Peptide Vaccines against the HER-2/neu Dimerization Loop

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by

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Abstract

Studies indicate that many tumors overexpress normal self-proteins. Multiple regulatory mechanisms of the body minimize the immune response to antigens perceived as self allowing the unregulated growth of cancerous cells expressing self-proteins. Different from active immunization for infectious diseases, which triggers an immune response to foreign antigens, vaccination for cancer treatment and prevention often targets antigens that are perceived by the immune system as native, and therefore must be capable of circumventing the body's defenses. HER-2 (Human epidermal growth factor receptor-2) is a self-protein overexpressed in 20-30% of breast, as well as, ovarian, lung, stomach, bladder, and salivary cancers. HER-2 is a member of the ErbB family of receptors, which convey signals by homo- or heterodimerization of the extracellular domain. Dimerization causes transphosphorylation of the receptors, which triggers downstream signaling and results in increased development, proliferation and differentiation of cells. The overexpression and the preference of HER-2 as a dimerization partner make it an ideal target in cancers for active and passive immunotherapy. Active immunization with tumor vaccines can be used to employ the patient's own immune system to destroy mammary tumor cells, leading to sustained anti-tumor resistance due to immunological memory, which would eliminate the requirement for continual, repetitive cycles of therapy. In this study, the specific HER-2 B-cell epitope evaluated was the extracellular dimerization loop region. The specific HER-2 B-cell epitope was co-linearly synthesized with the promiscuous T-helper epitope MVF (288-302), which originated from the measles virus fusion protein (MVF). The B-cell and promiscuous T- cell epitopes were connected by a flexible four-residue turn (GPSL)

allowing the B-cell and T-cell epitopes to fold independently. A portion of the B-cell epitope synthesized was altered to form a disulfide bond between two cysteines to mimic the structure of the native HER-2 receptor dimerization region, giving a cyclized (Cyc) construct (containing the disulfide bond) and a linear (NC) construct. These rationally designed peptide vaccines were analyzed for their ability to induce antibodies with anti-proliferative capabilities. Both epitopes induced high-titered antibodies in outbred rabbits. Analysis by flow cytometry revealed antibody recognition and binding of the native HER-2 protein in human HER-2 overexpressing BT-474 cells, as well as the rat *neu* homolog of human HER-2 in mouse NT2.5 cells. These antibodies caused antibody-dependent cell mediated cytoxicity of HER-2 positive breast cancer cells *in vitro*. Passive and active immunotherapeutic experiments were carried out in transgenic mice. Inbred Neu2-$5^{+/-}$ mice vaccinated with the target peptide showed delayed onset of tumor development and a decrease in tumor volume. Double transgenic ($VEGF^{+/-}$ Neu2-$5^{+/-}$) mice were tested by passive immunotherapy due to rapid tumor onset in these mice. $VEGF^{+/-}$ Neu2-$5^{+/-}$ mice injected with peptide antibodies had smaller average sized tumors demonstrating the ability of the induced antibodies to inhibit HER-2-dependent proliferation and dimerization. These synthetic peptide vaccines have therapeutic potential to elicit antibodies that will successfully prevent or limit the growth of HER-2 overexpressing cancers.

~ Dedicated to my loving family ~

Melvin G. Jones I, Audrey L. Jones, Gary M. Jones II

and

my wonderful grandmothers

Daphene Jones and Lucy Dooley

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Chapter 1

Introduction

1.1 Cancer Issue

The second leading cause of death in the United States is cancer. In 2003, there were 556,902 deaths due to cancer which contributed to 22.7% of all deaths that year. Cancer contributes $180 billion in direct medical costs and one out four deaths is due to cancer (1). Four of the most common cancers account for over 50% of the estimated new cases in the US in 2006 (Table 1). The prognosis for cancer patients is not hopeless; there were nearly 9 million people living in the United States in 1999 who survived cancer (1).

Types of Cancer	Estimated number of new cases (both sexes)	Estimated deaths
Breast	214,640	41,430
Prostate	234,460	27,350
Lung and bronchus	174,470	162,460
Colon	106,680	55,170
Stomach	22,280	11,430
Ovarian	20,180	15,310

Source: American Cancer Society, Cancer Facts & Figures 2006 (http://www.cancer.org/downloads/stt/CAFF06EsCsMc.pdf)

Table 1.1. Estimated new cases of cancer and deaths in 2006 in the U.S. The first four are the most common cancers. HER-2 overexpression found in all.

This suggests that current treatments are effective; however the options that exist such as chemotherapy, surgery, and radiation therapy have detrimental and debilitating side effects.

1.2 Current Cancer Treatment

Cancer occurs as a result of cells that continually divide and grow without normal control. Cancer treatments are used to remove, control or kill the abnormal growing cells. Some treatment options for cancer that exist now include chemotherapy, surgical resection and radiation therapy.

Chemotherapy

Chemotherapy is a treatment for cancer that uses powerful drugs to stop abnormal cancerous cell growth. Because cancer cells divide frequently they are more sensitive to chemotherapy treatment than normal cells. However, rapidly dividing healthy cells such as skin cells and normal blood cells can be affected by chemotherapy. The harmful side effects from this treatment arise when healthy cells are destroyed along with the cancerous cells. Chemotherapeutic agents can cause a decrease in red blood cells that carry oxygen to the lungs, white blood cells that fight infections and platelets that help blood clot. A low red blood cell count results in anemia, which causes one to feel tired and short of breath. When the white blood cell count is low, it is hard for the body to fight infections this condition is known as neutropenia. A decrease in platelets results in thrombocytopenia. This condition can result in excessive external and internal bleeding, which can result in strokes or death.

Surgical Resection

Curative surgery is a procedure used to remove a tumor that is confined to one area. A problem with this method of treatment does not ensure that all of the tumor will

be removed. There are different risks associated with surgery. Possible risks include excessive bleeding, which may require blood transfusion, and damage to internal blood vessels or organs. Problems with major organs such as the heart, lungs or kidneys can occur and become life-threatening. After surgery pain and infection are common side effects, but are usually not life threatening. Some long-term side effects occur as a result of specific surgical procedures. For example, men undergoing prostatectomy (removal of the prostate) sometimes lose control of urination or become impotent.

Radiation Therapy

Radiation therapy is the treatment of cancer with X-ray or other radiation beams. It is used to treat localized solid tumors. Ionizing radiation delivers energy to and destroys the target tissue (cancer) by damaging the genetic material, thus stopping cell growth. The risk involved is that radiation damages both normal and cancerous cells. However, normal cells are able to repair themselves and function properly. Aside from carcinogenic characteristics of radiation, side effects of this treatment include nausea, hair loss, genetic effects and low blood count. Genetic effects include abnormal changes, mutations and failure to reproduce. Radiation can directly damage lymphocytes in the blood stream, which are important components of the body's immune system

1.3 Cancer Research

The failure of current treatments is generally due to the rapid growth of tumor cells that are resistant to standard treatments. These observations demonstrate the need for a different approach that can overcome or circumvent the resistance mechanisms of

tumor cells to standard treatments. Maximizing cancer treatment by using the host's immune system is a highly attractive alternative approach to disease management.

Studies indicate that many tumors overexpress normal self-proteins. HER-2/ *neu* (human epidermal growth factor 2), a growth factor receptor, is overexpressed in 30% of breast cancers as well as, ovarian, lung, colon, stomach and prostate cancers (see Table 1.1)(2). The idea of vaccination against such cancers was derived from the detection of weak immune responses in cancer patients who overexpress self-tumor antigens. Thus these antigens (HER-2/*neu*) have become excellent targets for active and passive immunotherapies.

Passive immunotherapeutic strategies include the transfer of antigen-specific T lymphocytes and the injection of monoclonal antibodies that target a specific tumor antigen. Traztuzumab (Herceptin) is a monoclonal antibody specific for HER-2/*neu*, as a passive immunotherapeutic it is effective against the biologically distinct set of breast cancers that overexpress HER-2/*neu* oncoprotein (3). Active immunization with tumor vaccines can be used to employ the patient's own immune system to destroy mammary tumor cells. The theoretical advantage of active immunization is the potential for sustained anti-tumor resistance due to immunological memory, which would eliminate the requirement for continual, repetitive cycles of therapy.

Different from active immunization for infectious diseases, which triggers an immune response to foreign antigens, vaccination for cancer treatment and prevention often targets antigens that are perceived by the immune system as native (3). Therefore, multiple regulatory mechanisms of the body minimize the immune response to antigens

perceived as self. In order to develop an effective cancer vaccine it is vital to form strategies for circumventing such immune tolerances.

Clinical Trials

Trastuzumab and pertuzumab are recombinant humanized monoclonal antibodies that target different regions on the extracellular domain of HER-2. Trastuzumab is directed against the extracellular subdomain IV of the HER-2 tyrosine kinase receptor. Clinical studies have shown trastuzumab (Herceptin) to be active against HER-2 overexpressing breast cancer, leading to its FDA approval (Fig.1.1) (4). Current treatment protocols combine trastuzumab with other drugs to successfully increase time to tumor progression, and survival of patient. Pertuzumab is currently undergoing Phase II clinical trials in cancer patients with solid tumors. Pertuzumab sterically hinders HER-2 dimerization with other HER-receptors and effectively blocks ligand-activated signaling from the HER-2/HER-1 and HER-2/HER-3 heterodimers (Fig. 1.1) (4).

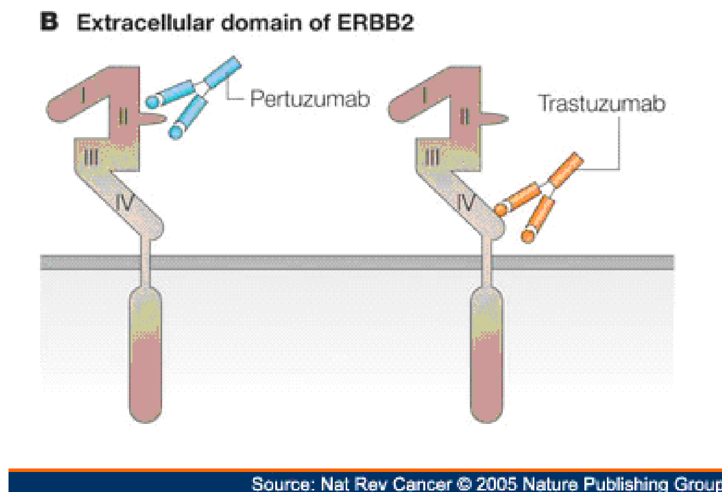


Fig.1.1. Schematic diagram of Trastuzumab's blockage on subdomain IV and of Pertzumab's blockage on subdomain II of HER-2 (ERBB2). Source http://www.medscape.com.

Trastuzumab and pertuzumab in combination effectively inhibited HER-2 positive cell (BT474) survival, partly because of increased apoptosis (4). Trastuzumab augments pertuzumab mediated interference of HER-2 dimerization with HER-3. The combined drug treatment reduced levels of total HER-2 and of phosphorylated HER-2 protein, and it also inhibited receptor signaling. This suggests that an effective therapeutic strategy for breast caner would involve a combination of HER-2 targeting agents as opposed to treatment with a single HER-2 monoclonal antibody (4).

HER-2 Background

In normal cells, activation of the HER-2 receptor family starts a network of signaling pathways that control normal cell growth, differentiation, motility and adhesion in several cell lines. Studies have shown the crucial role of the HER receptor family in development of the cardiovascular system and the nervous system(5). HER-2 is a transmembrane phosphoglycoprotein that is the product of the epidermal growth factor receptor B-2 (*erb*B-2) gene (Fig.1.1).

