with the immune system, sometimes queasiness and rarely radiation fibrosis where an affected portion of the lung becomes fibrous. Most side effects disappear after treatment.

While the afore mentioned treatments work in a defined area, adjuvant systemic treatment is an attempt to destroy tumor that may have passed through the blood stream or lymphatic system and embedded in other tissue. This is termed metastasis. Systemic
treatments include hormone treatment to reduce the amount of estrogen in the body, or drugs such as Tamoxifen which blocks the stimulatory effect of estrogen on breast cells. Chemotherapy utilizes drugs that are toxic to cancer cells. Since this is not a perfectly selective system, normal cells are affected as well. White blood cell counts need to be taken before each treatment to check Tamoxifen's toxicity to normal immune cells. Other negative effects include nausea, tiredness, loss of appetite, hair loss and diarrhea. More powerful antiemetic drugs can alleviate some of these symptoms.

The availability of dietary fuel controls cell multiplication and growth. Antioxidants and the free radical scavenging properties of various foods are being explored (Kohlmeier 1995, Trichopoulous 1996). Support for the influence of diet on cancer has come in recent years. 'Westernized' diet takes a large percentage of its calories from animal fat and the tendency is to consume fewer fruits, vegetables and less dietary fiber (Schatzkin, 1995). It is thought that fiber acts by inhibiting reabsorption of bile estrogens in the intestines (Hunter 1996) reducing the proliferative effects of estrogen.

Immunotherapy of cancer began in the 1800s when doctors noticed that tumors sometimes regressed after a patient contracted bacterial infection (Old 1996). In the previously mentioned example of antigen recognition, a gastric cancer patient received an incompatible blood transfusion. The patient developed an antibody response against an antigen expressed on the tumor. The tumor regressed and the patient remained tumor free for 25 years (Botstein p100). The first illustration demonstrates nonspecific action where an antigen on the invading bacteria was probably similar to the one expressed on the tumor. This primed the immune system to target the tumor. In the second example (specific immunity) the antigens were identical. The BacillusCalmette-Guerin (BCG) vaccine shows
how nonspecific immunity can work in cancer. Superficial bladder cancer usually recurs after surgery and invades the bladder wall. If BCG is placed in the bladder it evokes an inflammatory response that kills preexisting/developing cancer cells in the bladder wall (ibid). This type of treatment is not effective if metastasis has occurred.

Several cancer therapies target the signal transduction cascade activated by growth factor. Points of intervention include:

1. neutralization of growth factors by antibody or competition with an antagonistic chemical
2. blockade of growth factor receptors by antibody or recombinant toxins
3. inhibition of tyrosine kinases by competing for ATP binding site or substrate binding site
4. activation of phosphotyrosine phosphatases
5. phospholipase C inhibitors
6. phosphatidylinositol 3' kinase inhibitors
7. protein kinase C inhibitors
8. p21 ras inhibitors
9. nuclear transcription factor inhibitors by antisense DNA (reviewed in Langdon 1995).

Biological therapies for breast cancer have included antihormones, natural cytotoxic compounds, adoptive cellular therapy and immune based approaches. Antihormones inhibit the effects of steroid hormones by either inhibiting their synthesis or more commonly stimulating their receptor. A gonadotropin releasing hormone (GnRH) agonist is being
tested for its ability to prevent pregnancy and its cancer preventative abilities (Henderson 1993). Tamoxifen is widely used because of its antiestrogenic properties (Hulka 1995).

Taxol and ricin are plant based products along with abrin, gelonin and saproin. Bacterial toxins include diptheria toxin (DT) and pseudomonas toxin (PT). Ricin and abrin act by inactivating ribosomes and although gelonin and saporin accomplish the same task, they are unable to bind cells on their own. DT and PT halt protein synthesis (Abeloff 1995). Other agents tested have been enzymes and inflammatory molecules i.e. tumor necrosis factor (Old 1996). The problem with a toxin approach is that it must be internalized to have effective cell killing power. Additionally, the nonspecific binding of most toxins allows them to bind normal cells creating severe consequences. They are potent at even undetectable physiological levels (Muto 1995).

In adoptive cellular therapy lymphokine activated killer (LAK) cells or tumor infiltrating lymphocytes (TILs) are administered to an individual with tumor. LAK cells are derived from peripheral blood induced with high IL-2 levels. Results in mice have been promising but human trials have been highly variable. TIL’s are isolated from the infiltrate surrounding tumors. They include CTLs and NK cells but their specificity is not well established (Abbas 1994, p. 372, Ikarashi 1994).

Genetic vaccines or naked DNA are plasmid vectors containing DNA of a foreign protein injected in aqueous solution or on DNA coated gold beads using a gene gun. The DNA is taken up by the cells, transcribed and translated. The resulting protein is able to enter the endogenous (Class I) pathway and activate CTLs. A characteristic of this immunization method when tested in animals is a slow antibody response that remains for the lifetime of the animal. Transfected muscle cells, which are poorly recognized by CTL
because of their low MHC Class I expression, may serve as a reservoir of antigen giving a constant low level stimulation to the immune system. In vivo, no calcium precipitation or liposomes are needed to achieve transfection. Even mucosal application can achieve an immune response (Ertl 1996). Some advantages of DNA immunization are ease of construction, modification and heat stability. The erbB2 gene has been used as an immunogen in mice. Resulting antibodies could bind human HER-2 and inhibit tumor cell growth in vitro (Concetti 1996).

Passive immunotherapy makes use of unconjugated monoclonal antibodies (MAbs), radioimmunoconjugates and antibody-drug conjugates. Unconjugated antibodies work through indirect methods such as complement fixation and antibody-dependent cell mediated cytotoxicity (ADCC) or the idiotype/anti-idiotype process. They can activate complement, attract LAK and NK cells, stimulate non-specific activation of cells that release cytokines and initiate various tumor cell killer functions, behave as agonists or mimic ligands causing internalization, degradation, differentiation to the mature phenotype or tumor growth retardation (Drebin 1986, Harwerth 1993, Mellstedt 1990, Wada 1990, Katsumata 1995). In complement fixation, an enzymatic cascade is triggered by the Ig Fc region. Complement proteins polymerize in the target cell's membrane causing perforation and cell death. ADCC uses antibodies to target host effector cells.

Antibodies targeting growth factor receptors can block growth stimulation and retard tumor growth. Most human trials use injected mouse antibodies to tumor antigens. Since the mouse antibodies are foreign, the body manufactures human antimouse antibodies (HAMA) and infusions can only be given once unless they are humanized. These HAMA recognize only a portion of the mouse antibodies including the antigen binding site-
idiotype. About one hundredth of one percent of antibody reaches the tumor. Low tumor localization occurs because antibody is diluted in the total plasma volume and it must overcome being metabolized or excreted. It may encounter antigen in the circulation or human antimouse antibodies. Antibody must also traverse the capillary's endothelial lining, its disadvantage being its large size of 150kD. Other impediments are tumor size, location, vascularity, cellular composition, antigen accessibility, vascular permeability and antibody avidity (Abeloff 1995).

Radioimmunoconjugates act by damaging DNA. Isotopes used are $^{131}$I, $^{90}$Y and $^{212}$Bi. The emissions of radioisotopes affect distant tissues causing undesirable damage. Drug conjugates are used to target cytotoxic drugs to a tumor. Agents such as methotrexate and doxorubicin are employed. The efficacy of antibody conjugates is diminished by the alterations physical conjugation makes on both the antibody and the drug.

In order to combat cancer cells, the immune system must be able to recognize and react to them. This means they must express specific antigens, in this case cell surface molecules. It has become apparent that many 'tumor associated antigens' also exist on normal cells or the region of interest may be intracellular. How can an immune response be raised to 'self' proteins? Evidence is accumulating that the immune system does not react against every antigenic epitopes in the body but only the most immunodominant ones (Melief 1996), therefore some self epitopes i.e. those expressed by cancerous cells, may be recognized.

T cells are able to recognize peptides from normal endogenous and sometimes mutant self proteins. CD8+ T cells have been used to select for tumor antigens of human melanoma. These epitopes are not expressed in normal adult tissue with the exception of the testis and
retina which are sequestered tissues (reviewed by Nanda 1995). Solid tumors continue to be a significant obstacle in antibody therapy. Antibody-dependent cellular cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC) are the most effective mechanisms by which unconjugated antibodies exert their effect. Antibody regimens sometimes show a cytostatic instead of cytocidal effect indicating that a tumoricidal effect is necessary for complete remission (Mellstedt 1990, Pietras 1994, Xu 93).

The immune system is composed primarily two types of lymphocytes: B cells and T cells. B cells, so named for their discovery in the bursa of Fabricus in birds. The bursal equivalent in mammals is the bone marrow. Their function is to produce antibodies or immunoglobulin (Ig) receptors. Igs are heterodimers of 2 heavy and 2 light protein chains connected by disulfide bonds. Each heavy chain is about 50kd and the light chain 20kd giving a total molecular weight of about 150kd after glycosylation. Enzymatic cleavage (Figure 3) breaks the antibody into 3 domains- antigen binding, hinge and Fc region. Variability and antigen specificity are achieved by gene splicing giving different antigen binding sites. Each B cell produces Igs of only one specificity. Binding of target antigen (any molecule bound by an antibody) results in differentiation of the B cell and finally secretion of antibody with identical specificity as the receptor.

The term B-cell antigenicity refers to the ability of a protein or peptide sequence to bind specifically to an antibody in vitro while immunogenecity refers to the ability of an antigenic site to elicit antibody production. Antigenicity is largely a characteristic of the peptide while immunogenecity relies on the quality of cytokine response, immunization protocol, the nature of B and T cell interactions, and the genetics of the immunized animal. Although the only way to unequivocally identify antigenic determinants is through x-ray
crystallographic studies of antigen-antibody interactions, methods for predicting antigenic determinants in a protein have been developed. These include analysis of protein hydrophilicity, flexibility, mobility, solvent exposure, amphiphilicity, reverse turns, alphahelical propensities, and protrusion. These only predict a subset of B cell determinants that have a high chance producing protein reactive antipeptide antibodies.

The shift of B cells from resting to active antibody secreting cells requires cytokines. Cytokines can be provided by T cells which are divided into T helper (T<sub>H</sub>) CD4<sup>+</sup> cells and cytotoxic (T<sub>C</sub>) CD8<sup>+</sup> cells. A B cell that has bound and endocytosed antigen on an antibody receptor processes and presents its fragments on Major Histocompatibility (MHC) Class II molecules. Helper T cells that recognize the complex produce cytokines that stimulate B cell proliferation and differentiation into antibody secreting cells. The specific complement of cytokines determines which isotype (IgA, IgG, IgM, IgE) of antibody is produced.

Cytotoxic T lymphocytes (CTLs) recognize and kill cells that present antigen on MHC Class I molecules. Unlike the epitopes recognized by B cells, T cell epitopes are usually amphiphilic α-helices of approximately nine amino acids in length and are the product of proteolytically processed proteins derived from endogenous proteins (Van den Eynde 1995, Garcia 1996). Antigen is degraded by the proteasome and threaded through the endoplasmic reticulum membrane and onto a new Class I MHC molecule. Different allelic forms of MHC molecules have discrete peptide binding motifs with pockets for critical anchor residues. The peptide-MHC complex is then transported to the cell membrane through the Golgi apparatus. Naive CD4<sup>+</sup> T cells recognize this complex and are stimulated to produce the autocrine growth factor interleukin 2 (priming). Proliferation and differentiation of the T cell to effector cells occurs.
The main effector cell is the antigen-activated CD8\(^+\) T cell. The CTL binds to the target cell using its T cell receptor (Tcr) which is specific for the particular lineage of MHC molecules that triggered its maturation. Clustering of the Tcr accessory molecules initiates delivery of its pore-forming proteins perforin and cytolyisin to the target cell. The target is released and undergoes cell death. CTLs recognize single amino acid changes and may target mutated oncogenic products. This knowledge is being exploited to target tumor cells of viral or melanoma origin. A protective anti-viral response induced by vaccination with MHC Class I binding peptides was shown in 1991 by Shulz et al. (Aichele 1990). In another study peptides from a melanoma induced complete remission in two patients (reviewed in Melief 1996).

In the case of HER-2 where the protein is overexpressed, there may be a high enough concentration of peptide binding MHC to evoke immune response (Tuttle 1996, Yoshino 1994). Researchers have shown existent T cell and antibody immunity in breast cancer patients (Pupa 1993, Jerome 1991) and a significant CTL response to intracellular sequences of HER-2/neu \textit{in vitro} (Disis 1994, 1996). With animal models, T cell immunity did not prevent morbidity but if immunity was removed death was more rapid. Immune T cells in such cases can be made more effective by \textit{in vitro} activation and growth then treating the animal with the expanded cultured T cells (Disis, 1994). Some antigens are shared between tumors (Peoples 1995a and 1995b, Fisk 1995) that express HER-2 which may indicate efficacy of treatment in more than one cancer type.