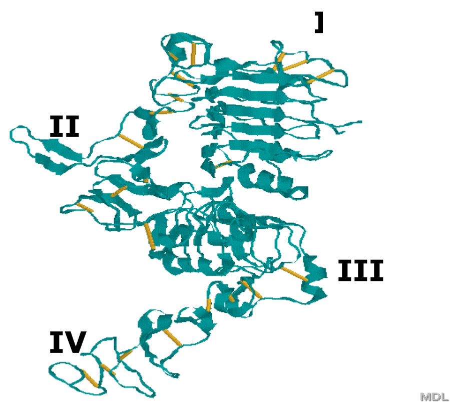


Fig. 1.2. Three-dimensional structure of the human epidermal growth factor 2 (HER-2) protein monomer with subdomains I-IV labeled. Picture from Protein Databank http://www.pbd.org.

It is the human version of the rat oncongene *neu*. HER-2 is classified as an epidermal growth factor receptor (EGFR/*erb*B-1) family member. The ErbB family members and their ligands are present in eukaryotic organisms and have been involved in different cancers because of dysregulation or mutation of the ErbB genes (5). The extracellular domain (ECD) consists of multiple glycosylation sites and is very cystein-rich. The intracellular domain contains a highly conserved tyrosine kinase (6). The ErbB family members ErbB1(HER-1/EGFR), ErbB2 (HER-2), ErbB3 (HER-3) and ErbB4 (HER-4) have ligand binding extracellular regions that consists of four domains (I, II, III, and IV) as well as a number of tyrosine phosphorylation sites on a C-terminal tail (with varying length dependent on the family member). The tyrosine phosphorylation sites serve as a platform for connecting molecules and enzymes to facilitate downstream signaling (7).

There are about a dozen known ligands that bind mammalian ErbBs. Structural comparisons of ErbB3 monomer and the EGFR ligand-bound dimer have shown that the ligand binds at a site between domains I and III, and this has been shown in ErbB4 as well (8)(Fig.1.3b). This binding results in a conformational change in the receptor ECD (9). However, HER-2 is the only ErbB family member for which no ligands have been found. The structure of the ECD of HER-2 reveals a closed binding site where positions in domains I and III are in direct contact with each other (10) (Fig.1.3). Therefore, the binding site residues present in the rest of the ErbB family is no longer conserved in HER-2 indicating that HER-2 is incapable of ligand binding.

Ligand-induced dimerization permits normal downstream signaling for the ErbB family (11) (Fig.1.3). In the absence of a ligand, the extracellular domain of EGFR, ErbB3, and ErbB4 has a β hairpin loop in domain II that contacts a region of domain IV.

This is designated as the “closed” confirmation (8). In the “open” form the domain II β hairpin extends outward from the body of the molecule, and is involved in most of the contacts seen in the EGFR dimer (Fig.1.3b) (11). HER-2 is always in an open confirmation (10), which gives a possible reason as to why it is the favored dimerization partner of the other ErbB family members (12).

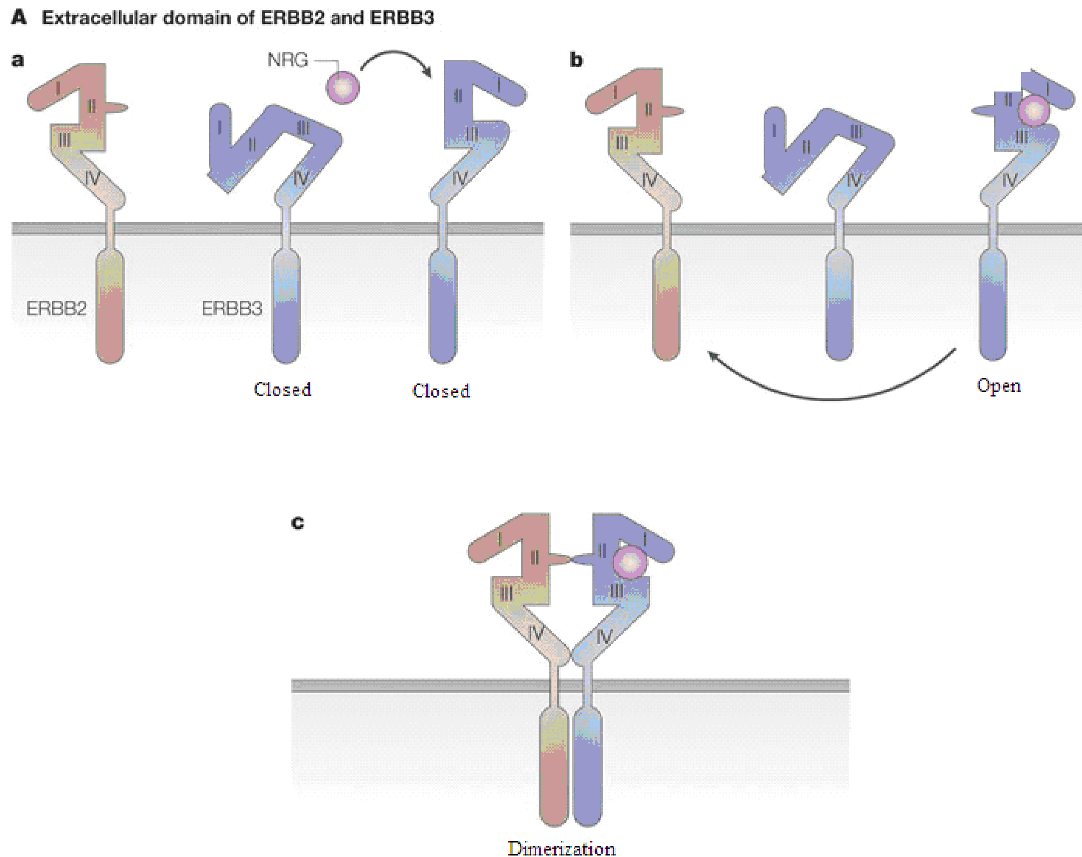


Fig. 1.3. Dimerization process of HER-2 and HER-3 (ERBB3). **a.**HER-2 and the closed confirmation of HER-3 **b.** Ligand (NRG) bound HER-3 in open form. **c.** Dimerization of HER-2 with opened HER-3. Source http://www.medscape.com.

Dimerization allows the intracellular kinase domains of the two ErbBs to associate intimately, thus stimulating tyrosine kinase activity and resulting in transphosphorylation of tyrosine residues on the C-terminal tail of one ErbB by the other’s kinase domain (7).

The phosphorylated residues serve as a scaffold for signaling molecules involved in the regulation of intracellular signaling cascades (13). These cascades play roles in development, proliferation and differentiation in a variety of tissues where ErbB receptors are present.

Mouse Model of Human Cancer

Most of the genetic changes observed in human breast cancer can be categorized as: 1) gain of function mutations in genes (proto-oncogenes) that are involved in cell growth, division and survival or 2) loss of function mutations in "tumor suppressor" genes responsible for regulating unrestrained cellular growth. Genetically engineered mouse (GEM) models of human cancers are developed by modifying the mouse genome to dysregulate the expression of a specific gene in targeted tissues or by introducing specific genetic mutations that change gene function, causing tumor development (14). These mouse models help to assess potential cancer preventative methods *in vivo*. Such screenings are important steps towards the selection of methods for clinical trials in humans. These studies have defined the biological functions of hundreds of genes and have provided insight into many aspects of cancer research (15). Mouse models have been used to test cancer vaccines, target therapies, preventative agents and combinations of chemopreventive and/or therapeutic agents (14).

<u>Neu2-5$^{+/-}$ Mouse Models</u>

Several mouse models have been developed for specifically testing HER-2 overexpressing tumor growth. A majority of these models use the MMTV promoter to

overexpress the neu protein resulting in mouse mammary tumors similar to human breast cancer. Transgenic mouse models have shown that the rat proto-oncogene HER-2/neu, with an amino acid change in the extracellular domain is highly tumorigenic in mice (5). The transgene is expressed in the mammary and salivary glands of female mice and in the salivary and reproductive organs in males. These transgenic mice show a late onset of mammary tumors that is restricted to females. The Neu2-5^{+} mice develop spontaneous tumors around the age of 177 days.

VEGF$^{+/}$- × Neu2-5$^{+/-}$ Mouse Models

HER-2 signaling leads to the increased expression of VEGF, an angiogenic (new vessel formation) factor. Double transgenic mice (VEGF$^{+/-}$ Neu2-5$^{+/-}$) have accelerated tumor development around the age of 56 days due to the combination of VEGF and *neu* proteins in mammary tissue. The two transgenes have been found to have overlapping expression patterns.

Advantages and Disadvantages of Transgenic Mouse Models

Advantages:

There are many advantages in developing such models for human cancers. These models progressively develop tumors in stages, while also interacting with the surrounding tissues. Like in humans, metastasis occurs in the presence of a working immune system (16). There are histological similarities between the mammary tumors in mice and human breast cancer(14), and many of the mouse tumors that develop are hormone independent, like human breast cancers.

Disadvantages:

Most transgenic females can not carry and support babies, which requires the use of non-transgenic females as breeders. This results in a 25% chance of getting transgenic females that are expected to develop tumors. Lack of mutation in human breast cancers suggests that the primary cause is due to over-expression of the wild-type HER-2/neu and not mutational activation, which is used in mouse models. Despite the 88% homology (similarity in structure and function) of human and rodent HER-2/neu, the monoclonal antibody Pertuzumab only has affinity for the human HER-2/neu and not the rodent homolog.

1.4 Value of an Immunotherapeutic Approach

Surgical excision or ablations by use of radiation are primary cancer treatments that work for some patients. However, for other patients relapses are common and tumors may reoccur at the same site or metastasize to different sites. For this reason, preventative measures have become a popular area in clinical oncology in recent years. Different studies have shown that the cellular and the humoral branches of the immune system are capable of generating an antigen specific response against tumor antigens. There is a vast array of potential advantages affiliated with active immunization as opposed to current chemotherapy and radiation therapy. A key advantage is the specificity that is offered by active immunotherapy. Specificity leads to tumor rejection with fewer side effects that include autoimmunity. Another advantage of active specific immunotherapy is the production of an immunological memory, which is not associated with passive treatments such as the introduction of antiviral or anti-tumor antibodies.

The subject of this thesis is the design and application of synthetic peptide based vaccines in the treatment or prevention of cancer.

1.5 Synthetic Peptides as Vaccines for Cancer

Cancers such as breast, ovarian and skin are due to the overexpression of normal growth regulatory proteins, known as tumor antigens (2). The concept of designing vaccines that target such cancers comes from the detection of weak immune responses in cancer patients against self-tumor antigens. For instance, patients who overexpressed the self-protein HER-2 in their cancers demonstrated both humoral (B-cell) and T-cell immune responses (2). Therefore, it is hypothesized that constructing vaccines to stimulate the already present immune response could aid in the treatment of cancer by enhancing the hosts immunity to the specific tumor antigen.

T-Cell Vaccines

Cancer researchers have largely focused their efforts on vaccines designed to trigger cytotoxic T-cell responses. T-cells (T-lymphocytes) target and attack infected foreign or cancerous cells in the body. They also help regulate other immune responses by signaling other defenders in the immune system. The rationale behind this focus mainly stems from the belief that a cell-mediated immune response is the most promising mechanism for controlling viral replication and destroying tumor cells. Cell based vaccination using either allogenic (genetically different but from same species) or syngenic (genetically identical) tumor cells expressing rat *neu* have been shown to elicit T-cell and antibody responses in mice (2). However, a disadvantage to using cell-based

vaccines is their potential to cause complex immune responses that can result in tolerance or hypersensitivity and the additional complications from auto immune reactions.

B-Cell Vaccines

There is evidence that humoral immune responses (antibodies) are probably intimately involved in controlling tumor growth. B-lymphocytes (B-cells) mature into antibody (immunoglobulin) secreting plasma cells. Antibodies recognize and bind to foreign substances known as antigens. Each type of B-cell is unique and makes one specific antibody that recognizes one specific antigen, like a lock and key. B-cell vaccines stimulate the immune system in an attempt to indirectly eliminate cancerous cells by marking them for destruction.

1.6 Project Overview

The immune system is a complex network of cells; organs that operate together to protect the body from foreign or non-self antigens. The immune system works to fight disease and cancer by differentiating between healthy and non-healthy (i.e. cancerous) cells in the body and working to destroy the cell that become cancerous (Fig.1.3). When the immune system is not functioning properly or breaks down cancer can occur. Immunotherapy is designed to stimulate the body's immune system to directly or indirectly fight cancer and lessen or eliminate detrimental side effects experienced by most cancer treatments.

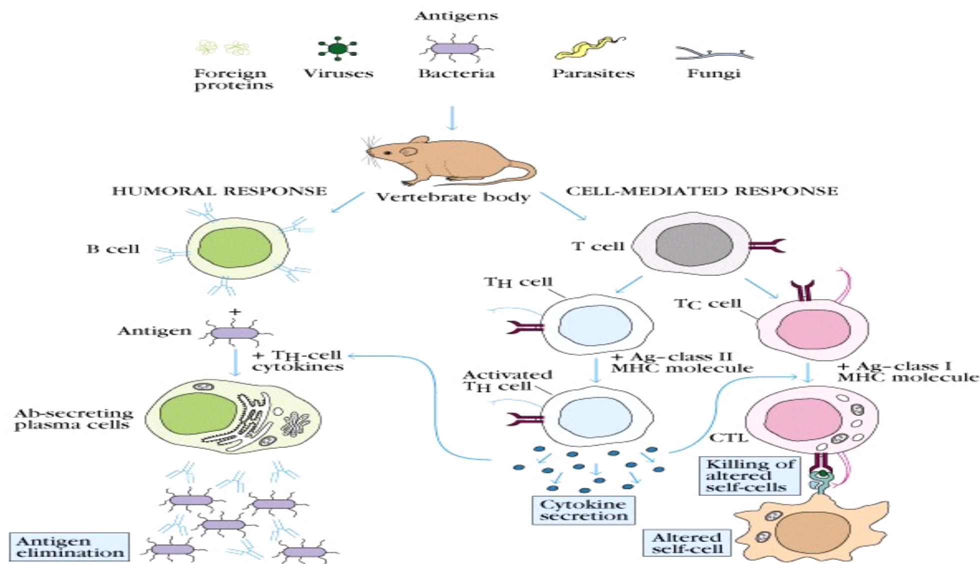


Fig.1.4 Immune system responses to antigens. Source: *Cellular and Molecular Immunology*, Abbas

There are compelling discoveries that support the significance of the humoral immune responses (antibodies) in controlling tumor growth. This approach to immunotherapy involves passive injection of humanized monoclonal antibodies. Antibody based approaches are supported by tumor regressions observed in cancer patients given Herceptin (humanized monoclonal antibody). Other anticancer antibodies like pertuzumab and bevacizumab are presently in phase II and III clinical trials.

The focus of this thesis is to develop a peptide vaccine that can stimulate both cell-mediated and humoral immune responses including antibodies and helper T-cells. Antibodies have an affinity to certain regions in proteins on tumor cell surfaces. A vaccine design that targets a portion of the protein rather than the whole protein domain of the antigen may prove effective in circumventing the immune tolerance of the native protein. The rational for subunit peptide-based vaccines comes from studies showing that immunizations of rats with multiple T-helper peptides derived from the rat neu protein

elicited strong humoral and $CD4^+$ (T-helper cell) responses, whereas immunizations with purified whole rat *neu* protein failed to cause detectable immune responses (17).

We hypothesized that a subunit peptide-based vaccine targeting a specific B-cell epitope on the HER-2 extracellular domain could induce antibodies capable of inhibiting the growth of HER-2 overexpressing cancers.

Chapter 2

Material and Methods

2.1 Rationale of Peptide Construct

B-cell Epitope Selection

The selection of the B-cell epitope located within the human HER-2 extracellular domain was accomplished by analysis of a 3.2 angstrom X-ray picture of HER-2 in complex with pertuzumab(18) (Fig2.1).

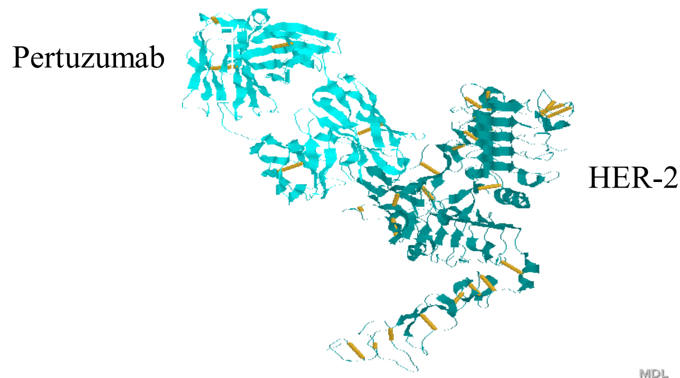


Fig.2.1 Pertuzumab complexed with HER-2 protein's extracellular subdomain II.

Pertuzumab is an anti-HER-2 monoclonal antibody that is currently used in clinical trials to treat HER-2 overexpressing cancers.

2.2 Peptide Construct Synthesis and Purification

Peptide Synthesis and Purification

B-cell epitope HER-2 pertuzumab loop was co-linearly synthesized with a promiscuous T_H epitope (MVF) derived from the measles virus fusion protein (amino acids 288-302) (Fig.2.2). Peptide synthesis was performed on a Milligen/Biosearch 9600 solid-phase peptide synthesizer (Bedford MA) using a Fmoc/t-But chemistry. Preloaded

Fmoc-Val-CLEAR ACID resin (0.36 meq/g) (peptide International, Louisville, KY) was used for the peptide synthesis using PyBop/HoBt coupling method. The B-cell epitope was assembled by choosing regioselective side chain protection on Cys residues as: Cys(trt) (19). Also, MVF T_H epitope with four residue linker (GPSL) was incorporated for independent folding and was assembled on B-cell epitope. All peptides were cleaved from the resin using global deprotection reagent B (TFA: Phenol: Water: TIS, 90:4:4:2). The protecting group from Cyc (Trt) comes off in the global cleavage reaction as confirmed by electrospray ionization mass spectroscopy (ESI-MS) analysis at the Campus Chemical Instrumentation Center, The Ohio State University Columbus, Ohio. Crude peptides were purified on preparative RP- HPLC using C-4 vydac column in water: acetonitrile (0.1%TFA) gradient system. Pure fractions were analyzed using analytical HPLC, pooled together and lyophilized in 1% acetic acid solution.

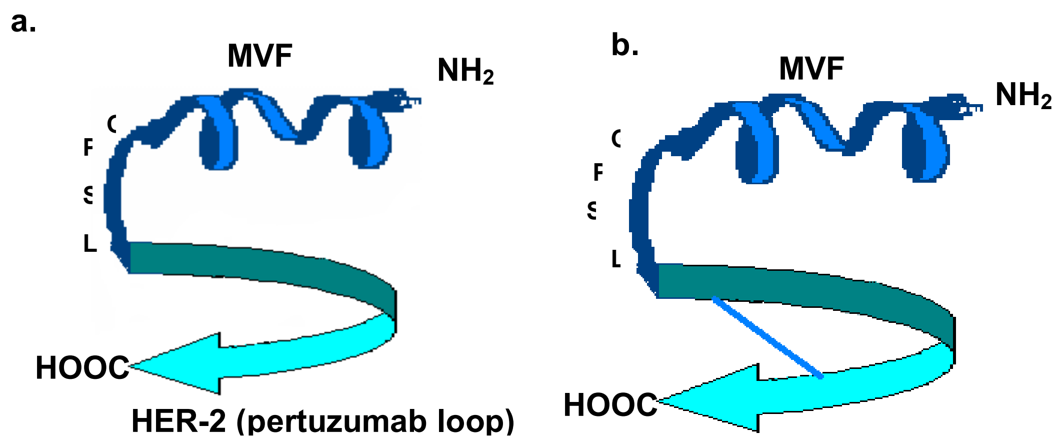


Fig.2.2. MVFHER-2 pertuzumab loop chimeric peptide constructs **a.** Non-cyclized (NC) linear construct **b.** Cyclized (Cyc) construct

Disulfide Pairing of Cyclization

Intramolecular disulfide bonds were formed using iodine oxidation as reported by (20) (Fig.2.3). Linear peptide was generated by dithiothreitol (DTT) reduction. Cyclic

and linear peptides were further purified by semi-preparative RP-HPLC and characterized by electrospray ionization mass spectroscopy (ESI-MS). Disulfide bond formation was confirmed by PEO-maleimide reaction (Fig. 2.3).

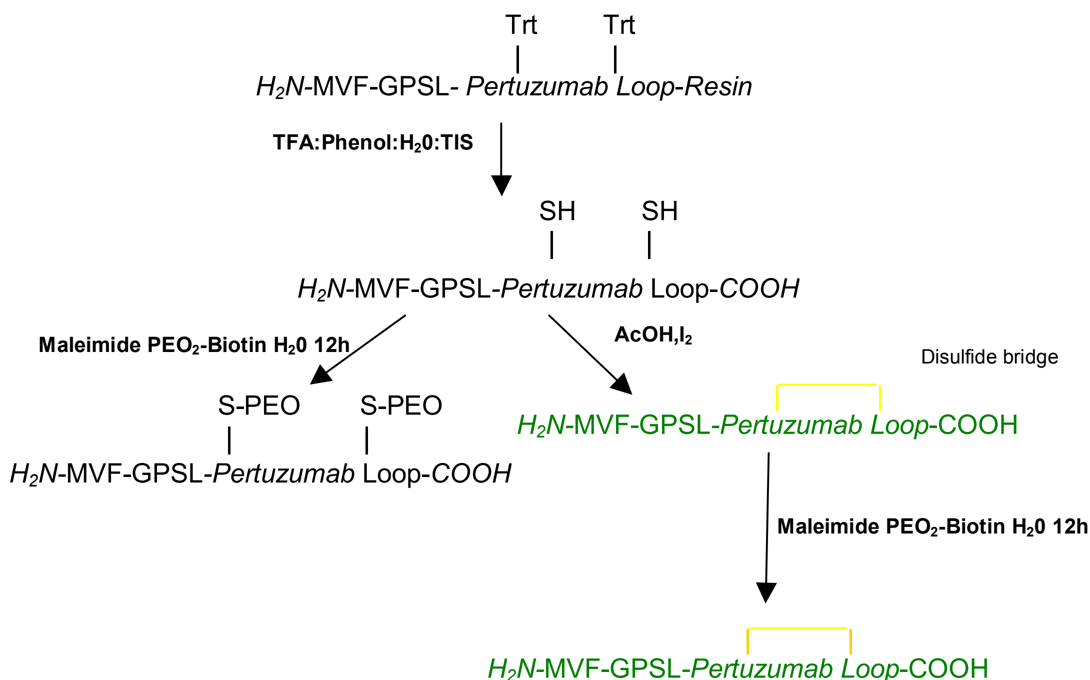


Fig. 2.3 Disulfide pairing between twp cysteines and malimide treatment after synthesis of MVFHER-2(pertuzumab loop) Cyc and NC (see Fig2.2). (J.Peptide Sci. 6:387)

2.3 Characterization of Peptide Construct

Mass Spectrometry

The purified peptides were identified by electrospray ionization spectrometry (Campus Chemical Instrumentation Center, Ohio State University, Columbus,OH).