In contrast with the T cell approach, other investigators have used antibodies to combat tumors. Several HER-2 MAbs have been generated by immunizing animals with whole tumor cells. The disadvantages of immunizing with whole tumor cells are twofold.
First, it has been shown that this method produces antibodies, some of which are stimulatory (Stancovski, 1991). Hurwitz, et. al determined that tumor stimulatory antibodies remain at the cell surface while tumor inhibitory antibodies are targeted to endocytic vesicles. The antagonistic effect between stimulatory and inhibitory antibodies is undesired. Second, results are not reproducible in that each individual develops antibody to only those portions of the proteins that are immunogenic for them. A way must be devised to generate reproducible results and consistently inhibitory antibodies.

The overall conclusion from studying passive immunotherapy is that the methods are ineffective. (1) Less than one hundredth of a percent of infused antibody is localized to the tumor. This value cannot be improved by a second infusion because of the HAMA response. (2) The production of anti-idiotype antibodies decreases effectiveness since anti-idiotype antibodies can bind to infused antibody in the circulation and prevent their transit to tumor. (3) There is no immune memory. (4) Effects are transitory often lasting less than three months.

In my studies on Her-2/neu I have focused on raising antibodies to the extracellular domain that would theoretically cause receptor internalization and slowed tumor growth. The key difference in this approach is immunizing with peptides to generate highly specific antibodies. My goal has been to develop a vaccine for breast cancer. Attempts at designing effective vaccines have yielded unsatisfactory results largely because of the inability to: 1) define tumor-associated antigen, 2) target vaccines towards the relevant arm(s) of the antitumor response 3) utilize appropriate animal models and 4) design and perform appropriate clinical trials. Immunologically based strategies are probably the most effective ways to prevent and control disease.
Recently, several T cell epitopes have been described which are broadly reactive in multiple MHC haplotypes. These "promiscuous" T cell epitopes are found in tetanus toxin(TT), measles virus and malaria peptide (reviewed in Kaumaya, 1994). I have engineered the MVF sequences into chimeric constructs with HER-2 B cell epitopes with much success and raised high titered antibodies. These antibodies are able to specifically recognize HER-2 and selectively inhibit tumor cell proliferation in vitro. The anti-peptide antibodies also retard tumor growth in a nude mouse model. My work suggests that antibodies raised to a peptide vaccine in humans may offer an effective, selective and less toxic system of HER-2 positive tumor management. Further studies incorporating the CTL response will explore the efficacy of CTL involvement in this form of therapy.
CHAPTER 2
PRINCIPLES OF THE METHODS

Chemical Procedures

In order to generate specific antibodies capable of affecting HER-2 biology, peptides corresponding to HER-2 were synthesized and used to immunize rabbits. Antibodies elicited by these animals were then screened for biologic activity by the following assays: immunoprecipitation, tritiated thymidine proliferation and \textit{in vivo} tumor reduction.

\textit{Solid phase peptide synthesis}

Peptide synthesis involves the linkage of amino acids to form a chain. The linkage is a secondary amide moiety (peptide bond) formed from the carboxyl and alpha-amine functions of adjacent amino acids. The peptide bond forming reaction thus involves the isolation of the functional side chains by the blockade of reactive groups.

The chain is extended in the C to N terminal direction by the stepwise addition of individual residues, a strategy demanded for the retention of enantiomeric purity in the product, and in contrast to the biosynthetic reactions which suffer no such synthetic restraint. The innovation of solid phase peptide synthesis (SPPS) was the attachment of the first amino acid in the chain to an insoluble, chemically resistant compound, essentially and insoluble protecting group, that facilitated the retention of the developing product in an appropriately.
designed vessel. Peptide is retained above a fritted glass disk allowing repetitive performance of individual amide link formation steps. Reagents are successively washed away leaving the polymer-supported protected peptide product.

The solid support (usually cross-linked polystyrene) provides an insoluble anchor for the C-terminal amino acid. It must be mechanically resistant and inert enough to survive the conditions of peptide synthesis. Derivatization, most commonly of the hydroxy moiety creates a site at which a wide variety of 'linkers' can be introduced. The 'linkers' are moieties which allow for variety in cleavage mechanisms and in cleavage products when inserted between the protected peptidyl chain and the polystyrene support. Thus peptide product may be in the form of free carboxyl peptides, sidechain protected or free peptides, C-terminal carboxamides ester chains.

**Coupling**

The peptide bond forming reaction is a condensation reaction between two amino acids one N-terminal protected and the other C-terminal protected. In order to form a peptide bond between the free amine and the carboxyl group of the former, the -COOH must be converted to an acylating species by replacing the -OH moiety with a superior leaving group. The variations on the theme are numerous but the purpose is the same- to create an environment for nucleophilic substitution by amine at an acyl carbon. The general reaction scheme of peptide synthesis is depicted as follows:
where P is the protecting group

Anhydrides and substituted aryl esters are commonly explored. The myriad 'coupling agents' (such as DCC, BOP, HBTU) and derivatives (such as HOBt) represent the range of tools by which these acylating species are generated.

Protecting groups/Deprotection

In the Merrifield method, the question arises concerning the method of blocking of the incoming amino acid’s N-terminus (and the side chains it presents). The blocking group used to protect the alpha amine must be removed in a selective fashion to allow peptide bond formation. Side chain protecting groups must be resistant enough to survive the sequential deprotection steps but labile enough to be removed in the final steps of synthesis without destroying the peptide product. (The same applies to the linker attaching the peptide to the solid support since it can be thought of as blocking a functional group).

In addition to simply protecting the alpha amine from unwanted acylation, the blocking group prevents racemization of the alpha carbon chiral center (Figure 4). This necessitates the use of a bulky structure in the protecting group. Acylating species bearing bulky groups do not cyclize to form oxazolones as do those with protecting groups of simple
acyl structure. Oxazolone formed from urethane protected acylating species are far less susceptible to proton abstraction. Peptides cannot be synthesized in the N to C terminal direction for this reason- loss of optical integrity in the C-terminal residue.

Choice of protecting group depends on the synthetic chemistry. Two chemistries are in use: tBoc and FMOC. In tBOC strategy the alpha amino protecting group is cleaved by acid. This means side chain protecting groups and the anchoring linkage must remain unreactive in all but the most powerful acids. Deprotection or cleavage thus depends on the strength of the acid. FMOC chemistry can also use acid for cleavage. Alpha amino deprotection is accomplished by the base piperidine while TFA cleaves side chain protecting groups and the peptide-resin link.

Purification

The final peptide product must be purified from the mixture of deletion sequences, scavengers and resin. A fritted disk in the reaction vessel retains the resin and the solubilized peptide is rinsed into a new container. Ether/water extraction removes scavengers and the product is lyophilized and purified by column chromatography.

Gel Filtration

Separation on the basis of molecular size is accomplished with gel filtration. A sample is applied to the top of a column filled with porous, insoluble beads. Three kinds of gels (beads) are available: Sephadex (dextran), Bio-Gel P (polyacrylamide) and Sepharose or Bio-Gel A (agarose) gels. Agarose is a linear polymer of D-galactose and 3,6-anhydro-1-galactose which is held together without a cross linking agent. Pore size is determined by
agarose concentration. Pore size determines the molecular weight range for separation and in dextran and polyacrylamide gels it is a function of cross linking. Small molecules can enter the pores of the gel matrix but large ones cannot and are rapidly eluted from the column. Therefore molecules are eluted in decreasing molecular weight order (Friefelder 1982, p.240).

**Reversed phase HPLC**

In liquid chromatography solvent immobilized to a solid support is the stationary phase and an organic solvent runs through a glass column making up the mobile phase. The solid support is usually silica gel and water attaches by hydrogen bonding. It is relatively inert. Sample is dissolved in a small volume of mobile phase and injected into the column. Compounds are eluted from the column by stepwise or gradient elution and spectrophotometry is used to visualize concentration of sample components as they are eluted over time. In stepwise elution, mobile phases of increasing elution power are added in distinct time periods. With gradient elution, a gradual continuously changing flow of mobile phase is added with increasing elution power.

There are five major components of an HPLC system: a pump which supplies pressure to the mobile phase, an injector system, the chromatographic column, a detector and a recorder. The choice of column depends on the polypeptide’s characteristics and the impurities to be chromatographed. Column diameter is chosen based on sample load and desired flow rate. Standard analytical columns have an internal diameter of 4.6mm and a recommended flow rate of 0.5 - 1.5 ml/min. Semi-preparative columns are 10mm internally with a 2.5 - 7.5 ml/min suggested flow rate and can practically resolve up to 40mg of protein.
Column length is usually 5 - 15cm and column life is extended by using a guard column between the injector and the column to trap insoluble particles.

RP-HPLC separates polypeptides based on subtle differences in the hydrophobic fold anchoring the peptide to the solid phase. Polypeptides adsorb to the hydrophobic surface after entering the column where they remain until the organic phase reaches sufficient concentration to displace or desorb them. Differences in the binding folds or 'hydrophobic foot' are the result of different amino acid sequences and conformations.

The organic phase serves to solubilize and desorb the polypeptide from the solid support. The most common is acetonitrile (ACN) because of its volatility, low viscosity which minimizes back pressure, low UV adsorption and reliability. Ion pairing agents set the pH and enhance separation by interacting with the polypeptide. The most common agent is trifluoroacetic acid (TFA) because of its low UV absorption at lower wavelengths and its volatility. The concentration is usually 0.1% (w/v) and it is placed in both the aqueous and organic solvents. A wavelength of 214 - 216nm is best used with this agent.

*Capillary Zone Electrophoresis (CZE)*

In electrophoresis electrically charged particles move through a buffered electrolyte under the influence of an applied field. Molecules migrate differently based on their size, shape, charge and the properties of the solvent. When a mixture of molecules is injected into a migration channel it separates into zones that migrate at different rates depending on their mobility.

Some differences exist between chromatography and CZE. First, in chromatography the retention time of molecules differs. In CZE there is no retention of molecules only
differing rates of movement thus the term used in migration time. No stationary phase is
required, therefore resistance to movement caused by interaction with both the stationary and
mobile phases is a problem. In a pressurized system such as HPLC, there is friction at the
interface between the mobile and stationary phases causing fluid velocity to be slow at the
edges of the tube and increase quadratically toward the center. This is called parabolic flow.
In electrical systems endoosmotic flow (EOF) is uniform through the entire length of tubing
giving a flat or 'plug front' flow. (See Fluid front in HPLC and CZE). This gives the sharp,
narrow peaks of CZE (Figure 5).

A capillary electrophoresis system consists of a fused silica capillary 10 - 100cm in
length, a power supply, detector and recorder. The power supply provides voltages of 20 -
30 kV. The capillary is placed in electrolyte reservoirs and filled with a buffer. Sample is
dissolved in the same buffer and injection is made by hydrostatic pressure, vacuum, gravity
or electricity. Sample volume is usually in the nano to picoliter region.

Before use, capillaries are conditioned with 1N NaOH. The capillary wall is a highly
cross linked polymer of silicon dioxide. Base ionizes free silanol groups giving the wall a
net negative charge. This surface charge is called the zeta potential. Anions are repelled and
cations bind tightly creating a positive internal surface. The charge becomes increasingly
neutral towards the center. When an electric current is applied the positive charges migrate
toward the cathode (negative) electrode. This is termed electroendoosmotic flow (EOF)
(Gordon 1988).

Capillary diameter is usually 20 - 200μm. This small diameter increases the surface
to volume ratio allowing heat generated by the current to be efficiently dissipated through
the capillary walls. CZE separations are critically dependent on pH therefore the choice of
buffer is very important. The buffer should: have good pH control and buffer capacity, low conductivity, good UV transparency in the lower wavelengths. Frequently used buffers include phosphate, citrate, borate, acetate and their combinations (Schwartz, et al. Beckman 1992).

Mass Spectrometry

Mass spectrometry molecules in the gaseous state under low pressure are hit by a barrage of high energy electrons. The energy of the beam (usually 70 eV) is sufficient to dislodge one of the electrons of a molecule creating a molecular ion. The beam also imparts sufficient energy to break covalent bonds and the molecule 'explodes' or fragments in different ways depending on the molecules’ structure.

\[ \text{H :N: H + e}^{-} \rightarrow [\text{H :N: H}]^{+} + 2e^{-} \rightarrow \text{H :N:+} + \text{H+} , \text{etc} \]

\[
\begin{array}{c}
\text{H} \\
\text{H} \\
\text{H}
\end{array}
\]

Since the charge on all the cations produced is essentially +1, the spectrophotometer can sort the cations on the basis of their mass by accelerating them through a magnetic field. The particles are deflected as an arc in the magnetic field. The arc’s radius of curvature is related to the m/e ratio of the ions, the momentum (mass x velocity), magnetic field and accelerating voltage. Since all particles have a charge of +1, their molecular weight can be determined.

Amino Acid Analysis

The amino acid sequence of a protein can be determined by first hydrolyzing the protein or peptide in acidic conditions, normally 6N HCl, and the individual amino acids resulting from that hydrolysis are derivatized using phenylisothiocyanate (PITC). The
generated the PITC derivatives can be separated using reversed phase HPLC. Individual
PITC-amino acids are identified based on their retention time.