Circular Dichorism (CD) Measurements

The CD spectra of both peptides were obtained on an AVIV model 62A DS CD instrument. The spectral measurements were taken at 25ºC and under continual nitrogen purge of the sample chamber, using a 0.1-cm path length quartz cuvette. Each peptide

was dissolved in water and concentrated at 25µm, 50 µm, 75µm, and 100µm. The mean residue ellipticity ([θ]), which is recorded in (deg cm^2 / dmole), was calculated according to the equation: [θ] = (millidegrees)/ [10 x (l) x (n) x (c)]. Here n is the number of amino acids in the peptide, c is the peptide concentration in molarity, and l is the path length of the cuvette. The circular dichroism (Δε) = [θ]/ 3298. The helicity of each peptide was calculated according to Chen *et al*.,1969, which uses the mean residue ellipticity of polylysine for 100% α-helix θ_{222} = -35,700 as a reference. Therefore, the actual percent helicity is calculated as follows: %helicity = $[\theta]_{222}/ X_H^n$ where X_H^n is the max helicity and $X_H^n = X_H^\infty$ (1- K/n). X_H^∞ is the ellipticity for 100% helix of a chain of infinite length, and K is a wavelength dependent constant (2.57 at 222nm).

Cross Reactivity ELISA

Six columns of 96-well plates were coated with 100µL of 2µg/mL peptide solutions of MVFHER-2 (pertuzumab loop) Cyc or NC in 1X PBS and the remaining four columns were coated with the matching B-cell epitope HER-2 (pertuzumab loop) Cyc or NC and refrigerated overnight at 4°C. Serum dilutions, starting at 1/8000 of the cyclized antibodies were tested against the non-cyclized peptide and serum dilutions of the non-cyclized antibodies were tested against the cyclized peptide. The plates were then developed as described above.

2.4 Immunogenicity

Peptide Immunization

Female New Zealand White rabbits, FVB/n and BALB/c mice were used to obtain antibodies. Two rabbits per peptide were immunized subcutaneously per peptide at multiple sites with a total of 1mg of each chimeric peptide in 100µg nor-MDP adjuvant and emulsified (50/50) in ISA 720. Additional booster injections were given every 3 weeks after the primary immunization with 1mg of peptide and 100µg nor-MDP adjuvant for 12 weeks. Rabbit serum was collected every week and complement was inactivated by incubation for 30minutes at 56º C. High-titered sera was purified on a protein A/G-agarose column (Pierce, Rockford,IL), and eluted antibodies were concentrated in PBS. The antibody concentration was elucidated by the Commasie plus protein assay reagent kit (Pierce).

Direct Enzyme Linked Immunosorbent Assay (ELISA)

The 96-well plates were coated with100µl of a 2µg/ml concentration of antigen in PBS and refrigerated over night at 4ºC. Wash buffer used was PBT/HS, (100mL of 10X PBS, 0.50mL Tween 20, 10mL Donor Herd Horse Serum and was brought up to 1L with ddH_2O). Plates were washed with PBT/HS and non-specific binding regions were blocked for 1 hour with 200µl of PBS/1%BSA and 0.02% sodium azide. Plates were washed, then 200µl of serum dilution starting at 1/4000 was added to the antigen-coated plates in duplicate wells, and serially diluted 1:2 in PBT/HS wash buffer. The plates were then incubated for 2 hours at room temperature. After washing, 100µl of a 1/500 dilution in PBT/HS of Pierce goat-anti-rabbit IgG with horse radish peroxidase was

added as a marker to each well and incubated for 1 hour. The plates were washed with PBT/HS and water, afterwards 50µl of substrate solution (50µl of 30% H_2O_2, 10mL of citrate phosphate buffer with 0.5mg/ml 2,2-aminobis (3-ethylbenzthiazoline-6-sulfonic acid) as the chromophore) was added to each well for bound antibody detection. The plate was then incubated for 10minutes in the dark at room temperature. The reaction was stopped by adding 25µl of 1% SDS to each well. The absorbance was read at 415nm on a BioRad microplate reader. Titers were defined as the highest dilution of sera with an absorbance reading of greater than 0.2 excluding the background.

2.5 Cell Culture

All cell lines were kept according to the manufacture's guidelines. Both HER-2 overexpressing and normal human cell lines were used, as well as, Neu^+ and Neu^- mouse cell lines (Table.2.1).

Cell-line	Human Cells	Mouse Cells	$HER\text{-}2^+$ levels	Neu^+ levels
BT474	Yes	No	High	-
SK-BR-3	Yes	No	High	-
MDA-468	Yes	No	Normal	-
MCF-7	Yes	No	Normal	
NT2.5	No	Yes	-	High
TUBO	No	Yes	-	High
TS/A	No	Yes	-	Low

Table.2.1. Cell line characteristics.

HER-2 overexpressing cancer cell lines

BT-474

BT-474 is a human breast adenocarcinoma cell line that overexpresses the HER-2 protein. These cells were taken from a patient's solid invasive ductal carcinoma of the

breast. All cell lines were purchased from the American Type Culture Collection and were kept according to the supplier's guidelines. The cells were split 2-3 times a week depending on confluence. The culture media consisted of 500mL DMEM-F12, 75mL (15%) fetal bovine serum (FBS), 5mL (1%) penicillin/streptomycin (pen/strep) and 0.01mg/mL (5mg) of insulin.

SK-BR-3

SK-BR-3 is another human breast adenocarcinomal cell line that overexpresses HER-2. However, these cells were taken from a metastatic site of a pleural effusion in the mammary gland of a patient with adenocarcinoma. The culture media consists of 500mL McCoy's 5A Modified Medium, 50mL (10%) FBS and 5ml (1%) pen/strep.

Human HER-2 non-over expressing cancer cell line

MDA-468

MDA-468 is a human breast adenocarcinoma cell line that does not overexpress the HER-2 protein. This cell line was isolated from a pleural effusion of an African American female with metastatic adenocarcinoma of the breast. The culture media consisted of 500mL RPMI 1640, 50mL (10%) FBS, and 5mL (1%) pen/strep.

MCF-7

MCR-7 is a human breast adenocarcinoma cell line with normal HER-2 levels. It was derived from a metastatic site of a Caucasian female. The culture media consist of 500mL DMEM, 50mL (10%) FBS and 5mL (1%) pen/strep.

Mouse neu^+ overexpressing cancer cell line

NT2.5

NT2.5 is an FVB/n mouse cancer cell line that overexpresses the neu protein. These cells were a kind gift from Dr. R. Todd Reilly. The culture media is the same as the BT-474 cell line.

TUBO

TUBO is a Balb/c cell line taken from a Balb-neuT mouse (neu^+). The culture media was the same as MCF-7. These cells were a kind gift from Dr. John Morris.

Mouse non-neu overexpressing cancer cell line

TS/A

TS/A is a Balb/c mouse cancer cell line that is neu^-. The culture media is the same as MDA-468.

2.6 Antibody Binding

Flow Cytometry

This procedure used is from that described in (21). Cells were trypsinized and resuspended in 10 mL of appropriate culture media. The cells were counted by trypan blue staining. A single cell suspension of about 1×10^6 cells per 100µL was placed in polysterene 5mL tissue culture tubes. Initially primary (peptide) antibody concentrations of 1, 20, and 50µg were used to find optimum concentration. The primary antibody was added to each test tube and gently vortexed. The tubes were then incubated at 4°C for 2

hours. The tubes were then washed with 1mL of ice cold PBS and spun at 1500rpm for 5min at 4°C and decanted. This was repeated twice. 100µL of the secondary antibody, (anti-rabbit, anti-mouse or anti-human IgG-FITC conjugate) made up at 1:50, was added to each tube with minimal light exposure. The tubes were then gently mixed by vortexing and incubated at 4°C for 30minutes and then re-washed 2 times as described above. After incubation 500µL of 1% formaldehyde made in PBS was added to each tube. The samples were analyzed by a Coulter ELITE flow cytometer. About 10,000 events were counted per sample for statistical significance. Selection of healthy cells was gated by light scattered assessment before single-parameter histograms were drawn.

HER-2 ELISA

Plates were coated overnight at 4°C with 100µL of 10µg/mL of Herceptin, washed four times with 0.1% Tween/PBS, and blocked with of 100µL of PBS-1% BSA for 4 hours on a rocker. Plates were washed four times with 0.1% Tween/PBS. Wells were then coated overnight at 4°C with 50µL of either PBS-1% BSA or SK-BR-3 cell lysate (1 x 108 cells in 20 mL lysis buffer). Lysis buffer was composed of 1% Triton X-100, 10% glycerol, 150 mM NaCl, 50 mM HEPES, 1.5 mM $MgCl_2$, 1 mM EDTA, 10 mM pyrophosphate, 100 mM NaF, 0.2 mM Na_3VO_4, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM PMSF. Plates were washed four times with 0.1% Tween/PBS and serial dilutions of rabbit sera (starting at 1:100) were added and the plates were incubated for 2 hours on a rocker. Antibody binding was detected using goat-anti rabbit IgG HRP.

2.7 Antibody Characterization

Mouse Isotyping

FVB/n mouse sera from immunization of mice were typed using a Mouse Typer Sub-Isotyping Kit (Bio-Rad). The manufacturer's instructions were followed with the exception of substrate addition and stopping buffer reagent. ABTS substrate and 1% SDS stopping buffer reagent as described above was used for this experiment.

2.8 Antibody Inhibition of cell function and growth

Cell Proliferation Assay

1×10^4 MCF-7 cells were plated in 96-well flat bottom plates and incubated at 37°C overnight. Growth media was then replaced with low-sera (1% FCS) growth media and cells incubated overnight. Media was removed from the wells and replaced with antibody made up in 1% growth media. Plates were incubated for one hour at 37°C, and then 10ng/mL HRG was added in 1% growth media. Plates were incubated an additional 72 hrs at 37°C before 5mg/mL of MTT was added to each well. Plates were incubated for 2hr at 37°C, then 100µL extraction buffer (20% SDS, 50% dimethylformamide, pH 4.7) was added to the plates. Plates were incubated overnight at 37°C, and read on an Biorad microplate reader at 570nm with 655nm background subtraction. % Lysis calculated as 100*(HRG Treated Cells-Antibody Treated Cells)/(HRG Treated Cells).

HER-2 Phosphorylation Assay

MCF-7 cells were plated at $1x10^6$ cells/well in 6-well plates and incubated overnight at 37°C. Culture media was removed from the wells, and cells were washed

once with PBS. 1% culture media was added to the wells and plates were incubated overnight at 37°C. Culture media was removed and the cell layer was washed with PBS. Next, 50μg of antibody in Binding Buffer (0.2% w/v BSA, RPMI medium with 10mM HEPES (pH 7.2)) was added to the wells and incubated at room temperature for one hour. 5nM of HRG/well was added to the plate, and the plate was incubated an additional 10 minutes at room temperature. Binding Buffer was removed and the cell layer washed with PBS. PBS was removed and 1mL lysis buffer (1% NP-40, 20mM Tris (pH 8.0), 137mM NaCl, 10% glycerol, 2mM EDTA, 1mM Na_3VO_4, 10μg/mL aprotinin, 10μg/mL leupeptin) was added to the plate. Plates were rocked at 4°C for 30min. Lysates were removed from wells and spun at 13000× and supernatants were transferred into clean tubes. Protein concentration of each sample was measured by Comassie plus protein assay reagent kit (Pierce). Lysates were frozen immediately at -80°C and phosphorylation of HER-2/*neu* was determined by the DuoSet IC for Human Phospho-ErbB2. The manufacturer's directions were followed (R&D Systems, Minneapolis, MN).

2.9 <u>Antibody dependent cell death</u>

Antibody dependent cell-mediated cytoxicity (ADCC) Assay

Procedures previously described in (22) were used to perform ADCC. PBMC's were separated from heparinized whole blood from human donors by density gradient sedimentation using Ficoll. The purified PBMC's were washed three times with culture medium and serially diluted into a 96-well plate to give effector:target ratios of 100:1, 20:1, and 4:1. On day two target cells are prepared and added to the effectors. The target cell lines (BT474 and MDA468) were labeled with 200μCi of radioactive Na $^{51}CrO_4$ and

incubated with 50μg of antibody for one hour at 37°C and then washed three times in cultured medium. Targets were incubated with PBMC's without antibodies to determine nonspecific lysis. The plates were incubated for four hours at 37°C. The supernatants were then harvested and radioactivity was determined using a gamma counter. The percent lysis was determined by the following calculations: %lysis = (A-B)/ (C-B) x 100, where A is ^{51}Cr (cpm) from test supernatants, B is spontaneous release (^{51}Cr from target cells without antibody treatment) and C is maximum release (^{51}Cr from target cells lysed with 5%SDS detergent). Triplicate measurements were performed on each treatment and were averaged prior to the calculation of percent lysis.

3.0 *In vivo* Mouse Models

Passive Immunotherapy: Double Transgenic Mice

VEGF$^{+/-}$Neu2-5$^{+/-}$ double transgenic mice were screened by PCR at 21 days old. On day 28 they were treated twice a week with 750μg purified IgG peptide induced rabbit antibodies for four weeks. At 45 days old the mice were monitored for tumors twice a week until 69 days old when they were sacrificed (see Table2.2).

Active Immunotherapy: Transgenic Mice

Neu2-5$^{+/-}$ transgenic mice were screened by PCR at 21 days old. On day 30 they were immunized with chimeric peptide and boosted every three weeks. Blood was drawn by retro-orbital bleeding prior to immunization and every week after the first boost. Tumor onset in untreated mice is about 177 days. At age 145 days mice were monitored

twice a week for tumor development until age 200 days when mice were sacrificed (see Table 2.2).

Tumor Challenge

NT2.5 Challenge

Mice were immunized with 0.1mg peptide emulsified in ISA720 with 100µg nor-MDP. Mice were boosted at 3 week intervals. Blood was drawn prior to immunization and weekly after the first boost. Ten days after the third immunization, mice were challenged with 3×10^6 NT2.5 cells. Mice were monitored twice weekly for the presence of palpable tumors for a total of 24 days. Tumors were measured with calipers in a blinded fashion and tumor volume calculated by the formula (long measurement x short measurement ^{2})/2 (see Table 2.2).

TUBO Challenge

Mice were immunized with 0.1mg peptide emulsified in ISA720 with 100µg nor-MDP. Mice were boosted at 3 week intervals. Blood was drawn prior to immunization and weekly after the first boost. Fourteen days after the third immunization, mice were challenged with 1×10^5 TUBO cells. Mice were monitored twice weekly for the presence of palpable tumors for a total of 35 days. Tumors were measured with calipers in a blinded fashion and tumor volume calculated by the formula (long measurement x short measurement ^{2})/2 (see Table 2.2).

Mouse strain	Genotype	Treatment	Tumor origin
1. FVB/n	Neu2-5$^{+/-}$VEGF$^{+/-}$	pure IgG antibodies	spontaneous
2. FVB/n	Neu2-5$^{+/-}$	peptide vaccine	spontaneous
3. FVB/n	wild type	peptide vaccine	NT-2.5 challenge
4. Balb/c	wild type	peptide vaccine	TUBO challenge

Table.2.2. Mouse treatment. #1 passive immunotherapy #2 active immunotherapy #3 and #4 tumor challenge.

Chapter 3

Results

3.1 Design and Characterization of Chimeric HER-2 Peptide Constructs

We relied on the crystal structure of HER-2 complexed with pertuzumab to predict the B-cell epitope (Fig.2.1)(18). The HER-2 B-cell epitope was co-linearly synthesized as a chimeric peptide containing a promiscuous T-helper cell epitope (a.a. 288-302) from MVF at the amino terminus end. The T-helper epitope from MVF has been shown to help bypass the haplo restrictive immune response in humans (23). The cyclized chimera contained a cysteine bridge in the B-cell epitope to mimic the native structure of the HER-2 protein. The chimeric peptides contained a four-residue (GPSL) linker between the T-helper and B-cell epitope. The flexible nature of the linker allows the T-helper and B-cell epitope to fold independently into their native secondary structures (Table 3.1).

Table 3.1 Synthetic peptide vaccines characteristics

Chimeric peptide constructs	Molecular Weight	Disulfide Bridge	T_H epitope	GPSL linker
MVFHER-2 (pertuzumab loop) NC	5759.72	No	Yes	Yes
MVFHER-2(pertuzumab loop) Cyc	5757.72	Yes	Yes	Yes

NC- non-cyclized ; Cyc- cyclized

Mass spectroscopy is the technique used to identify the molecular mass of purified peptide. The matrix-assisted laser desorption/ionization (MALDI) mass spectroscopy technique was used. The peptide is dissolved in a solvent and a UV absorbing agent is added. In a vacuum the solvent evaporates and the peptide is dispersed in a matrix of the UV absorbing compound. The sample is then hit with a UV

laser in the 330-360nm range. The UV absorbing material absorbs the energy from the laser and passes the energy onto the peptide, which then evaporates. The matrix material interacts with the peptide in such away that the peptide becomes charged ions. Electrical force is applied on each peptide molecule that has a different molecular weight. The output of the mass spectrometer plots the relative intensity vs. the mass to charge ratio, which is usually equivalent to the molecular weight. Theoretical molecular weight and observed molecular weight should be in agreement (Fig3.1).

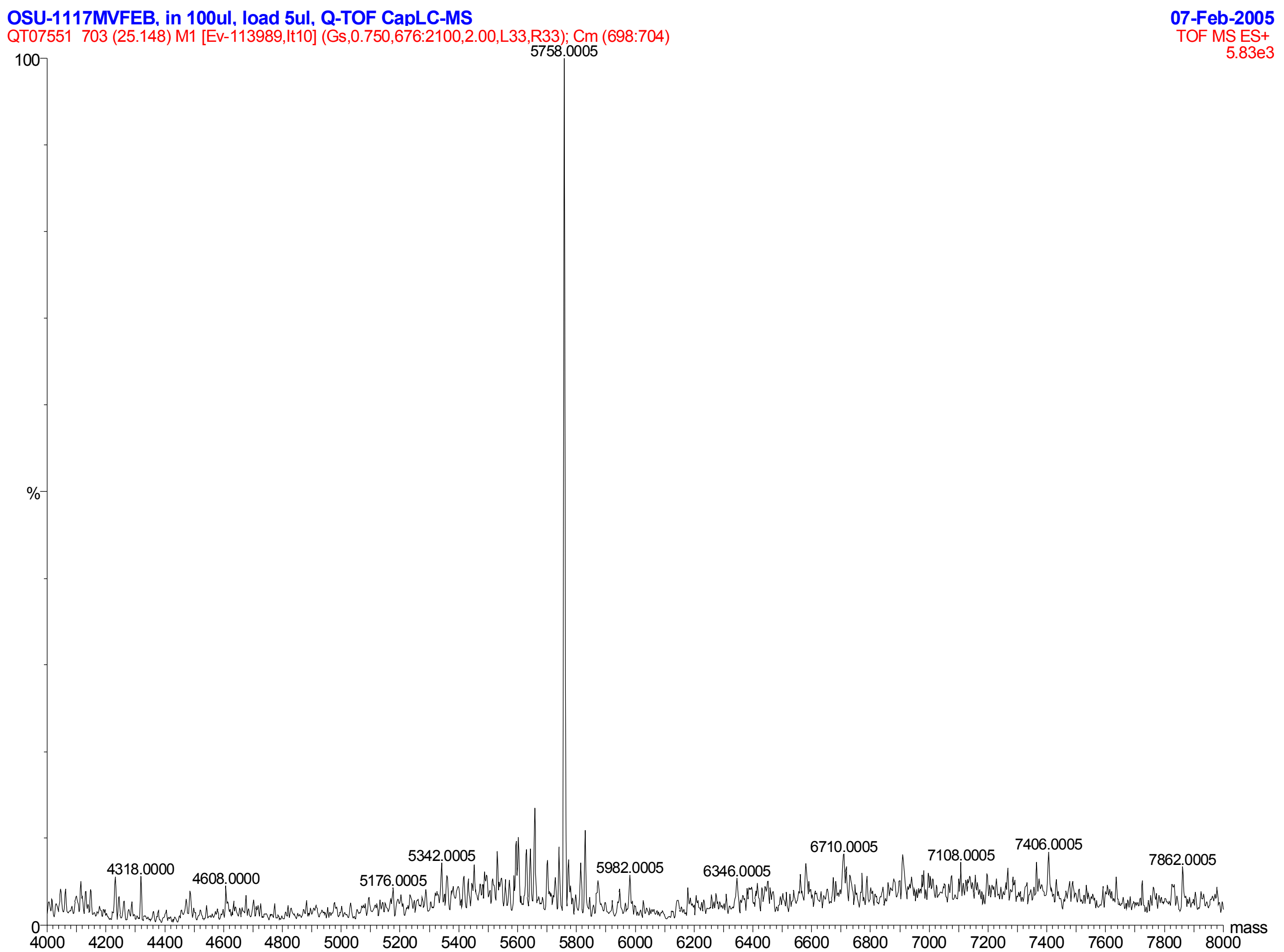


Fig. 3.1 Mass spectrometry reading for MVFHER-2(pertuzumab loop) Cyc

The molecular weight of the non-cyclized construct (not shown) is 5,759.72 and 5,757.72 for the cyclized construct. The difference arises due to the disulfide bond in the cyclized construct which results in the loss of two hydrogen atoms. To determine if the disulfide bond was correctly formed, both constructs were treated with PEO-Maleimide. PEO-Maleimide attacks free sulfhydryl groups and therefore can be used to determine the completion of disulfide pairing by ESI-MS. Therefore, the linear peptide with two free Cys (SH) group shows the addition of PEO groups (Mr =525.62), while the cyclized peptide shows no addition of PEO-maleimide and molecular weight of the peptide remains the same (see Fig 3.2).