Circular Dichroism

Circular dichroism (CD) measures the differential absorption of left and right
polarized light by molecules. Visible light contains electric and magnetic fields which run
perpendicular to each other. In plane polarized light the electric waves point in only one
direction while they oscillate in multiple directions in ordinary light. If two perpendicular
plane polarized waves of equal amplitude who differ by 1/4 wavelength are superimposed
the vector of propagated waves follows a helical course. This is called circularly polarized
light.

A compound that can rotate the plane of polarized light are termed ‘optically active’
The difference between left and right waves caused by a compound is called ellipticity (θ).
Graphs are plotted as θ vs. Wavelength and the particular characteristics of secondary
structure (α- helix, β- helix, random coil) become apparent (Friefelder 1982 p. 589) See
Figure 6.

Biological Procedures

Immunizations and Animals The choice of animal for antibody studies depends on 1) the
quantity of sera needed, 2) the availability of the host, 3) the experiments to be performed
and 4) if necessary, specie compatibility with ultimate recipient. Our studies were carried
out in outbred rabbits. Rabbits are readily available and provide sufficient sera for common
experiments such as ELISAs and Western blotting, while requiring less housing costs than larger animals. Commercial kits for antibody purification and characterization can be purchased from several suppliers. Primary immunizations are given in Complete Freund's Adjuvant (CFA) which recruits effector cells to the injection site(s). Subsequent boosts are given in phosphate buffered saline.

**Antibody Purification.** Whole blood can be divided into cellular constituents, serum and serum components. Centrifugation separates the large particles (cells) from the clear serum. Serum contains two major proteins—albumin and globulin—which can be separated by electrophoresis. Globulins consist of α, β and γ fractions, the last of which contains the antibodies or immunoglobulins (Ig). It is necessary to chemically define an agent of interest to remove uncertainties and unwanted activities. Hence, purification of Ig from serum.

Though there are several ways to accomplish this, the most common is saturated ammonium sulfate (SAS) precipitation due to its ease, reliability and high yield of stable IgG. This process relies on the differential solubility of proteins in the presence of certain ions. Proteins remain in solution because of their ability to interact with water. Added salts attract more and more water until the proteins must interact with each other. This causes the formation of large protein complexes that fall out of solution as a precipitate.

The Iggs of different species precipitate ideally at different salt concentrations (Herbert 1973). Rabbit Antibodies achieve best precipitation at about 35% SAS. Precipitated antibodies can be solubilized and subjected to several rounds of precipitation to increase yields.
Affinity chromatography is based on the ability of protein to bind ligand. To purify an antibody, antigen or protein A/G specific for Ig F\textsubscript{c} region is attached to a solid support such as agarose, polyacrylamide etc., of appropriate pore size. A column is packed with this material which becomes the stationary phase. The sample solution is placed on the column. Only the protein bound by the stationary phase remains on the column. All other proteins are eluted in the void volume. To remove and collect the desired protein the ionic strength of the buffer is increased by changing pH, adding NaCl or both. This displaces bound ligand which then comes off the column.

**ELISA**  
Enzyme linked immunosorbent assay (ELISA) is a method used to determine antigen or antibody concentration on a solid phase. Immunoadsorbent material is usually a multi-well plate made of polycarbonate, polypropylene, polystyrene or polyvinyl. This allows for better separation of complexes from free antigen or antibody. ELISAs contain some common steps (1) antigen and antibody are permitted to combine (2) the complex formed is separated from free components (3) activity is determined in bound or free fractions.

ELISA is used to plot the change in specific antibody titer over time. Fraction of polyclonal sera recognizing immunogen is resolved in this assay. Antigen is adsorbed onto the plastic by nonspecific binding. Better coating can be achieved by precoating with poly-L-lysine which gives a positive charge to the surface. Negatively charged molecules bind. Unoccupied binding sites are blocked by binding an irrelevant protein usually serum albumin in solution. This prevents subsequent proteins used in the assay from adsorbing to the plate. Next, unlabeled test antibody is added and serially diluted. At the end of the incubation time
unbound antibody is washed away. In the last step, an enzyme-labeled antibody directed against the first antibody is added. After incubation excess antibody is washed away. Enzyme substrate is added and relative concentration of test antibody found by intensity of color in test samples compared with control. (See Figure 7 ELISA procedure flow diagram)

The most common enzyme conjugated to the secondary antibody is horseradish peroxidase. The enzyme converts hydrogen peroxide to water in the presence of an electron donor such as diaminobenzidine or 2, 2'-aminobis (3-ethylbenzthiazoline -6-sulfonic acid). Spectrophotometer determines the absorbance which is plotted against antibody dilution.

\( ^{3} \text{H} \text{thymidine proliferation assay} \). Cells grow and divide in a defined sequence known as the cell cycle. It is divided into four phases: DNA synthetic phase (S), postsynaptic gap (G2), a mitotic phase (M) and a presynaptic gap (G1) phase. DNA replication can be monitored in the S phase by addition of radioactively labeled DNA precursors such as tritiated thymidine. As new DNA is synthesized, labeled precursors are incorporated. Proliferation of test samples is determined by scintillation counting. The drawback of in vitro \( ^{3} \text{H} \) thymidine assays is that only cells in the S phase of the cell cycle are tagged. It ignores the remaining cell population which may differ markedly between test samples (Wada 1990).

**Immunoprecipitation** In immunoprecipitation an antibody specific for one protein antigen is used to isolate it from a mixture of proteins. An antibody attached to a solid particle or matrix is incubated with a mixture of antigens. The solid particle can be an agarose bead or protein A or G from staphylococcal bacteria which has high affinity for the Fc region of Ig
molecules. Antibody - antigen- particle complexes are separated from the free molecules in solution by centrifugation. Antigen is eluted from antibody by a pH change, detergent, etc., The pure antigen can then be analyzed by electrophoresis.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS- PAGE) separates proteins on the basis of their molecular weight. Using 1% SDS and 0.1M mercaptoethanol proteins are denatured, chains dissociated and disulfide bridges broken. The protein binds SDS (about 1.4g SDS to 1g polypeptide) causing them to behave more like rods of uniform shape. SDS imparts a negative charge to the protein causing it to migrate toward the positive electrode. The constant charge to mass ratio allows the MW separation. Smaller molecules move quickly through the gel so proteins of higher molecular weight move more slowly and show up as bands closer to the negative electrode. Molecular weight accuracy is about ±10% (Friefelder 1982).

Blotting refers to the transfer of electrophoretically separated macromolecules from one solid medium to another. In Western blotting, the separated molecules are transferred electrically onto nitrocellulose paper giving a replica of the SDS gel on paper. The position of antigen is determined by binding labeled antibody. After developing, the antigen-antibody band becomes visible.

*Phosphotyrosine assay*  The phosphotyrosine assay relies on the specificity of antibodies. Phosphorylated tyrosine or serine residues can be detected with a labeled monoclonal antibody. In this assay cells are subjected to various test conditions, i.e. incubation with antipeptide antibody, and lysed. The relevant protein is immunoprecipitated and resolved.
on an SDS-PAGE gel. After blotting a change in the amount of phosphorylation when compared with untreated control is determined by the intensity of the phosphotyrosine labeled protein band.

**CTL Assay** The goal of the CTL assay is to determine whether CD8+ T cells stimulated with a short peptide will recognize and destroy cells presenting the same antigen. T cell stimulation: Since T cells recognize peptide in the context of MHC, peptides used for stimulation must be bound in the cleft of an MHC molecule. To accomplish this, peptide is pulsed with an antigen presenting cell (APC), usually P815s from a mouse mastocytoma (Brichard 1995, Carbone 1988), for a short time. Cells are then irradiated to halt cell division. Pre-CTLs are isolated from the lymph nodes and spleen of a mouse immunized with the same peptide. The CTLs are cocultured with the target cells where they proliferate and mature in the presence of added IL-2.

Cytolytic assay: The CTL targets are prepared by pulsing with appropriate peptide and labeling with $^{51}$Cr. This radioactive metal binds intracellular proteins and is released into the medium upon cell lysis. Mature CTLs are cocultured with the targets at different effector to target ratios. If the CTLs recognize the target it releases molecules such as perforin which polymerizes in the target cell's membrane and cause lysis (Rose 1997).

**FACS** In flow cytometry or fluorescence activated cell sorting, cell populations with homogeneous characteristics are grouped together. Labeled cells in a fluid stream are passed through a laser beam that determines the intensity, color and polarization of its fluorescent
label. The cells then pass through an electric field which diverts the cells to the appropriate collection vessel. The sorter can thus separate cells showing different fluorescence intensities and group them by the quantity of antigen expression.
CHAPTER 3
MATERIALS AND METHODS

Chemical Procedures

Peptide Synthesis and HPLC Purification. Peptides were synthesized as previously described (Kaumaya 1994). Briefly, peptides were synthesized on a Milligen/Biosearch 9600 peptide synthesizer, using a 4-methylbenzhydramine resin as the solid support (substitution 0.54mm/g). The Fmoc/t-butyl synthetic method was employed using 4-(hydroxymethyl)phenoxyacetic acid as the linker. After the final deprotection step, protecting groups and peptide resin bond were cleaved with 90% TFA, 5% anisole, 3% thioanisole, 2% ethaneditiol. Crude peptide was purified by semipreparative HPLC using a Vydac C₄ (10mm x 25cm) column at 32.5° C. Buffers were 0.1% TFA in H₂O and 0.1% TFA in acetonitrile. Peptides incorporate a "promiscuous" T cell epitopes MVF 288-302 (Kaumaya 1994): DW1MVF (Her-2 376-395), MVFDW4 (628-647), DW5MVF (115-136), DW6MVF (410-429).

Gel Filtration. 20mg/ml acidified peptide solution (.1mg/ml in DTT) was loaded onto a Sephadex G-25 column and 5ml fractions eluted with 0.1M HOAc. Peptide samples were measured spectrophotometrically at 235nm and absorbance values plotted vs. time. Samples
with absorbance values above 0.1 and eluting before DTT were pooled and lyophilized. The reaction was monitored for completion by Ellman's reagent at 410nm.

**Capillary Zone Electrophoresis**  CZE was performed on a Beckman P/ACE System 2100 interfaced with an IBM computer. Sample was voltage separated (15kV) in 100mM sodium borate using a 50cm capillary over 20min. Eluant was monitored at 214nm.

**Circular Dichroism and mass spectrometry**  Measurements were performed on a JASCO J-500 spectropolarimeter interfaced with an IBM computer. The instrument was calibrated in 0.06% (w/v) solution of ammonium-d-10-camphorsulfonate. The CD spectra of the peptides (62.5-250μM by dilution of peptide stocks in water) were measured at ambient temperature in a 0.1cm path length cylindrical quartz cuvette (Hellma). Mean residue ellipticity (mdeg) was calculated using the relationship \( \Theta = 100 \frac{\theta}{cnl} \) where \( \theta \) is the ellipticity, \( c \) is the peptide concentration (mM), \( n \) is the number of amino acids in the peptide, and \( l \) is the path length (cm). Fast atom bombardment (FAB) mass spectrometry measurements were carried out on a FinneganMat-900 instrument.

**Mercuric Acetate**  Peptide was dissolved in a minimal amount of water and 100mg/mm S-tBu solution (2-10 fold excess) added. Peptide was placed under vacuum and precipitated by 2-Mercaptoethanol in a 55°C water bath under stirring. After filtering through dampened Celite, the filtrate was rotary evaporated, acidified with 0.1% TFA in water and lyophilized.
Biological Procedures

*Immunizations and animals*  Female New Zealand white rabbits were obtained from Mohican Valley Rabbitry (Loudenville, OH). Rabbits were immunized subcutaneously at multiple sites with a total of 1mg of peptide emulsified in CFA. Subsequent booster injections (1mg and 500μg in PBS) were given three and six weeks after the primary immunization. Sera were collected and complement inactivated by heating to 56°C for 30min. Sera aliquots were stored at -5 to -15°C. Antibodies were purified by ammonium sulfate precipitation: A stock solution of saturated ammonium sulfate solution (SAS) was prepared, autoclaved and cooled to 4°C. Antibody was allowed to precipitate by slowly adding SAS to 35% v/v under stirring in cold room. Samples were centrifuged 14,000 xg 20min and the supernate stored at -20°C. The pellet was dissolved with 0.1M PBS in ½ original volume. Fractions were then placed in Slide-a-lyzer cassettes (Pierce) and dialyzed against frequent changes of >200 volumes pH 8, 0.15M NaCl. The saline was brought to pH 8 with a few drops of 0.1M NaOH. IgG concentration was determined by radial immunodiffusion (RID) (The Binding Site, UK). Monoclonal antibodies were purchased from Oncogene Science.  

*Direct ELISA*  U-bottom polyvinyl chloride plastic assay plates were coated with 100μl of antigen at 2μg/ml in PBS overnight at 4°C. Nonspecific binding sites were blocked for 1 hour with 200μl PBS-1% BSA and plates were washed with PBT(phosphate-buffered saline containing 0.05% Tween 20 and 1% horse serum). Rabbit antiserum 1/500 or mouse antiserum 1/50 in PBT was added to antigen coated plates, serially diluted 1:2 in PBT, and incubated 2hr at room temperature. After washing the plates, 50μl of 1/500 goat anti-rabbit
or goat anti-mouse IgG conjugated to horseradish peroxidase (Pierce Chemical Co.) was added to each well. Excess antibody conjugate was removed, and bound antibody was detected using 50μl of 0.15% H₂O₂ in 24mM citric acid, 5mM sodium phosphate buffer, pH 5.2, with 0.5 mg/ml 2,2'-aminobis(3-ethylbenzthiazoline-6-sulfonic acid) as the chromophore. Color development was allowed to proceed for 10min and the reaction was stopped with 25μl of 1% sodium dodecysulfate. Absorbance was determined at 410nm using a Dynatech MR700 ELISA reader. Results are expressed as the mean absorbance of duplicate wells after subtraction of background.

**Cell Culture.** Stock cultures were maintained at 37°C in a 5% CO₂ incubator. All cell culture media, FCS, and supplements were purchased from GIBCO (Grand Island, NY). The human breast adenocarcinoma cell lines SKBR-3 and MCF-7 were obtained from the American Type Culture Collection and was subcultured in McCoy's 5A or DMEM supplemented with 10% FCS and L-glutamine. Cav-1 was maintained in RPMI 1640 with 10% FCS and L-glutamine. Cav-1 was derived from a fresh colon tumor specimen which was cryopreserved and subsequently cultured; it does not express detectable levels of HER-2/neu. SKBR3 is a breast tumor cell line which overexpresses the HER-2 protein while MCF-7 expresses the normal concentration of protein.

**Immunoprecipitation and Western Blotting.** On Day zero 1.0 x 10⁷ SKBR3 cells were plated in 75cm² cell culture flasks and allowed to adhere overnight. Anti-peptide antibodies were added (10 μg/ml) for 4 hrs. The reaction was stopped by aspirating the media and immediately adding ice cold 0.1M phosphate buffered saline (PBS). Cells were trypsinized
and washed twice with cold Hank's Balanced Salts Solution (HBSS). Cold lysis buffer (150mM NaCl; 50 mM Tris, pH 8; 10mM EDTA, 10mM sodium pyrophosphate, 10mM sodium fluoride; 1% NP-40, 0.1% SDS) containing 3mM Na3VO₄, 10μg/ml each aprotinin and leupeptin was added to cells resuspended in 100μl HBSS. Lysis was achieved by gentle rotation at 4°C for 20min. After centrifugation (14,000xg, 20min) to remove cell debris, lysates were incubated with 3-5μg antibody and 30μl Protein A/Protein G (Oncogene Science) overnight. Beads were pelleted by centrifugation (14,000xg 30sec), washed twice in lysis buffer containing 1mM Na3VO₄ and boiled in SDS sample buffer 5 min.

Proteins were resolved by 7.5% SDS-PAGE, transferred to nitrocellulose and probed with antibody. Protein transfer was monitored with prestained molecular mass standards (BioRad). Immunoreactive bands were detected using horse radish peroxidase conjugated goat anti rabbit immunoglobins by enhanced chemiluminescence (Amersham).

**Indirect Binding Assay** SKBR3 cells or MCF-7 cells were plated at 5,000 cells/well in V-bottom plates (Linbro, McLean VA). The cells were incubated with various concentrations of antibodies. After being washed with Hank's Balanced Salts Solution (HBSS) the cells were incubated for one hour with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit or goat anti-mouse antibody and fixed with formalin. A mouse monoclonal Ab (Oncogene Science, Cambridge, MA) was used as the positive control and an anti-CD3 Ab as the negative control. The cells were analyzed by a Coulter ELITE flow cytometer (Coulter, Hialeah, FL), which has an argon laser for excitation at 488nm, and a 525nm band pass filter for FITC fluorescence. 5.0 x 10³ cells were counted for each sample and final
processing was performed. Debris, cell clusters and dead cells were gated out by light scattered assessment before single parameter histograms were drawn.

**Effect of Abs on cell proliferation**  
SKBR3, MCF7 and CAV1 cells were plated 5,000 cells/well in V-bottom plates along with various concentrations of Ab on day zero. On day 3 cells were pulsed with [3H] thymidine (1μCi/well) at which time they were placed in a -20°C freezer for 1h. After thawing at room temperature cells were harvested an a PHD cell harvester (Cambridge Tech, Inc.). Samples were incubated in 5ml Ready Safe liquid scintillation cocktail (Beckman) and radioactivity determined by beta counter. Results are expressed as the mean CPM +/- the standard deviation (SD).

**CTL Assay: In vitro stimulation:** Inguinal and periaortic lymph nodes (LN) are removed 7 - 10 days after immunization. LN cells (4 x 10^6 - 5 x 10^6) are then stimulated *in vitro* by coculturing with 1.5 x 10^5 irradiated (10 000 rad) P815 cells prepulsed for 1h with 1μM of the appropriate CTL peptide. The culture medium used is cDMEM (DMEM supplemented with 10% FCS). Supernatant containing 30 U/ml (final) of IL-2, 2mM L-glutamine, 10mM Hepes and 5 x 10^-5 M 2-mercaptoethanol).

Seven days after *in vitro* stimulation, the CTL activity is tested in a standard chromium-release assay. P815 cells (10^6) are labeled with 150μCi sodium [^{51}Cr] chromate for 1h at 37°C in the presence or absence of the appropriate peptide (1μM) and washed three times. Labeled targets (2 x 10^5) are co-incubated with stimulated LN cells at predetermined ratios in 200μl volumes in V-bottom 96 well plates. After a 4h incubation at 37°C, the
supernatants (100μl) are harvested for γ-counting. The % specific lysis is calculated as 100 x [(experimental-spontaneous release)/(total-spontaneous release)] (Valmori, et al. 1994).

Effect of antibodies in vivo. HER2 cells (3 x 10⁶) were suspended in 250ul PBS, mixed with 250ul MATRIGEL (Beckton Dickinson) on ice and injected s.c. into mice. Polyclonal antibodies to a total concentration of 2mg/mouse, were injected i.p. on days 9 and 11. Tumor volume was measured twice weekly with calipers and calculated by the formula (length x width x height).
1. Identification of B cell epitopes in p185 Her-2/neu.

The profiles of chain flexibility and mobility of individual sequences were calculated according to Karplus and Schultz. Hydropathy profiles were generated using the scale of Kyte and Doolittle, over a seven residue span setting, and were finally smoothed with a three residue span. Hydrophilicity profiles were generated using the program of Hopp and Woods using a 6-residue window. Analysis of the exposure of an amino acid residue to water (1.4Å probe) was carried out by Rose solvent exposure algorithm. Protrusion indices were calculated by the method of Thornton that predicts portions of proteins that are accessible and protrude into the solvent. The probability that a five-residue sequence is an antigenic epitope was determined by the method of Welling. Computer programs by Chou and Fasman, Novotny were used to predict the secondary structure (α-helix, β-strand/sheet, β-turn/loop, random coil) and helical amphiphilic moment (Kaumaya, 1992).

The best vaccine candidates according to our analyses were deduced as follows: 1) computer generated profiles for individual algorithms were analyzed, and sequences were scored on their respective index values and were assigned a priority value 1-6; 2) Sequences were ranked by comparing the joint predictions involving the combination of several different empirical predictive algorithms. Highest ranking sequences have the highest
individual score for the maximum number of analyses examined (6/6), and successive candidates have the next highest score (5/6) and so on. Table 4 lists Her-2 B cell epitopes predicted to be immunogenic.

3) The best scoring epitopes were further ranked by correlation with their secondary structural attributes. For example, an amphiphilic alpha-helical sequence or a beta-turn loop region is preferred over a random coil fragment; 4) Finally, consideration was given to the individual amino acid sequence (e.g. hydrophobic/hydrophilic balance). Those sequences (from the above prediction table) receiving the highest score were arranged into Table 5 which lists their secondary structural characteristics and probable post translational modification. For example the amino acid sequence Asn-X-Thr/Ser often represents an N-linked glycosylation site. Six B-cell epitopes from the published amino acid sequence of Her-2/neu were chosen for peptide synthesis. The peptides are: DW1 376 -395, DW2 391 -399, DW3 376 - 399, DW4 628 - 647, DW5 115 - 136 and DW6 410 - 429.
2. Evaluation of the immunogenicity of predicted B cell epitopes by peptide synthesis and ELISA.

*Peptide DW1(376-395)MVF*

*Rationale:* HER-2 B cell epitopes 376 - 395 appeared to be the most straightforward synthesis and was carried out first. There are no cysteine residues or putative N-linked glycosylation sites. The epitope, named DW1, is predicted to be α-helical with a slight β-turn propensity.

*Synthesis:* The 20 amino acid HER-2 sequence was attached to the N-terminus of MVF 288 - 302 by the four amino acid linked sequence Gly-Pro-Ser-Leu. The resulting peptide was named DW1MVF indicating DW1 placement at the N-terminus as opposed to MVFDW1 which would represent C-terminal position (Figure 8).

The first amino acid was joined manually and completion monitored by Kaiser ninhydrin test. Subsequent couplings were performed on the Milligen/Biosearch 9600 peptide synthesizer. After final deblocking the peptide was cleaved from the resin with Reagent PU. An extended cleavage time is necessary since the peptide contains Arg - 2,2,5,7,8-pentamethylchroman-6-sulfonyl(PMC) and His. (A yellow cleavage solution is observed when histidine is present).

*Purification and characterization:* The peptide was rotary evaporated to remove TFA, precipitated with cold ether, and water/ether extracted. Extraction was fairly easy and HPLC analysis of the gel filtered lyophilized peptide showed few deletion peptides. (Figure 9 Absorbance values for G-25 column purified DW1MVF or DTT). The peptide eluted as 2 peaks prior to DTT. These were pooled and subjected to analytical HPLC (Figure 10).
Analytical HPLC of semiprep purified DW1MVF (Figure 11) identified one major peak while CZE identified three species of differential charge (Figure 12). The complete peptide contains 6 negative and 4 positively charged species at neutral pH. Mass spectrometry results (Figure 13) confirmed the major peak as the epitopes of interest with a molecular weight of 4472. The CD spectra of peptides in dilute aqueous acetic acid indicate slight random coil. In TFE, the conformation shifts to a slight α-helix with a strong maximum in the 190 - 195nm range and minima at 208 and 222nm (Figure 14). Helicity of peptide $[\theta_{222} = -5,000]$ was calculated using Chen’s equation with reference to the mean ellipticity of polylysine for 100% helix $\theta_{222} = -33,000$.

Peptide MVFDW4(628-647)

Rationale: At the time this peptide was being considered there were several putative HER-2 ligands in the literature. From published crystal structures the motif for ligand binding to TK receptors was through the most N-terminal folds. Assuming that this could be the case with HER-2, a peptide was chosen that would raise antibodies to a more C-terminal, membrane proximal region. Receptor aggregation could make ligand binding more difficult of prevent HER-2 from interacting with other family members thereby blocking its activation. B cell epitopes 628 - 647 was synthesized with this in mind.

The native sequence (Figure 15) contains 3 cysteine residues whose disulfide bonding pairs are unknown. I hypothesized that the cysteines at position 634 and 642 might form a bridge, therefore Cys 630 was substituted with Gly. Substituting glycine for cysteine is one way to preserve the relative size of the R group at that position. A bulkier amino acid may change the natural fold of the peptide while substitution using a charged specie may
perturb the native peptide conformation. Synthesis proceeded by first making the DW4 (628-647) peptide attached to the linker then extending the sequence N-terminally by addition of the MVF (288-302) T helper cell sequence. This produced the MVFDW4 peptide.

In order to create the disulfide bond, the tBut protecting group was cleaved giving the free thiol form. The mercuric acetate/2-mercaptoethanol procedure reduces production of disulfide bonded multimers. Analytical HPLCs of the crude product and samples were compared. In the crude sample, two sharp peaks are immediately followed by a broad ill-defined shoulder. The treated sample (Figure 16) showed a reduction in the size of the leading peak and a broader second peak. The correct fraction was later identified by mass spectrometry (Figure 17) which confirmed the molecular weight of the peptide as 4612.

To deduce the identity of the new peaks hydrogen peroxide or dithiothreitol (DTT) was added to the crude samples. Addition of hydrogen peroxide causes oxidation.

\[ 2RSH + H_2O_2 \rightarrow R-S-S-R + H_2O \]

Figure 18 shows that this reaction produced a single major peak that eluted at 11.5 minutes. This corresponds with the leading peak of the crude sample. DTT treatment causes reduction by the following reaction scheme:

\[ R-CH_2-S-S-CH_2 + DTT \rightarrow R-CH_2-SH + HS-CH_2-R' \]

The profile of the DTT treated product (Figure 19) resembled the starting material. These profiles reveal that the starting material is a mixture of reduced and oxidized peptide. Mercuric acetate treatment shifts the concentration in favor of the reduced specie.
Peptide DW5(115-136)MVF

**Rationale:** Sequence alignment of EGFR, HER-2, HER-3 and HER-4 reveals a unique span of amino acids in the amino terminal of HER-2. I speculated that this could confer special characteristics upon HER-2 and wanted to test the effects of an antibody to this region.