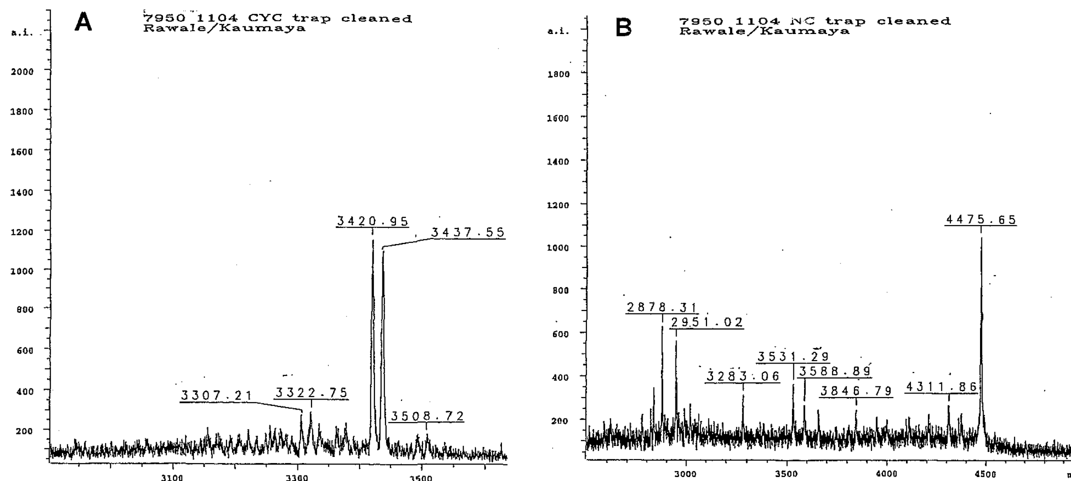


Fig 3.2 Maleimide treated mass spectrometry reading **a**. HER-2(pertuzumab loop) Cyc **b.** HER-2 (pertuzumab loop) NC

The secondary structure of the B-cell epitope was analyzed by circular dichroism (CD) spectroscopy. CD was used characterize the secondary and tertiary structures of the protein. In the "far-uv" spectral region (190-250nm) the secondary structure can be evaluated. At these wavelengths the peptide bond is the chromophore and a signal arises when it is detected in a regular, folded environment. Protein secondary structures (α-helix, β-sheet, random coil) give rise to unique CD spectra. Analysis of the far-uv CD

spectrum can give an approximate fraction of each secondary structure present in the protein. The CD spectrum of a protein in the "near-uv" spectral region (250-350nm) is sensitive to tertiary structural features. If the protein does not contain a well defined three-dimensional structure the signals in the near-uv region will be close to zero.

The B-cell epitope analyzed has a β-sheet secondary structure in the native HER-2 protein. The CD spectra from 190-290nm of both chimeric peptides in water were analyzed (Fig 3.3).

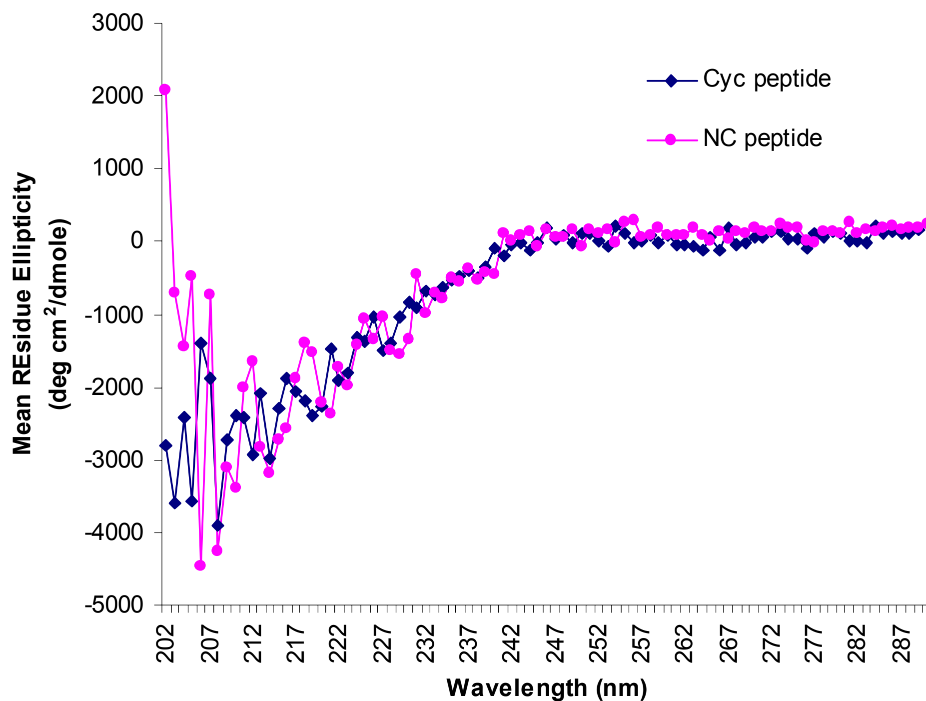


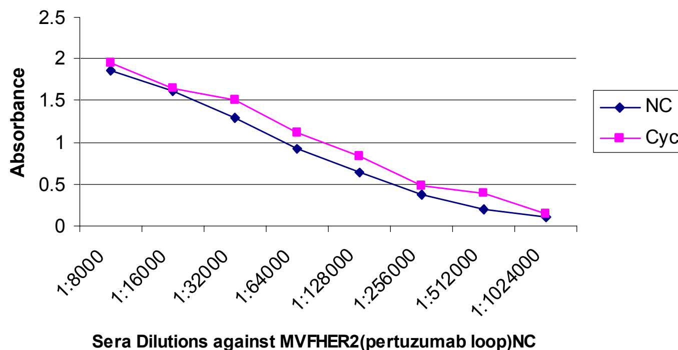
Fig 3.3. Circular Dichroism spectrum of HER-2(pertuzumab loop) Cyc and HER-2(pertuzumab loop) NC at 100μM

Non-cyclized peptide showed CD ellipticity minima around 206nm and the cyclized peptide showed minima around 209nm. These minima are characteristic of beta sheet secondary structures in the B-cell epitopes similar to the native protein. In the near uv

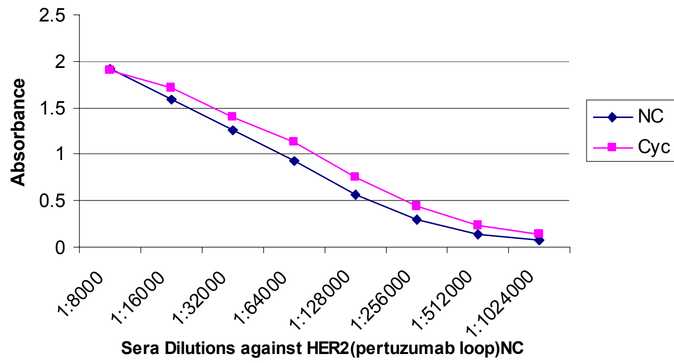
region (250-290nm) of both peptides give signals near zero indicating their lack of well defined three-dimensional conformations.

The cross-reactivity ELISA analyzed antibody binding of both the cyclic and linear structures of the synthetic HER-2 B-cell epitope. This experiment allowed us to determine how significant a role the disulfide bond in the B-cell epitope played in antibody recognition. The antibodies were tested against the peptide immunogens (Fig. 3.4a and c.) and the non-cyclized and cyclized HER-2 B-cell epitopes (Fig.3.4b and d). Both cyclized and non-cyclized antibodies recognized the linear and cyclized chimeric peptides as well as both B-cell epitope constructs. This shows that the structural difference, removal of the disulfide bridge, in the synthetic B-cell epitope does not significantly inhibit antibody binding. The cyclized antibodies had a slightly higher affinity for binding to both chimeric constructs than the non-cyclized antibodies. The affinity of the antibodies to the peptide was nearly identical to that of the B-cell epitope, this demonstrates the inability of the antibodies to significantly bind to the MVF T-helper epitope that is incorporated in the synthetic peptide vaccines.

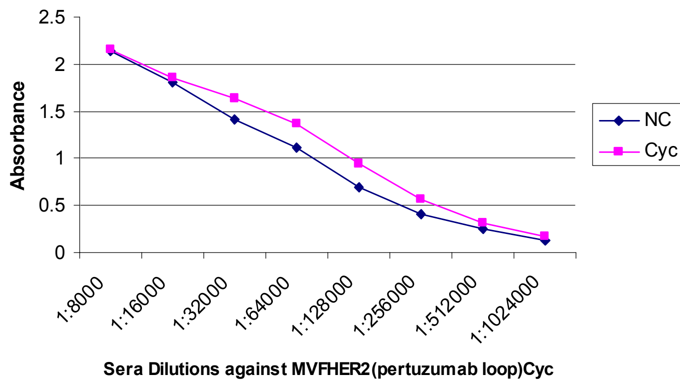
a.



b.



c.



d.

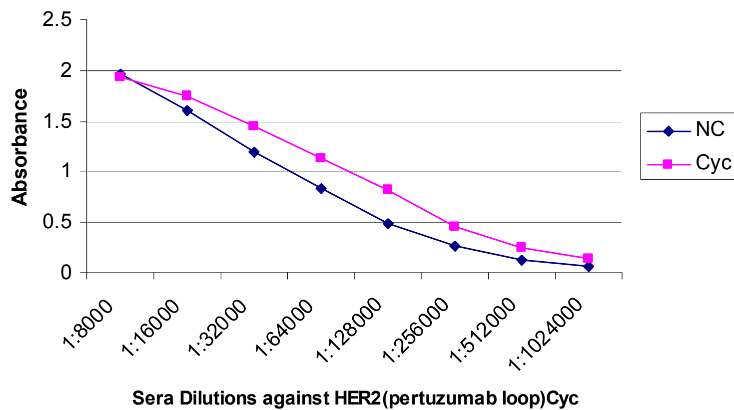


Fig.3.4. Cross-reactivity ELISA antibody affinity to chimeric peptides and B-cell epitopes. **a**. Antibody titers against the MVFHER-2 (pertuzumab loop) NC peptide construct **b.** Antibody titers against non-cyclized B-cell epitope **c**. Antibody titers against the MVFHER-2 (pertuzumab) Cyc peptide construct **d.** Antibody titers against cyclized B-cell epitope

3.2 Immunogenicity of the Peptide Constructs in outbred Rabbits

Both HER-2 peptide constructs elicited high titered antibody responses in pairs of immunized outbred rabbits (Fig.3.5). The rabbits were bled every week for 10 weeks and boosted every third week after the initial immunization. The NC peptide was more immunogenic between the two constructs and elicited slightly higher antibody titers. The direct ELISA experiment provides a way to measure the anti-peptide antibody concentration in sera.

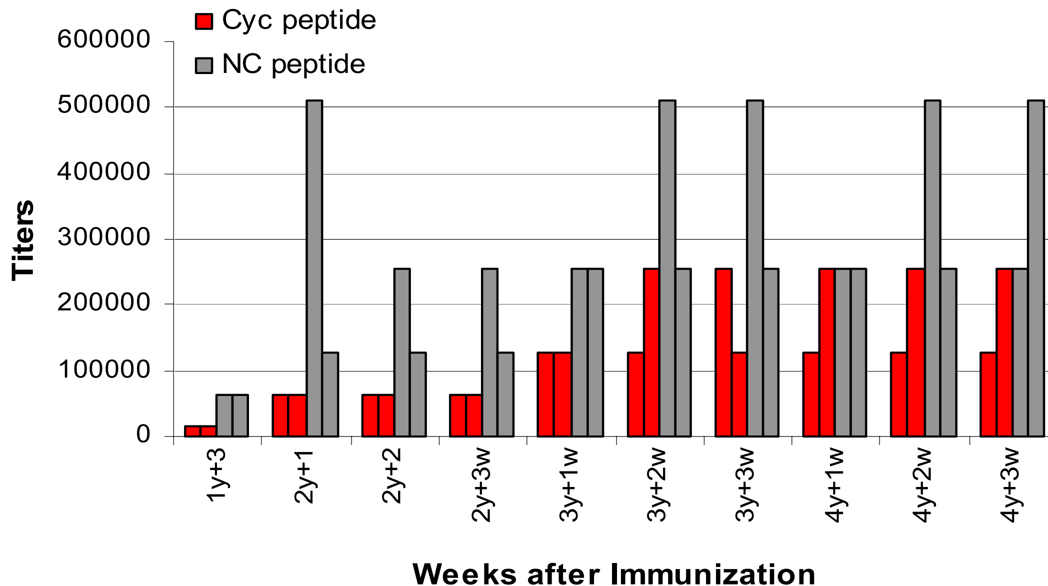


Fig. 3.5. Direct ELISA. Antibody titers in immunized outbred rabbits. 1y+3w indicates the titers obtained from sera 3 weeks (3w) after the first (1y) immunization.