**Synthesis:** MVF 288-302 plus the four residue amino acid linker was connected to the resin as before and the sequence continued with HER-2 B cell epitopes 115-136. This produced the peptide DW5MVF (Figure 20). The sequence is predicted to be a β-turn with high aggregation potential. This necessitated double coupling critical residues A115, V116, T127, V129 and S133.

**Purification and characterization:** DW5MVF was cleaved and extracted with ether and water. Extraction was quite difficult as the peptide formed dense, sticky aggregates which were only minimally soluble by addition of acetic acid. Analytical HPLC of the crude sample showed one predominant peak with a minor doublet. Semipreparative HPLC was used to separate the doublet. The lyophilized sample was readily dissolved in dilute acetic acid for analytical HPLC (Figure 21). A sample was subjected to time of flight mass spectrometry (Figure 22) and yielded a molecule of the correct molecular weight 4431. The peptide elutes as a single peak at 15.5min.

Peptide DW6(410-429)MVF

**Rationale:** Residues 410-429 of HER-2, named DW6, represent a potential immunogenic epitopes from the same region as DW1MVF. Due to the success of DW1MVF in early
FACS experiments we wished to raise additional antibodies to this region. Residues 410-429 were synthesized as previously described by N-terminal addition to the MVF/4-residue-linker sequence. The final product was DW6MVF (Figure 23). It is predicted to be a β-turn with moderate to high aggregation potential at its C-terminus.

**Synthesis:** The DW6 sequence was attached to the C-terminal resin linked MVF sequence using FMOC chemistry. Computer algorithms predicted high aggregation potential therefore extended coupling times and/or double coupling was used to try to minimize aggregation.

**Purification and characterization:** TFA cleavage yielded DW6MVF peptide which was ether/water extracted. This workup as well proved to be difficult due to the high degree of aggregation. There was a significant concentration of deletion peptides as shown by HPLC. Semipreparative HPLC separated the major peak which eluted at 13-14.5 minutes. Analytical HPLC showed a single peak that eluted at 17 min. The difference in retention time for semipreparative HPLC and analytical HPLC (Figure 24) is due to a 3 minute difference in the time of sample injection. Amino acid analysis (Figure 25) confirmed its identity as the peptide of interest. Derivatized amino acids were analyzed as their phenylthiohydantoin derivatives, (observed (theory): Asp (2.99(3)), Glu (4.32 (4)), Ser (4.77(5)), Gly (3.53 (4)), His (1.62(2)), Arg (1.08 (1)), Thr (.02 (0)), Ala (0.97(1)), Pro (3.02 (3)), Tyr (0.95(1)), Val (3.86(4)), Met (0.09 (0)), Cys (0(0)), Ile (2.87(3)), Leu (9.34 (9)), Phe (0.93(1)), Lys (2.28 (2)) and Trp (0(0)).

**DW2(391-399) and DW3 (376-399)**

**Rationale:** DW2, HER-2 (391-399) is a CTL epitope with an amino acid motif appropriate for binding the Class I MHC antigen HLA-A2.1. Two CTL sequences can be
found in the extracellular domain (ECD). A CTL response to these assessable epitopes may be able to aim a killing response at tumors over expressing HER-2.

**Synthesis:** Residues 391-394 were attached to the resin. Half of the product was reserved as DW2 peptide whose amino acid sequence is shown in Figure 26. Synthesis was continued using the remainder by addition of residues 376-390. This gave a final peptide (DW3) of 376-399 (Figure 27).

**Purification and characterization:** Semipreparative HPLC separated the major peak (eluting at 11 minutes) of a triplet from DW2. DW3 gave two major peaks of mixed composition. Mass spectrometry determined that the fraction eluting at 11 minutes in DW2 was the correct peptide with a molecular mass of 1052. No further characterization was done on DW3.
Immunogenicity of peptides

Peptides synthesized from the Her-2 sequence predictions are highly immunogenic as evidenced by high antibody titers as early as the third week post immunization. Sera obtained weekly was assayed for their ability to recognize and bind to the peptide sequence. DW1MVF showed a steady rise in antibody titers. Titers for DW5MVF were higher in one rabbit than the other. Rabbit 1 showed an immediate, vigorous response to peptide immunogen while Rabbit 2 gave a slow but steady rise in antibody titer. MVFDW4 produced the most immediate and vigorous response. These exceptionally high titers remained at maximal levels through four weeks after the tertiary boost. Peptide DW6MVF gave the lowest titers of the four antibodies but responses were stable and comparable between rabbits. (Table 6 Comparative immune responses to the various peptide immunogens). The polyclonal IgG sera did not cross react with the MVFT-cell sequence. All results were obtained in an outbred population indicating the broad immunogenicity of the peptides in rabbits.

3. Determination of the specificity of peptide antibodies for Her-2

FACS and Immunoprecipitation In a preliminary experiment (Figure 28) flow cytometric analysis determined that DW1MVF anti-peptide antibody directly targeted the HER-2 receptor. A commercially available mouse MAb to HER-2/neu was used as a control in SKBR3 cells. Negative control sera showed no binding to the receptor while an increase in fluorescence was seen with the immune sera. Fluorescence intensity of the polyclonal antipeptide serum was comparable to the monoclonal antibody. Therefore polyclonal serum can mimic the specificity and affinity of monoclonal antibodies.
Immune serum was serially diluted in Hank's Balanced Salts Solution and the mean cell fluorescence determined. Serum from the 'tertiary + 3 weeks' bleed gave a fluorescence value of 121.4 (diluted 1:320) while the MAb gave a value of 132. The following is a list of the mean cell fluorescence values at various serum dilutions.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Mean Cell Fluorescence (DW1MVF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.647</td>
</tr>
<tr>
<td>1:80</td>
<td>39.3</td>
</tr>
<tr>
<td>1:160</td>
<td>83.5</td>
</tr>
<tr>
<td>1:320</td>
<td>121.4</td>
</tr>
<tr>
<td>1:640</td>
<td>69.4</td>
</tr>
<tr>
<td>1:1280</td>
<td>34.4</td>
</tr>
<tr>
<td>1:2560</td>
<td>17.4</td>
</tr>
</tbody>
</table>

The peptide antibodies MVFDW4, DW5MVF and DW6MVF however did not give the same intensity of fluorescence as DW1MVF. I therefore used immunoprecipitation to verify specificity for Her-2. SKBR3 cells were immunoprecipitated with Protein A/G purified antipeptide antibodies (Figure 29). Antibodies are shown to be HER-2 reactive. Identical bands are evident in the Mab sample and the antipeptide antibodies.

4. **Identification of the biological effects of antipeptide antibodies**

*Proliferation in vitro* Once specificity for Her-2 was confirmed the next step was to determine biological activity, in this case a reduction in tumor growth such as that produced
by incubation of MAbs with Her-2 overexpressing cells. The \textit{in vitro} effects of antibody on tumor cells was determined by a standard tritiated thymidine proliferation assay (Figure 30). Antibodies DW1MVF, MVFDW4 and DW5MVF were able to reduce proliferation of SKBR3 cells \textit{in vitro}. MCF-7 cells expressing normal amounts of the receptor were not inhibited by the antibodies. In contrast, a commercial monoclonal antibody that reduces tyrosine phosphorylation and DW6MVF which also reduces phosphorylation \textit{in vitro} (Woodbine et al., submitted), stimulated cell growth in SKBR3 cells. These results show that polyclonal antibodies can behave like monoclonals in that biological effect is dependent on the epitopes recognized. Some antibodies may be stimulatory and others inhibitory (Stancovski 1991). Table 7 lists individual values for a representative tritium assay. Additionally, it is interesting to note that the inhibitory antibodies had negligible effect on cells expressing normal amounts of HER-2 (MCF7). This is also reported for some monoclonals and is advantageous for therapy of breast cancer where minimal toxicity of normal cells is desired.

\textit{Phosphorylation Assay} Since monoclonal antibodies have been shown to reduce Her-2 proliferation by a reduction in tyrosine phosphorylation, we wished to demonstrate this capability with antipeptide antibodies. The exact mechanism of action is unknown therefore epitopes spanning the entire extracellular domain were used. MVFDW4 (628-647) and DW6MVF (410-429) are immunogenic epitopes near the transmembrane region of p185. From the antiphosphotyrosine blot (Figure 31) it can be seen that reduction in phosphorylation was achieved with DW6MVF and to a lesser extent with MVFDW4. Reduction in HER-2 phosphorylation levels by MVFDW4 and DW6MVF are visible using
a monoclonal anti-phosphotyrosine antibody. Addition of sodium vanadate as the positive control and the untreated control show high levels of endogenous tyrosine kinase activity.

To determine HER-2 levels in each lane the blot was stripped and probed with a C-terminal antiHER-2 monoclonal. Figure 32 depicts low levels of HER-2 in the MVFDW4 sample while the monoclonal antibody and DW6MVF samples similarly show disappearance of HER-2. Figure 32 shows several bands recognized by a HER-2 monoclonal. These bands may be the result of differential phosphorylation or proteolytic cleavage of Her-2. Proteolytic cleavage of p185 and EGFR has been reported (Pupa 1993, Leitzel 1992). Degradation of receptor is inhibited in Tyr973 mutants (Decker 1992) which lack the internalization codes. Another group (Kiyokawa 1995) reports that the extent of p185 phosphorylation is cell cycle dependent and that a mobility shift on SDS/PAGE is mainly caused by the extent of phosphorylation.

*Proliferation in vivo* Three out of four antipeptide antibodies were successful at retarding tumor growth in a nude mouse model albeit to different extents (Figure 33). All instances of tumor reduction were not the result of a decrease in autophosphorylation. A summary of the results (Table 8) suggests that some antibodies (MVFDW4 and DW5MVF) may require host effector cells for maximum tumor suppression. A plot of tumor progression vs time (Figure 34) shows decreasing tumor volume with antibodies DW1MVF, MVFDW4 and DW5MVF. DW6MVF seemed to have negligible effects on growth.
5. Identification of cytotoxic T cell (CTL) epitopes

**CTL Assay**  Peptide DW1MVF (376-395) has an overlapping T cell epitope 391-399. We wished to test the efficacy of this epitope for incorporation into a HER-2 vaccine. Results showed that a specific CTL response could be raised to a HER-2 derived peptide (Figure 35). Cytotoxic T cells primed with Her-2 391-399 were able to specifically lyse autologous targets in a dose dependent manner. However, two *in vitro* re-stimulations were necessary to produce this result. Disis (1994) showed comparable specific lysis of Her-2 primed targets after four restimulations. At least 10 stimulations were necessary to achieve greater than 50% lysis in their study. Factors affecting these results are the number of responder T cells in the original culture, amount of CD4+ T-cell response and the number of restimulations.

Proper T cell help, such as production of the cytokine IL-2, is needed to achieve efficient target cell lysis when priming with a free peptide in vivo (LeClerc 1991, Fayolle 1991, Ishioka 1989). In this study IL-2 was added to the culture medium. Immunization with a peptide containing both T helper and cytotoxic T cell sequences may boost the results. This was the case in another study using ras peptides (unpublished observation). Increased specific lysis was demonstrated in animals primed with peptides incorporating a tetanus toxoid helper epitope.

Class I molecules bind intracellularly derived peptides. Intracellular Her-2 peptides produced more vigorous lysis in work done by Cheever et al (Disis 1996). The most C-terminal epitope gave the largest response. These results show that a CTL response can be raised to the self protein Her-2. The less vigorous response seen for extracellular sequences in our study and that of Cheever may indicate that an intracellular epitope would be more
favorable in a peptide based therapy. Further tests will be necessary to identify which Her-2
CTL epitope produces the best results when used in conjunction with the B-cell epitopes
synthesized for this study.
CHAPTER 5
DISCUSSION

The advantage of targeting HER-2 for an immune based therapy is that HER-2 is not a mutated protein thus antigenic determinants would not vary from tumor to tumor nor from patient to patient. The three dimensional structure of Her-2 is still unknown and the sequences bound by current Mabs have not been determined. It is here that active immunotherapy through antipeptide antibody generation becomes useful.

Active immunotherapy through the use of antipeptide antibodies offers a unique alternative to current cancer management.

(1) Vaccination with peptide can provide a safe, nontoxic, long-lived effect.

(2) The availability of tumor targeting antibody is continuous and boosts are given after a period of weeks or months instead of days.

(3) Antibodies are native to the host.

(4) Antibodies are highly specific and in the case of the HER-2/neu recognize nonmutated sequences.

(5) Antibodies ‘select’ for tumor cells expressing excess amounts of receptor.

The controversy among vaccine developers however is which of the two arms of the immune system must be turned on: one which depends on T lymphocytes to kill tumor cells or one that rests on antibodies to block or inhibit metastasis. We are striving to develop
synthetic peptide vaccines that not only offer a safe, stable alternative to most preparations currently under investigation but also the potential of optimizing specific B cell, T helper (Tk) cell and cytolytic T cell (CTL) responses, all of which appear to be requisite for inducing effective antitumor immunity.

Peptide vaccines have several advantages over conventional vaccines: they are safe, produce well-defined immune responses, have high reproducibility and purity. Problems might relate to the ability to define appropriate tumor antigens and variability of the host. An individual's response to an antigen is determined by their MHC polymorphism. Individuals will recognize different epitopes of a complex protein antigen. This is called MHC restriction (Lanzavecchia 1993). A vaccine must allow for recognition in at least one allele in each individual. So called 'promiscuous epitopes' which bind multiple MHC haplotypes have been identified in lymphocytic choriomeningitis virus (LCMV) amino acids 119-127 (Oldstone 1991).

• **Identification of B cell epitopes in p185 Her-2/neu**

The three dimensional structure of Her-2 is not known. Therefore, computer algorithms were used to predict the amino acid sequences likely to be immunogenic. From the crystal structures of other proteins it is known that antibodies are likely to bind regions that are hydrophilic, solvent exposed and flexible. Antibodies are more likely to bind regions with defined secondary structural characteristics such as α- helices, β- sheets and β or hairpin turns over those adopting a random coil conformation.
Evaluation of the immunogenicity of predicted B-cell epitopes by peptide synthesis and ELISA.

Historically, antibodies induced by immunization of short peptide sequences into animals showed poor affinity to the native protein. This is partly because the antibodies generated by peptide immunization are a collection of clones raised to the solution conformation of the peptide, denatured and differentially processed sequences. Antibody recognition sites are usually of the conformational type and the peptide sequences lack defined structure in solution. Peptides must mimic the native conformation of the protein for their respective antibodies to bind with high enough affinity to be biologically significant. Affinity is a direct consequence of achieving shape complementarity. We have previously engineered conformational epitopes with defined secondary structural characteristics in solution. We have also had much success in predicting potential immunodominant epitopes from proteins (Kaumaya 1992, 1994). These predictive methods were applied to the Her-2/neu protein.

Identification of the biological effects of antipeptide antibodies.

The cell surface is a dynamic structure and cells perceive their environment through proteinaceous extensions that are free to move within its plane. Recognition is accomplished by binding events that change the resting state of the receptor itself and/or the intracellular environment. Thus receptors need to be linked to both the external and internal milieu. In the case of the EGFR family, receptors span the membrane creating a direct link. The cytoplasmic region of EGFR contains the kinase domain along with seven tyrosine sites available for phosphorylation. Once phosphorylated, intracellular adaptor/catalytic proteins,
some of which contain shc gene products (SH2/3 domains) can bind to the receptor. Examples are phospholipase C-γ (PLC-γ), GTPase-activating protein and phosphatidylinositol 3-kinase (Emlet 1997). This kinase and phosphatase cascade eventually couples extracellular signals to nuclear events. Genes are turned on and protein synthesis follows as evidenced in this case by cell growth.

Tyrosine residues are important not only for docking of signaling molecules but for receptor trafficking. Several of these tyrosines are within a constitutive internalization motif, NPXY, found in constitutively endocytosed receptors (Chang 1993). Mutations or certain changes in targeting domains can cause aberrant distribution and disease (Opresko 1995). Truncation mutants have given insight into the role of small C-terminal regions in EGFR biology and can give clues about HER-2 mechanisms. Tyr\textsuperscript{973} mutants are unable to bind PLC-γ and GTPase activating protein SH regions and internalization is slower than wild type (Decker 1992). A longer mutant, Tyr\textsuperscript{992} binds PLC-γ. These receptors displayed increased phosphorylation upon treatment with EGF but were also endocytosed more slowly than wild type (Emlet 1997). This may be explained by lack of downstream internalization codes having affinity for AP-2 and coated pit proteins (Chang 1993, Gilboa 1995, Nesterov 1995).

p185\textsuperscript{c-neu} contains five known autophosphorylation sites with tyrosines within an NPXY internalization motif. Neu mutant Tyr\textsuperscript{1253} (Gilboa 1995) possesses only one phosphorylated tyrosine which is sufficient to immobilize the receptor to the cytoskeleton. Several researchers have reported significant internalization of EGFR 60-80% (Opresko 1995, Lamaze 1995, Decker 1992) in less than 1 hour while incubation with antibody for 5 hours (Hurwitz 1995) brought about comparable internalization of p185.

It has been proposed that receptor occupancy gives a more open conformation
exposing C-terminal trafficking domains (Opresko 1995). Nesterov and colleagues (1995) found that addition of urea resulted in a 10 fold increase in receptor binding to AP-2 which connects receptor to the cytoskeleton. The binding of antibody in contrast to that of growth factor may perturb receptor structure in such a way as to affect the availability of tyrosine residues for phosphorylation. Blocking of or interference with the normal signaling pathway can reduce the expression of Her-2 at the membrane thereby inhibiting tumor cell proliferation.

In this study MVFDW4 and DW6MVF produced reduction in phosphorylation and receptor degradation. This could be a result of proximity to the membrane. A definitive reason for the regulatory effect of membrane proximal antibody epitopes is unknown. It can be speculated that this proximity to the intracellular autophosphorylation sites allows ready transfer of conformational changes brought about by the antibody. The antibody can expose sites for anchoring to the cytoskeleton and internalization. Once internalized the receptor can be degraded in lysozomes. It can also be seen from the antiHer-2 blot that DW6MVF was more efficient at HER-2 down-regulation than MVFDW4. (The amount of HER-2 phosphorylation in SKBR3 cells is much higher than MCF7 cells due to greater numbers of receptors and their constitutive activation.) No visible change in phosphorylation was noted with antibodies DW1MVF and DW5MVF. This is not surprising since monoclonal antibodies to different epitopes have differential effects (Stankovski 1991). Some bind and display no activity while others cause internalization and tumor regression (Katsumata 1995, Maier 1991, Hurwitz 1995). An analysis of the literature reveals that all tumor inhibiting monoclonal antibodies raised to the extracellular domain of p185 do not behave identically (Shawver 1994). Once the ligand-receptor complex has reached the endosome it may be
retained attenuating the signaling cascade or the ligand dissociated and receptor recycled to the cell membrane. Alternately, fusion of the endosome with a lysozome degrades the receptor. These pathways however are not absolute (Felder 1990).

Antibody DW1MVF was able to reduce proliferation in the SKBR3 cell line but not significantly in the MCF-7 cell line. MCF-7 cells may not present an effective target for the antibodies. Perhaps a critical mass of antibody is necessary to achieve the desired results. Without sufficient receptor numbers the MCF-7 cells cannot recruit enough antibody for the inhibitory effects.

Adenocarcinoma of the breast is a prevalent and poorly understood phenomenon in the human population. Ethical questions preclude the use of humans to study the disease. Additionally, by the time a tumor is detectable it has progressed through most of its growth cycle. This leaves only a short interval, detection to initiation of treatment, for study. The mouse model provides a reproducible system where disease progression can be chronicled over the lifetime of the animal. It allows for studying malignancy in large test populations.

The difficulty in creating a mouse model that closely resembles human mammary carcinoma by overexpression of HER-2 is quite apparent in the literature (Pattegale 1989, Cardiff 1991, Bouchard 1989, Guy 1996). Animals either synchronously produce tumors, male and female, with absence of any normal epithelium or stochastically (multistep) produce tumors where nonuniform expression may be due to unequal expression of the transgene in the tissue (Muller, 1991). In the mouse, tumors are usually not metastatic and the cell lineages of rodent and human breast are not identical (Taylor-Papadimitriou 1993).

The immunocompromised status of the nude mouse has lent itself well to the study of human xenografts. The problem is that many breast cancers do not grow well in the nude
mouse. Matrigel, solubilized tissue basement membrane protein from the ECM of Engelbreth-Holm-Swarm mouse tumor, has been used to increase the take rate of human tissue in nude mice (Mullen 1996). Its major components are laminin, collagen IV, heparin sulfate proteoglycans, entactin and nidogen. It also contains TGF-β, fibroblast growth factor, tissue plasminogen activator and other growth factors. At physiological temperature Matrigel polymerizes into a biologically active matrix.

Angiogenesis, the formation of new blood vessels, is necessary for the establishment and nourishment of tumor masses. Ito, et al, 1996 demonstrated that Matrigel promoted angiogenesis *in vitro* and in athymic BALB/c *nu/nu* mice. An initial study in our lab tested the ability of SKBR3 and NIH/3T3 cells over expressing HER-2 (Cohen, 1996) to grow in nude mice. Neither cell line could be established in nude mice. Upon injection of SKBR3 cells with Matrigel, persistent tumors were apparent (unpublished observation). This may have been due to 1) the physical support afforded by Matrigel. Concentration of cells in a medium as opposed to an injection of single cells, increased the probability of forming a tumor mass  2) angiogenesis induced by matrigel hastened nutrient delivery to the cells and prevented substantial cell loss. The matrix, in oversimplified terms, provides a familiar environment for host cells and they infiltrate, proliferate and form blood vessels.

Tumor cells in general grow more slowly than normal cells. Growth occurs because cell production is greater than cell death. In normal tissue the rates are equal. Tumor life history can be understood by a graphical representation. Normal cells exhibit simple exponential growth. Simple exponential growth states that if a cell divides in two and each cell then splits in two, growth occurs by powers of two. From studies of animal tumors, the actual growth curve plotted on a logarithmic scale is sigmoidal. An initial lag period is
followed by exponential growth and finally a leveling off (Del Buono 1995). Breast adenocarcinomas have a mean volume doubling time of about 3 months.

I wanted to assess the ability of the antipeptide antibodies to suppress tumor growth \textit{in vivo}. Since the antibodies were raised in rabbits it was imperative that a host anti-rabbit response was avoided. The effective use of polyclonal rabbit sera in an athymic nude mouse model has been demonstrated (Christensen 1990, Aleksander 1986, Aleksander 1990). Antipeptide antibodies have been used successfully to protect against bovine papilloma virus-4 challenge (Campo 1997), induce pharmacological and morphological changes in acetochocholine receptors (Fu 1996), inhibit testosterone 6-beta-hydroxylation (Wang 1997), identify the MAGE-1 gene product (Chen 1994), differentiate between G-protein isoforms (Rouot 1996), and to screen for human papilloma virus (HPV) in cervical intraepithelial neoplasia (Dillner 1989).

Although the nude mouse lacks functional T cells, its B-cells, NK cells, LAK cells, macrophages and other hematopoietic cells do not seem to be affected. These cells are necessary for the mouse to mount an antibody mediated response to the transplant. VandePal et al. 1986 cited the recruitment and activation of nude mouse NK cells by rabbit antibody to vesicular stomatitis virus (VSV) induced tumors. This was demonstrated by injecting \textit{in vitro} NK resistant cells into nude mice. Rabbit antibodies recognizing a tumor antigen allowed killing of virus infected cells \textit{in vivo}.

MCF7 and SKBR3 cells were derived from metastatic cancers in the early 1970's. In choosing breast cancer cell lines for studying potential therapies it is important to compare the normal with the malignant cells. SKBR3 cells overexpress Her-2 while MCF-7 cells express normal quantities. The mammary gland consists of two major epithelial phenotypes:
basal and luminal epithelial cells. They differ in antigen and basement membrane expression. More aggressive tumors seem to have more basal cell malignancy. Luminal cells seem to be derived from stem cells in the basal layer (Taylor-Papadimitirou 1993). The difficulty remains of identifying and isolating this elusive stem cell.

- **Identification of cytotoxic T cell (CTL) epitopes**

  The crystal structure of MHC Class I and Class II molecules has been determined. These structures reveal a cleft of definite size and shape in which peptides bind. The Class I receptor binds 8 or 9mers while the open ended Class II can accommodate longer peptides. Surface topology defines two pockets at positions 2 and 9 of HLA-A2.1 which hold the dominant anchor residues L and V respectively. The Her-2/neu protein contains several potential Class I MHC HLA-A2.1 sequence motifs. Predicted CTL sequences matching the human Class I MHC motifs are listed in Table 10. Peptide DW2 was used in a standard chromium release assay. Results showed specific recognition of about 8%. This value can be increased by several restimulations with peptide as shown by Cheever et. al (Disis 1996).

**Summary**

Table 9 summarizes the data in this study. DW1MVF was able to reduce the proliferation of tumor cells both *in vitro* and *in vivo*. Antipeptide antibody alone could reduce cell proliferation *in vitro* but reduction was enhanced when placed *in vivo*. MVFDW4 gave slight
reduction in proliferation in vitro as well as slight reduction in phosphorylation and degradation. Results in vivo were more variable between animals. DW5MVF was most successful at reducing tumor proliferation in the nude mouse model. This antibody to a Her-2 specific sequence produced the best tumor inhibitory response in vivo. Its efficacy in vivo as compared with in vitro results probably reflects the need for accessory cells such as NK cells, macrophages and activation of complement for a tumor inhibitory response. DW6MVF behavior most closely matched that of the monoclonal antibody. It stimulated cell growth in vitro and in the preliminary in vivo assay while giving the most significant reduction in tyrosine phosphorylation.