3.3 Antibody Recognition of Native HER-2 Protein

Synthetic peptides elicit protein-reactive antibodies if the structural characteristics of the peptides correlate with the native protein. In order to determine the ability of the antibodies induced by the chimeric HER-2 peptide constructs to recognize the native HER-2 protein, the cross-reactivity of the antibodies was analyzed by flow cytometry, competitive ELISA assays and the HER-2 ELISA assays. Flow cytometry gives

information about a cell's physical characteristics based on the way it absorbs or reflects light. Antibodies bound to cells are shown by a shift in the peaks of the histogram. The more rightward shift in a peak corresponds to increased binding of the anti-peptide antibodies. Analysis by flow cytometry showed binding of the induced antibodies from both peptide constructs to a HER-2 overexpressing cell line (BT474). The cyclized construct induced antibodies showed slightly more than binding than the non-cyclized construct induced antibodies (Fig.3.6 a). The peptide induced antibodies were also tested against a neu overexpressing mouse cell line (NT2.5) to determine their ability to bind the HER-2 homolog *neu* (Fig. 3.6 b). This information is useful to determine whether or not to pursue *in vivo* mouse studies.

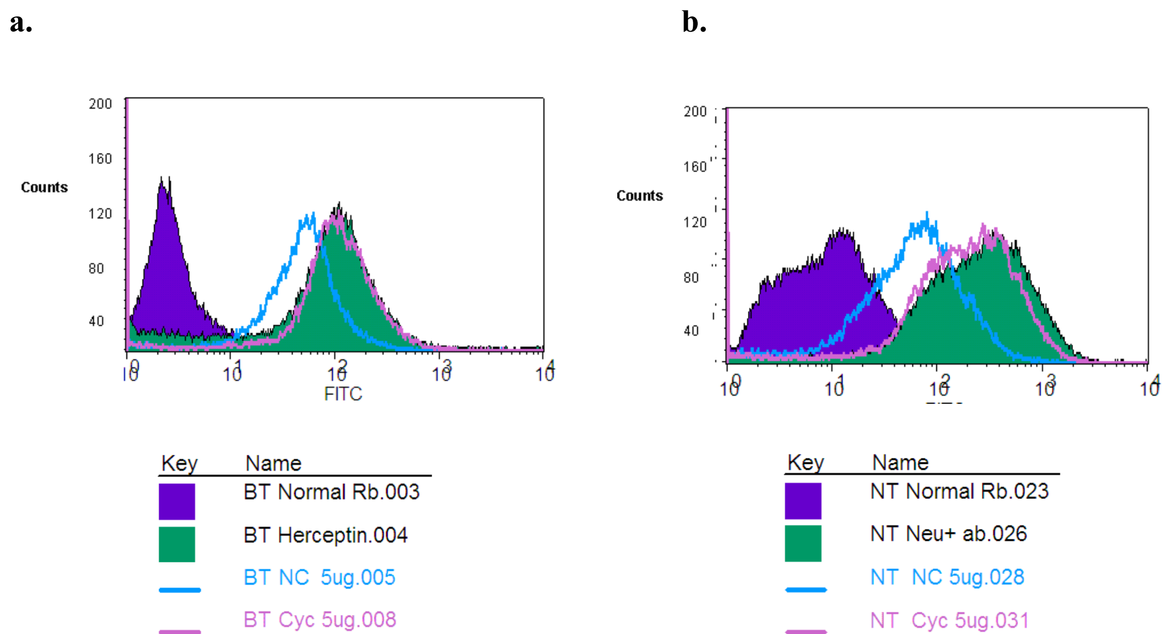


Fig. 3.6. a. Flow cytometry. Analysis of the binding of non-cyclized and cyclized rabbit induced antibodies to BT-474 (HER-2 overexpressing cells).

The HER-2 ELISA assed the ability of the anti-peptide antibodies to recognize and directly bind the native HER-2 protein. The high optical density (OD)

measurements correlate with a greater degree of antibody binding (Fig. 3.7). The Cyc antibodies showed slightly better binding of the native HER-2 protein.

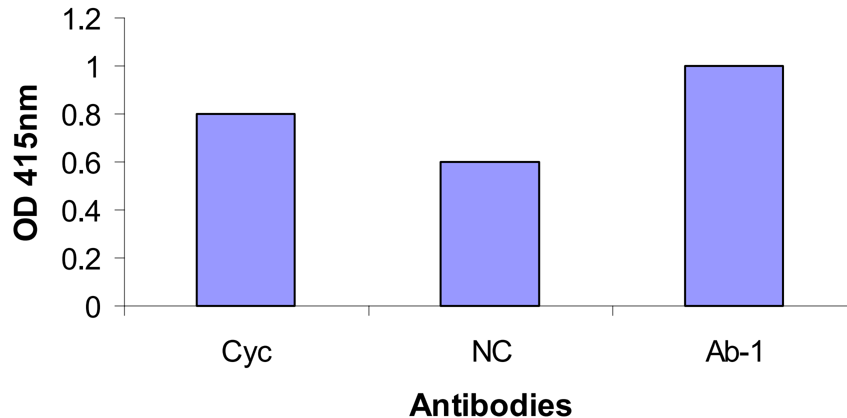
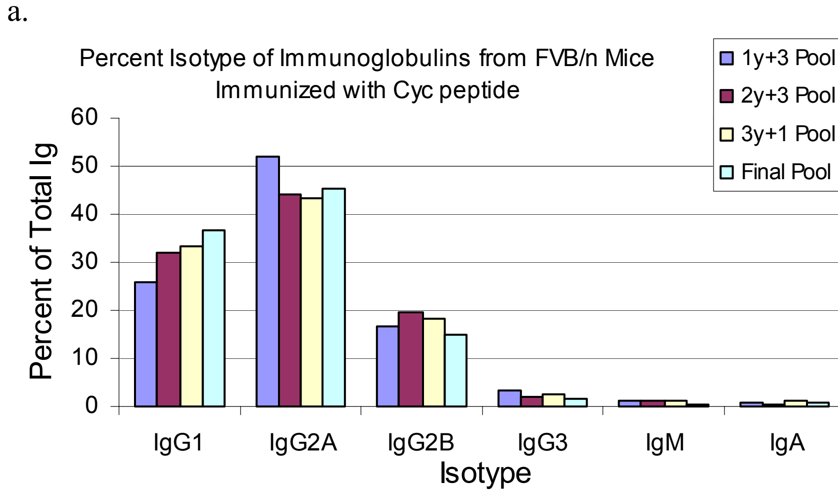


Fig.3.7 HER-2 ELISA. Antibody recognition of the native HER-2 protein.

3.4 Antibody Characterization

Several classes of mouse antibodies (immunoglobulins) exist and they are all structurally different depending on the heavy chain composition. Antibodies can kill tumor cells by engaging other cells of the immune system. IgG_1, and IgG_2 (both IgG_{2a} and IgG_{2b}) isotype antibodies are known for assisting in antibody dependent cell mediated cytotoxicity (cell death) using human peripheral blood mononuclear cells (PBMC) as effectors. The mouse isotype ELISA analyzed the light and heavy chains of the peptide induced antibodies (Fig.3.8). This experiment is convenient for identifying mouse immunoglobulin class and subclasses of IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgM and IgA as well as the light chain isotype of either κ or λ. The serum tested was from the different mouse bleeds. The light chain isotype of both the Cyc and NC antibodies was κ (not shown).

a.



b.

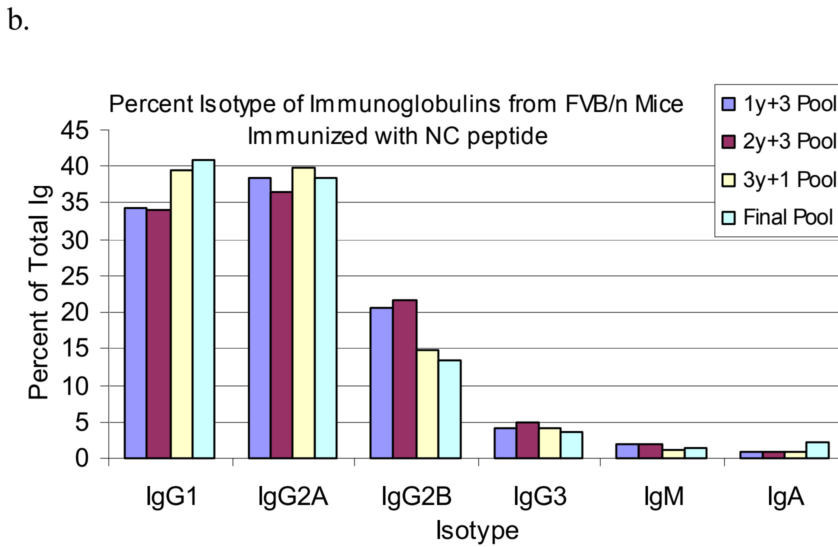


Fig. 3.8 Mouse Isotype ELISA. Determination of immunoglobulin class and sub-class. **a.** Cyc antibodies **b.** NC antibodies

Most of the antibodies typed from both peptide constructs were IgG class and specifically of IgG_1 and IgG_{2a} subclasses.

3.5 Antibody Inhibition of Cell Function and Growth

The ability of the peptide induced antibodies to effect tumor growth was tested *in vitro* by the MTT cell proliferation assay using human HER-2 normal expressing MCF-7

cells. Both Cyc and NC antibodies were capable of reducing the proliferation of MCF-7 cells in the presence of 10ng/mL heregulin (a HER-3 ligand) compared to presera treated cells (Fig3.9).

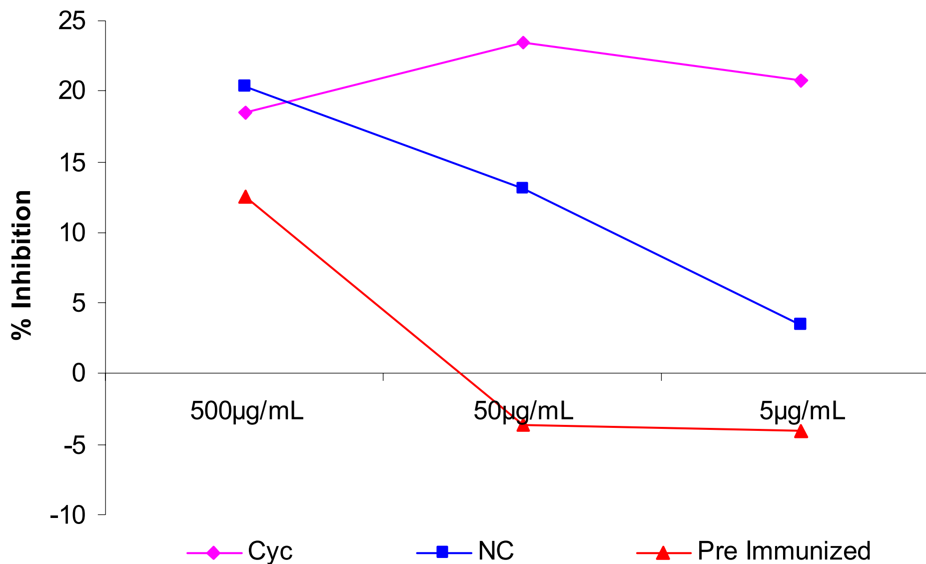


Fig. 3.9 Cell Proliferation Assay. Percent inhibition of HER-2 overexpressing human tumor cells (MCF-7).