These results indicate that

- peptides raised to Her-2 were immunogenic in an outbred population
- antipeptide antibodies reduced proliferation of tumor cells in vitro
- antipeptide antibodies decreased phosphorylation of the Her-2 receptor
- antipeptide antibodies suppressed tumor growth in a nude mouse model
- a specific CTL response was raised to the self protein Her-2
- gross reduction in the level of phosphorylation is not a good predictor of efficacy in vitro or in vivo.
- antibodies can give different results based on the epitope bound.

Implications

What are the implications of peptide immunogens for cancer therapy? Breast cancer incidence rates have continued to rise since 1980 thus there is a continually increasing
population that can benefit from improved cancer management methods. Current therapies are invasive and nonspecific with undesirable side effects. We have demonstrated the efficacy of peptide antibodies both in vitro and in an animal model. The approach discussed in this paper provides a highly specific method of care. Further, the availability of tumor targeting antibody is continuous and boosts can be given after a period of weeks or months instead of days. Her-2 positive cancer cells are located with the precision of antibodies and the body's own defenses are recruited to destroy tumor. Peptide preparations are pure, reproducible and immune responses are well defined.

The introduction of an antitumor peptide immunization would greatly simplify treatment procedures. There would be minimal financial investment for health care organizations and less medical supervision would be needed. Patients would require no hospital stays and the simple protocol would make long tiring trips to central cancer treatment facilities unnecessary. Considering the previously mentioned benefits, this method could revolutionize current cancer treatment raising the quality of life for cancer patients and significantly reducing medical costs.


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Appendix A
Figure 1  Ras signal transduction cascade. The growth factor receptor tyrosine kinase phosphorylates a protein complex termed ras activator complex (RA). The activated RA complex activates the ras oncoprotein which sequentially activates three serine protein kinases: RAF, MEK, and MAP-kinase. MAP-kinase can phosphorylate and induce cell division by activating transcription factors such as c-fos and c-jun. PKC induces c-fos and c-jun by unknown mechanisms.
Figure 2  EGFR and Her-2 internalization motifs. Tyrosine residues responsible for internalization are located in internalization motifs consisting of the amino acid sequence Asn-Pro-X-Tyr, where X can be any amino acid. Phosphorylated tyrosine residues in this environment can interact with the cytoskeleton, anchoring the receptor as the region invaginates and becomes internalized. TM, transmembrane region. TK, tyrosine kinase domain.
Figure 3 Antibody structure. Immunoglobulins are heterodimers of 2 heavy and 2 light protein chains connected by disulfide bonds. Each heavy chain is about 50kD and the light chain 20kD giving a total molecular weight of about 150kD after glycosylation. Enzymatic cleavage breaks the antibody into 2 or 3 domains. Papain yields 3 fragments while pepsin yields 2 fragments.
Figure 4  Cyclization of dipeptides bearing small protecting groups. In solid phase peptide synthesis, bulky protecting groups must be attached to the N-terminal of the incoming amino acids to prevent the cyclization illustrated above. X, protecting group.
Fluid front in HPLC and CZE. In the parabolic flow HPLC systems, friction between the mobile and stationary phases causes fluid velocity to be slow at the edges of the tube and faster toward the center. With capillary electrophoresis, the flow is uniform through the entire length of tubing giving a flat or 'plug front' flow.
Figure 6  Typical CD spectra of protein secondary structures: 1) alpha helical, 2) beta sheet and 3) random coil.
Figure 7 ELISA procedure flow diagram. Steps involved in performing a direct ELISA: 1) bind antigen, 2) serially dilute the primary antibody, 3) remove unbound antibody. Add labeled secondary antibody. Remove unbound antibody. Add substrate. Allow color development. 4) Plot absorbance as a function of Ab dilution.
Figure 8  Amino acid sequence of DW1MVF
Figure 9  Absorbance values for G-25 column purified DW1MV or DTT. Fractions were collected at λ235, pooled, lyophilized and subjected to analytical HPLC. DTT elution was monitored at λ410.
Figure 10 Analytical HPLC trace of G-25 column purified DW1MVF. A single major peak elutes at 16 minutes. Minimal amounts of deletion peptides are evidenced by the minor peaks. Semiperparative HPLC yielded a separated single entity as evidenced by analytical HPLC.
Figure 11 Analytical HPLC of reversed phase HPLC purified DW1MVF peptide. The peptide elutes as a single peak at 16 min. DW6MV (1 mg/ml) was chromatographed using a 30 min 10-90% linear gradient of 0.1% trifluoroacetic acid (TFA) in water and 0.1% TFA in acetonitrile. Eluant was monitored at 214 nm.
Figure 12  CZE profile of purified DW1MVF. Results seem to indicate 2 species with different charges. This could be from a minor contaminant of a peptide containing an amino acid substitution that cannot be resolved by reversed phase HPLC.
Figure 13  Mass spectrometry profile for HPLC purified DW1MV. Peaks with the greatest intensity confirm the compound mass as 4472.
Figure 14  Circular dichroism profile of purified DW1MVF peptide. The CD spectra of the peptide (250μM by dilution of peptide stock in water) were recorded in aqueous buffer (—) and with 50% TFE (—) at room temperature in a 1.0cm cuvette.
H₁N-Lys-Leu-Leu-Ser-Leu-Ile Lys-Gly-Val-Ile-Val-His-Arg-Leu-Glu-Gly-Val-Glu-Leu-
Ser-
Pro-
Gly-

HOOC-Arg-Gln-Glu-Ala-Pro-Cys-Gly-Lys-Asp-Asp-Leu-Asp-Val-Cys-Ser-His-Thr-Gly-An-Ile-

Figure 15  Amino acid sequence of MVFDW4
Figure 16 Analytical HPLC of MVFDW4 peptide. The peptide elutes as a jagged peak at 12.5 min. MVFDW4 (1 mg/ml) was chromatographed using a 30 min 10-90% linear gradient of 0.1% trifluoroacetic acid (TFA) in water and 0.1% TFA in acetonitrile. Eluant was monitored at 214 nm.
Figure 17  Mass spectrometry profile of HPLC purified MVFDW4. The peak with the greatest intensity confirms the compound mass as 4029.9.
Figure 18  Analytical HPLC of MVFDW4 and hydrogen peroxide. Addition of $\text{H}_2\text{O}_2$ to the peptide sample causes oxidation. The peak above corresponds to the leading peak of the crude sample.
Figure 19  Analytical HPLC of MVFDW4 and DTT. Addition of DTT to the peptide sample causes reduction. This profile reveals that the starting material is a mixture of reduced and oxidized material.
N-Ala- Val- Leu- Asp- An- Gly- Asp- Pro- Leu- Asn- Asn- Thr- Thr- Pro- Val- Thr- Gly- Ala- Ser- Pro- Gly-
Gly-
Gly-
Pro-
Ser-
Leu-

HOOC- Glu- Val- Gly- Glu- Leu- Arg- His- Val- Ile- Val- Gly- Lys- Ile- Leu- Ser- Leu- Leu- Lys-

Figure 20  Amino acid sequence of DW5MVF
Figure 21  Analytical HPLC of purified DW5MVF. The peptide elutes as a single peak at 15.5 min. DW5MVF (1 mg/ml) was chromatographed using a 30 min 10-90% linear gradient of 0.1% trifluoroacetic acid (TFA) in water and 0.1% TFA in acetonitrile. Eluant was monitored at 214 nm.
Figure 22  Mass spectrometry profile for purified DW5MVF peptide. The peak with the greatest intensity confirms the compound mass as 4431.
H₂N- Gin- Leu- Asn- Gin- Phe- Val- Ser- Leu- Asp- Pro- Leu- Ser- Asp- Pro- Trp- Ala- Ser- Ile- Tyr- Leu-
Gly-
Pro-
Ser-
Leu-
HOOC- Gul- Val- Gly- Glu- Leu- Arg- His- Val- Ile- Val- Gly- Lys- Ile- Leu- Ser- Leu- Leu- Lys-

Figure 23  Amino acid sequence of DW6MVF
Figure 24 Analytical HPLC of DW6MVF peptide. The peptide elutes as a single peak at 16 min. DW6MVF (1 mg/ml) was chromatographed using a 30 min 10-90% linear gradient of 0.1% trifluoroacetic acid (TFA) in water and 0.1% TFA in acetonitrile. Eluant was monitored at 214 nm.
Figure 25  Amino acid analysis of peptide DW6MVF. Peptide was hydrolyzed in 6N HCl for 24h at 110°C using the Waters Picotag system. Derivatized amino acids were analyzed as their phenylthiohydantoin derivatives, (observed (theory): Asp (2.99(3)), Glu (4.32 (4)), Ser (4.77(5)), Gly (3.53 (4)), His (1.62(2)), Arg (1.08 (1)), Thr (.02 (0)), Ala (0.97(1)), Pro (3.02 (3)), Tyr (0.95(1)), Val (3.86(4)), Met (0.09 (0)), Cys (0(0)), Ile (2.87(3)), Leu (9.34 (9)), Phe (0.93(1)), Lys (2.28 (2)) and Trp (0(0)).
NH₂-Pro-Leu-Glu-Pro-Glu-Gln-Leu-Gln-Val-COOH

Figure 26 Amino acid sequence of DW2.

Phe-Leu-Pro-Glu-Ser-Phe-Asp-Gly-Asp-Pro-Ala-Ser-An-Thr-Ala-Pro-Leu-Gln-Pro-Glu-Gln-Leu-Gln-Val-

Figure 27 Amino acid sequence of DW3.
Figure 28 Determination of antibody binding by flow cytometry. Antibodies raised to DW1MVF specifically bound the Her-2 receptor with comparable affinity as a commercially available monoclonal antibody. SKBR3 cells were incubated with antibody, washed and FITC-conjugated secondary antibody added. After being fixed in formalin, cells were analyzed by a Coulter ELITE flow cytometer at 488nm for excitation. 5.0 x 10^3 cells were counted for each sample.
Figure 28  Determination of antibody binding by flow cytometry
Figure 29 Immunoprecipitation of Her-2 by antipeptide antibodies. 10μg/ml antipeptide antibody was added to 1.0 x 10⁷ SKBR3 cells for 4h. Cold lysis buffer was added to cells with gentle rotation for 20min. Cells were centrifuged and 3 -5 μg antibody and 30 μl Protein A/Protein G were added overnight. Beads were pelleted, washed and boiled in SDS sample buffer 5min. Proteins were resolved by 7.5% SDS-PAGE, transferred to nitrocellulose and probed with antibody. Immunoreactive bands were detected using horseradish peroxidase conjugated goat anti rabbit immunoglobins by enhanced chemiluminescence.
Figure 30  \(^3\)H thymidine proliferation assay. Antibodies DW1MVF, MVFDW4 and DW5MVF were able to reduce proliferation of SKBR3 cells in vitro. MCF-7 cells expressing normal amounts of the receptor were not inhibited by the antibodies.
Figure 30   $^{3}$H proliferation assay
Figure 31  Antiphosphotyrosine blot. On day zero $1.0 \times 10^7$ SKBR3 cells were plated in 75cm$^2$ cell culture flasks and allowed to adhere overnight. 10μg/ml antipeptide antibody was added to $1.0 \times 10^7$ SKBR3 cells for 4h. The reaction was stopped by addition of ice cold 0.1M PBS, trypsinization and washing with cold HBSS. Cold lysis buffer was added to cells with gentle rotation for 20min. Cells were centrifuged and 3 -5 μg Mab to Her-2 and 30 μl Protein A/Protein G were added overnight. Beads were pelleted, washed and boiled in SDS sample buffer 5min. Proteins were resolved by 7.5% SDS-PAGE, transferred to nitrocellulose and probed with antiphosphotyrosine monoclonal antibody. Immunoreactive bands were detected using horse radish peroxidase conjugated goat anti rabbit immunoglobins by enhanced chemiluminescence.
Detection of Her-2 in SKBR3 cell lysates. Antiphosphotyrosine blots were stripped and reprobed with a monoclonal antibody to Her-2. Reduction in the level of intact protein is observed for MVFDW4, DW6MVF and the monoclonal antibody.
Reduction of tumor volume *in vivo* by antipeptide antibodies. 3 x 10⁶ SKBR3 cells were injected s.c. into groups of four nude mice. Peptide antibodies to a total concentration of 2mg/mouse were injected i.p. on days 9 and 11 after inoculation of the cells. The resulting tumors were measured twice a week with calipers and tumor volumes were calculated. Graph shows tumor sizes at 21 days after injection of cells.
Figure 34  Effect of antipeptide antibodies on tumor growth in nude mice over time. $3 \times 10^6$ SKBR3 cells were injected s.c. into groups of four nude mice. Peptide antibodies to a total concentration of 2 mg/mouse, were injected i.p. on days 9 and 11 after inoculation of the cells. The resulting tumors were measured twice a week with calipers and tumor volumes were calculated.
Specific lysis of Her-2 primed effector cells. Lymph node (LN) cells (4 x 10^6) from a peptide immunized mouse were stimulated in vitro by coculturing with 1.5 x 10^5 peptide pulsed, irradiated P815 cells for 7 days in cDMEM supplemented with 30 U/ml (final) IL-2, 2mM glutamine, 10mM HEPES, and 5 x 10^{-5} M 2-ME. At days 7 and 14, fresh peptide pulsed cells were added. Seven days after the final in vitro stimulation, 2 x 10^3 P815 target cells were ^51Cr labeled and coincubated with LN cells at ratios of 6, 25 or 100 to 1 in V bottom dishes. After 4hr at 37°C, the supernatants were harvested for γ counting. Percent specific lysis was calculated as 100 x [(experimental - spontaneous release) / (total - spontaneous release)]. At a peptide concentration of 100μg/ml, 8% specific lysis was achieved (at an effector to target ratio of 100:1) as compared with 4% lysis at 20μg/ml.
Appendix B
Table 4  B-cell epitope prediction for p185 Her-2/neu. At least two immunogenic sites were found for each 100 residue amino acid span. Four sequences (168-188), (376-395), (314-338), and (560-591) received a score of 6 out of 6. All were represented in the algorithms chosen for predicted immunogenicity. Of the remaining sequences, two received a score of 5/6, three received a score of 4/6 and three received a score of 3/6.
Table 4  B-cell epitope prediction

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<th>Algorithm/Sequence</th>
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<th>201-300</th>
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<th>401-500</th>
<th>501-600</th>
<th>601-700</th>
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<td>Hopp &amp; Woods</td>
<td>132-139 (1.0)</td>
<td>204-213 (1.6)</td>
<td>317-377 (1.5)</td>
<td>453-461 (0.95)</td>
<td>533-544 (1.2)</td>
<td>631-646 (1.7)</td>
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<td>181-190 (1.6)</td>
<td>273-286 (1.0)</td>
<td>376-383 (1.2)</td>
<td>493-500 (2.1)</td>
<td>576-581 (1.0)</td>
<td>671-694 (2.0)</td>
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<td>Kyte &amp; Doolittle</td>
<td>116-125 (2.6)</td>
<td>201-213 (2.6)</td>
<td>314-335 (2.5)</td>
<td>480-489 (3.0)</td>
<td>528-540 (3.2)</td>
<td>632-647 (3.1)</td>
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<td>Hydrophilicity</td>
<td>181-188 (2.5)</td>
<td>270-287 (2.3)</td>
<td>386-395 (3.4)</td>
<td>493-500 (1.6)</td>
<td>557-570 (2.4)</td>
<td>672-693 (1.7)</td>
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<tr>
<td>Rose</td>
<td>115-135 (3.2)</td>
<td>241-249 (3.4)</td>
<td>315-336 (5.2)</td>
<td>410-418 (3.0)</td>
<td>561-570 (3.3)</td>
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<td>376-395 (3.4)</td>
<td>492-500 (3.6)</td>
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<td>672-684 (3.65)</td>
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<td>167-180 (0.9)</td>
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<td>484-496 (1.3)</td>
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<td>201-214 (1.5)</td>
<td>316-334 (1.0)</td>
<td>419-429 (0.6)</td>
<td>516-527 (1.4)</td>
<td>637-655 (0.95)</td>
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<td>Mobility</td>
<td>171-177 (0.6)</td>
<td>236-247 (0.8)</td>
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<td>436-450 (0.8)</td>
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<td>314-338 (6/6)</td>
<td>410-429 (3/6)</td>
<td>560-591 (6/6)</td>
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### Table 5

Secondary structural prediction for p185 Her-2/neu. The amino acid sequence of each predicted B cell epitope is shown along with its predicted secondary structural characteristic. Potential N-linked glycosylation sites having the Asn-X-Ser/Thr motif are double underlined. Most epitopes are of the beta turn/loop type with a few short alpha helical segments. Epitopes 115-136 and 628-647 have putative disulfide bonding.
<table>
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<th>Residue #</th>
<th>A.A. Sequence</th>
<th>Secondary Structure</th>
</tr>
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<tbody>
<tr>
<td>115-136</td>
<td>AVLDNDGPLNTTPVTGASPQG</td>
<td>116-136 β turn/loop; 105117 α helix, potential N-linked glycosylation site, putative disulfide bond</td>
</tr>
<tr>
<td>168-189</td>
<td>LWKDJFHKNQLALTLDNTNS</td>
<td>164-184 α helix, N-linked glycosylation site</td>
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<tr>
<td>201-214</td>
<td>GSRCWGESSEDCQS</td>
<td>201-212 β turn or loop</td>
</tr>
<tr>
<td>270-288</td>
<td>ALVVTNYTDFESMPNPEGR</td>
<td>280-286 β turn/loop, N-linked glycosylation site</td>
</tr>
<tr>
<td>314-338</td>
<td>VCPLHNEVTAEDGTQRCENKCSKPC</td>
<td>323-337 β turn; 317-325 α helix, disulfide bond</td>
</tr>
<tr>
<td>376-395</td>
<td>FLPESFDGDPSNTAPLQPE</td>
<td>376-394 β turn; 372-380 α helix</td>
</tr>
<tr>
<td>410-429</td>
<td>LYISAWPSLDPSVFQNLQ</td>
<td>413-421 β turn/loop</td>
</tr>
<tr>
<td>480-500</td>
<td>VPWDQFLRNPHQALLHTANRP</td>
<td>494-500 β turn; 489-495 α helix, N-linked glycosylation site</td>
</tr>
<tr>
<td>516-540</td>
<td>Arrail.GSGPTQCVNCQSLRGQEC</td>
<td>520-530 β turn, N-linked glycosylation site</td>
</tr>
<tr>
<td>560-591</td>
<td>CLPCHPECQPQNSVTFCGPEADQCVAACHYK</td>
<td>587-593 β turn/loop disulfide bond, N-linked glycosyl site</td>
</tr>
<tr>
<td>628-647</td>
<td>INCTHSCVLDKKGCPAEQR</td>
<td>β turn/loop disulfide bond</td>
</tr>
</tbody>
</table>

Table 5  Secondary structural prediction of pl85 HER-2/neu
Table 6

Comparative immune responses to the various peptide immunogens. Antibody responses were titered against the peptide immunogen. ELISA titers are expressed as the serum dilution giving an absorbance of 0.2 above background at 410nm. Animals produced high titers of specific antibody which persisted for over 4 weeks following the third boost. MVFDW4 produced the most immediate and vigorous response of the four peptides.
<table>
<thead>
<tr>
<th>Immunogen:</th>
<th>DW1MVF</th>
<th>MVFDW4</th>
<th>DW5MVF10</th>
<th>DW6MVF10</th>
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<tbody>
<tr>
<td>Rabbit 1</td>
<td>250</td>
<td>250</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rabbit 2</td>
<td>250</td>
<td>250</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Primary + 1 week</td>
<td>8000</td>
<td>8000</td>
<td>32000</td>
<td>32000</td>
</tr>
<tr>
<td>Primary + 2 weeks</td>
<td>&gt;8000</td>
<td>&gt;8000</td>
<td>128000</td>
<td>128000</td>
</tr>
<tr>
<td>Primary + 3 weeks</td>
<td>32000</td>
<td>64000</td>
<td>&gt;128000</td>
<td>32000</td>
</tr>
<tr>
<td>Secondary + 1 week</td>
<td>64000</td>
<td>64000</td>
<td>&gt;128000</td>
<td>32000</td>
</tr>
<tr>
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<td>&gt;64000</td>
<td>&gt;64000</td>
<td>256000</td>
<td>256000</td>
</tr>
<tr>
<td>Secondary + 3 weeks</td>
<td>128000</td>
<td>128000</td>
<td>&gt;256000</td>
<td>&gt;256000</td>
</tr>
<tr>
<td>Tertiary + 1 week</td>
<td>128000</td>
<td>128000</td>
<td>&gt;256000</td>
<td>&gt;256000</td>
</tr>
<tr>
<td>Tertiary + 2 weeks</td>
<td>128000</td>
<td>128000</td>
<td>256000</td>
<td>256000</td>
</tr>
<tr>
<td>Tertiary + 3 weeks</td>
<td>128000</td>
<td>128000</td>
<td>&gt;256000</td>
<td>&gt;256000</td>
</tr>
<tr>
<td>Tertiary + 4 weeks</td>
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<td>256000</td>
<td>256000</td>
</tr>
<tr>
<td>Tertiary + 5 weeks</td>
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<td>32000</td>
<td>16000</td>
<td>32000</td>
</tr>
<tr>
<td>Tertiary + 6 weeks</td>
<td>128000</td>
<td>32000</td>
<td>16000</td>
<td>32000</td>
</tr>
<tr>
<td>Tertiary + 7 weeks</td>
<td>128000</td>
<td>16000</td>
<td>16000</td>
<td>32000</td>
</tr>
</tbody>
</table>

Table 6 Comparative immune responses
SKBR3

Cell line: NT* Control DW1 DW4 DW5 DW6 Mab
5323 4200 2739 3317 3165 6812 4630
5651 3393 3117 4877 4857 5277 5714
5096 5566 3675 3627 8120 6253 6548
Avg 5356.667 4386.333 3177 3940.333 5314 6114 5630.667
St. Dev. 279.0275 1098.418 470.8758 825.8531 2541.996 776.8829 961.7117

Cell line: MCF7

NT* Control DW1 DW4 DW5 DW6 Mab
2905 1537 2168 2314 2277 2960 1874
2700 1924 1990 2170 1917 3019 2733
2987 1562 1650 1391 1677 3183 2486
Avg. 2864 1674.333 1936 1958.333 1957 3054 2364.333
St. Dev. 109.3333 166.4444 190.6667 378.2222 213.3333 86 326.8889

Cell line: CAV-1

NT* Control DW1 DW4 DW5 DW6 Mab
9882 7292 7900 10267 11465 10698 8081
9272 7866 9011 9287 6328 12027 10207
9473 11077 6260 8595 12024 7130 5690
Avg 9542.333 8745 7723.667 9383 9939 9951.667 7992.667
St. Dev. 226.4444 1554.667 975.7778 589.3333 2407.333 1881.111 1535.111

NT* no treatment

Table 7 Individual ‘counts per minute’ values for tritiated thymidine assay. Samples were tested in triplicate and average values determined along with the standard deviation.
Table 8  Average tumor volume (cm$^3$) at days 15, 18 and 21 for each antipeptide antibody tested. Volume was calculated by the formula length x width x height.

<table>
<thead>
<tr>
<th></th>
<th>MUC1</th>
<th>DW1</th>
<th>DW4</th>
<th>DW5</th>
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<tbody>
<tr>
<td>day 15</td>
<td>2.605</td>
<td>1.97</td>
<td>2.27</td>
<td>2.277</td>
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<tr>
<td>day 18</td>
<td>2.61</td>
<td>1.303</td>
<td>1.98</td>
<td>1.73</td>
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<tr>
<td>day 21</td>
<td>2.055</td>
<td>1.297</td>
<td>1.703</td>
<td>0.91</td>
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Table 9 Summary of antipeptide antibody responses. (See text for discussion).

<table>
<thead>
<tr>
<th></th>
<th>DW1MVF</th>
<th>MVFDW4</th>
<th>DW5MVF</th>
<th>DW6MVF</th>
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<tbody>
<tr>
<td>376-395</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>628-647</td>
<td></td>
<td>(+)♦</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>115-136</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>410-429</td>
<td>+</td>
<td>(+)♦</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>reduction of proliferation <em>in vitro</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>reduction of phosphorylation</td>
<td>-</td>
<td>(+)♦</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Her-2 degradation</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>reduction of tumor growth <em>in vivo</em></td>
<td>+</td>
<td>(+)♦</td>
<td>+++</td>
<td>-</td>
</tr>
</tbody>
</table>

(♦) slight effect
<table>
<thead>
<tr>
<th>Her-2 peptide</th>
<th>Amino acid position</th>
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</thead>
<tbody>
<tr>
<td>48 - 56</td>
<td>H L Y Q G C Q V V</td>
</tr>
<tr>
<td>391 - 399</td>
<td>P L Q P E Q L Q V</td>
</tr>
<tr>
<td>650 - 658</td>
<td>P L T S I V S A V</td>
</tr>
<tr>
<td>661 - 669</td>
<td>I L L V V V L G V</td>
</tr>
<tr>
<td>662 - 670</td>
<td>L L V V V L G V V</td>
</tr>
<tr>
<td>689 - 697</td>
<td>R L L Q E T E L V</td>
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<tr>
<td>789 - 797</td>
<td>C L T S T V Q L V</td>
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<tr>
<td>845 - 853</td>
<td>D L A A R N V L V</td>
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<tr>
<td>851 - 859</td>
<td>V L V K S P N H V</td>
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<tr>
<td>1172 - 1180</td>
<td>T L S P G K N G V</td>
</tr>
</tbody>
</table>

Table 10  Predicted CTL epitopes in Her-2.