The HER-2 phosphorylation assay analyzed the amount of phosphorylated HER-2 present after reaction with the anti-peptide antibodies (Fig.3.10). Preventing phosphorylation of HER-2 is a way to stop signaling mechanisms. After HER-2 dimerizes, transphosphorylation of the dimer's tails occurs which initiates a signaling network involved in cell proliferation and differentiation, which can lead to tumor development. The prevention of phosphorylation of HER-2 by the peptide induced antibodies results in indirect inhibition of tumor growth. Both peptide induced antibodies showed the ability to inhibit phosphorylation. AG 825, a phosphorylation inhibitor of HER-2, was used as a positive control and the normal rabbit IgG antibodies were the negative control. The NC antibodies had a slightly higher percent of inhibition compared to the Cyc antibodies.

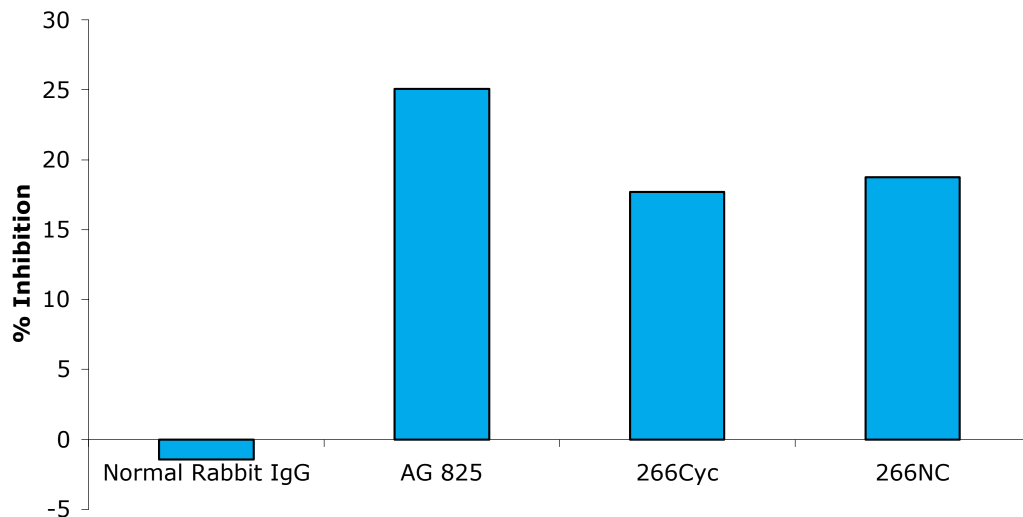


Fig.3.10 HER-2 phosphorylation Assay. Percent Inhibition of HER-2 phosphorylation *in vitro*.

3.6 Antibody Dependent Cell Death

The antibody-dependent cell mediated cytotoxicity assay (ADCC) shows the ability of the peptide induced antibodies to successfully cause cell lysis (Fig.3.11). The peptide induced IgG antibodies attach to the extracellular domain of the HER-2 protein and are recognized by the Fc receptor of natural killer (NK) cells leading to its activation and thus release of cytokines. These cytokines in return trigger events that result in lysis of the antibody-coated cells. The control IgG antibodies used for Herceptin were normal human IgG antibodies and for the anti-peptide antibodies normal rabbit IgG. In comparison to the percent lysis due to effectors and targets only the anti-peptide antibodies show antibody specific lysis at each effector target ratio.

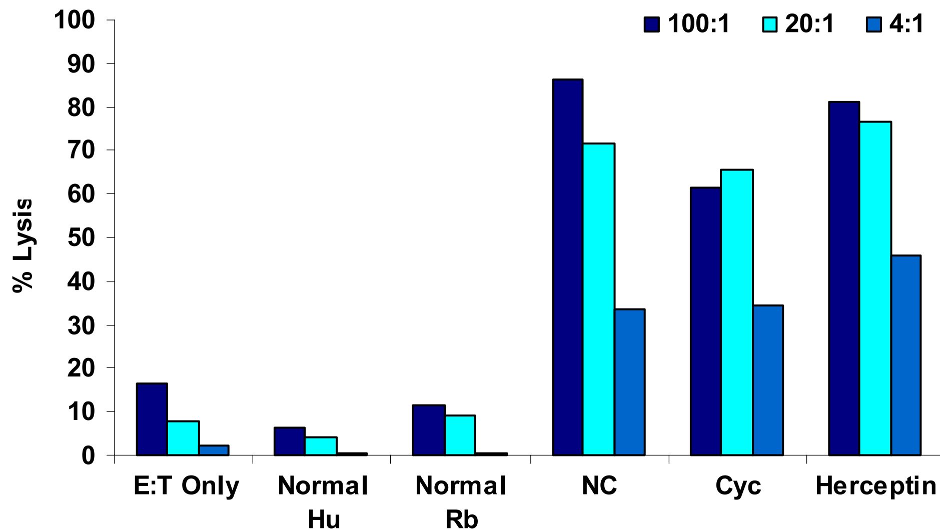


Fig 3.11 ADCC. Cyc and NC peptides antibody-dependent cell mediated cytotoxicity of ^{51}Cr-labeled mammary tumor target cell line BT474 assayed in presence of human PBMCs. (Effector: Target)

3.7 ***In vivo*** **Mouse Models**

Passive and active immunotherapeutic experiments were carried out in transgenic mice. Transgenic mouse models express mammary tumors similar to human breast cancer. Experimentation with these mouse models mimic tumor growth in a clinical setting. These genetically engineered mice develop spontaneous tumors similar to cancer patients. Spontaneous tumors effect surrounding tissue and create genetic changes due to tumor growth. Double transgenic (VEGF$^{+/-}$ Neu2-5$^{+/-}$) were tested by passive immunotherapy due to rapid tumor onset in these mice. These mice develop tumors around the age of 55 days due to the overexpression of VEGF protein. VEGF is a gene that causes angiogenesis (new vessel development), this in turn helps nurture the developing tumor, which results in early tumor development. These mice were passively treated with purified IgG rabbit antibodies to analyze the tumor inhibitory characteristics of the peptide induced antibodies. VEGF$^{+/-}$ Neu2-5$^{+/-}$ mice injected with purified IgG MVFHER-2(pertuzumab loop)Cyc or MVFHER-2(pertuzumab loop)NC antibodies

showed a statistically significant reduction in tumor size compared to mice treated with presera antibodies (p= 0.0001 and p=0.0008). This shows the capabilities of these antibodies to directly inhibit tumor growth (Fig. 3.12).

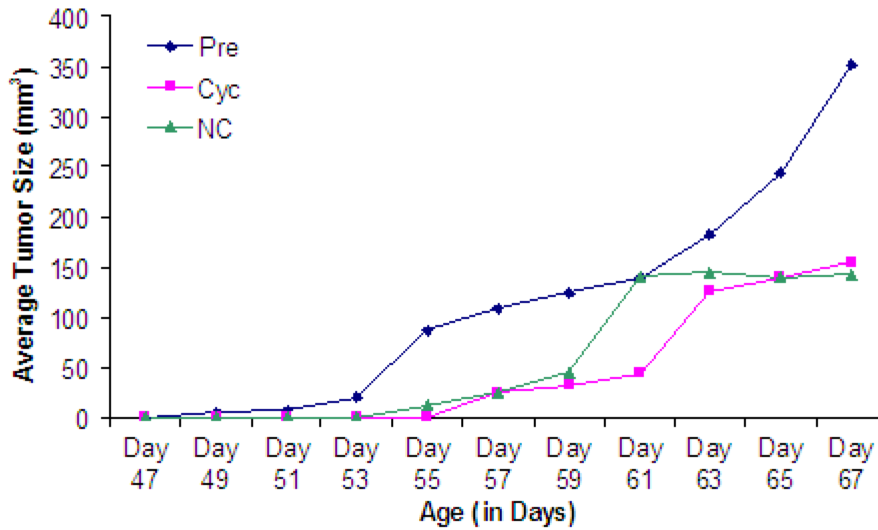


Fig 3.12 Passive immunotherapy. VEGF$^{+/-}$ Neu2-5$^{+/-}$ double transgenic treated with purified IgG MVFHER-2(pertuzumab loop) Cyc or NC or presera antibodies.

Neu2-5$^{+/-}$ mice were used to test the *in vivo* anti-tumor effects of the synthetic peptide vaccine. These mice develop tumors around day 177 days. The mice vaccinated with the target peptide showed a delayed onset of tumor and in general had a lower tumor volume compared to the pre-treated mice (Fig 3.13). These preliminary results and studies show the ability of the synthetic vaccines to enhance the mouse immune system and affect tumor development. However, ongoing studies are being conducted in order to reach statistical significance.

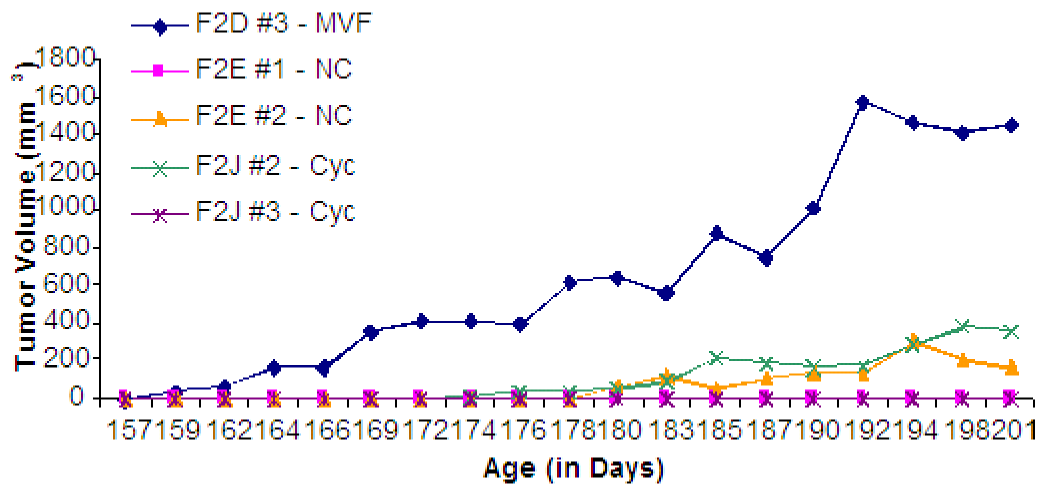


Fig 3.13 Active immunotherapy. Neu2-5$^{+/-}$ transgenic mice treated with MVFHER-2(pertuzumab loop) Cyc or NC chimeric peptide construct. (F2D#3, F2E#1, F2E#2, F2J#2 and F2J#3 are names of the mice)

Tumor challenge

Immunizing the mice with the synthetic peptide allows the mice time to mount an immune response. Their immune system will have already built up a defense before the mice are challenged with tumor cells. The mice are challenged with tumor cells that come from the same strain of mouse (same genotype). If the mice were challenged with tumor cells that are genotypically different the immune system would recognize the tumor cells as foreign antigens and cause the mouse to immediately reject the tumor cells and not allow tumor development. Tumor challenged mice develop tumors different from spontaneous tumors in that there is not as much genetic manipulation in the surrounding tissues and the tumor is of one cell type as opposed to multiple types. In the NT2.5 tumor challenge, FVB/n mice were challenged with tumor cells (NT 2.5) from the same mouse strain 10 days after their last immunization. The mice immunized with the synthetic vaccine elicited high titered antibody responses prior to the tumor challenge

(Fig.3.14a). Mice immunized with either the Cyc or NC peptide showed a statistically significant reduction in tumor volume compared to mice treated with MVF (p= <0.001 and p= 0.002) (Fig3.14b). The synthetic MVFHER-2(pertuzumab loop) Cyc vaccine demonstrated an overall lower tumor volume in comparison to the linear construct.

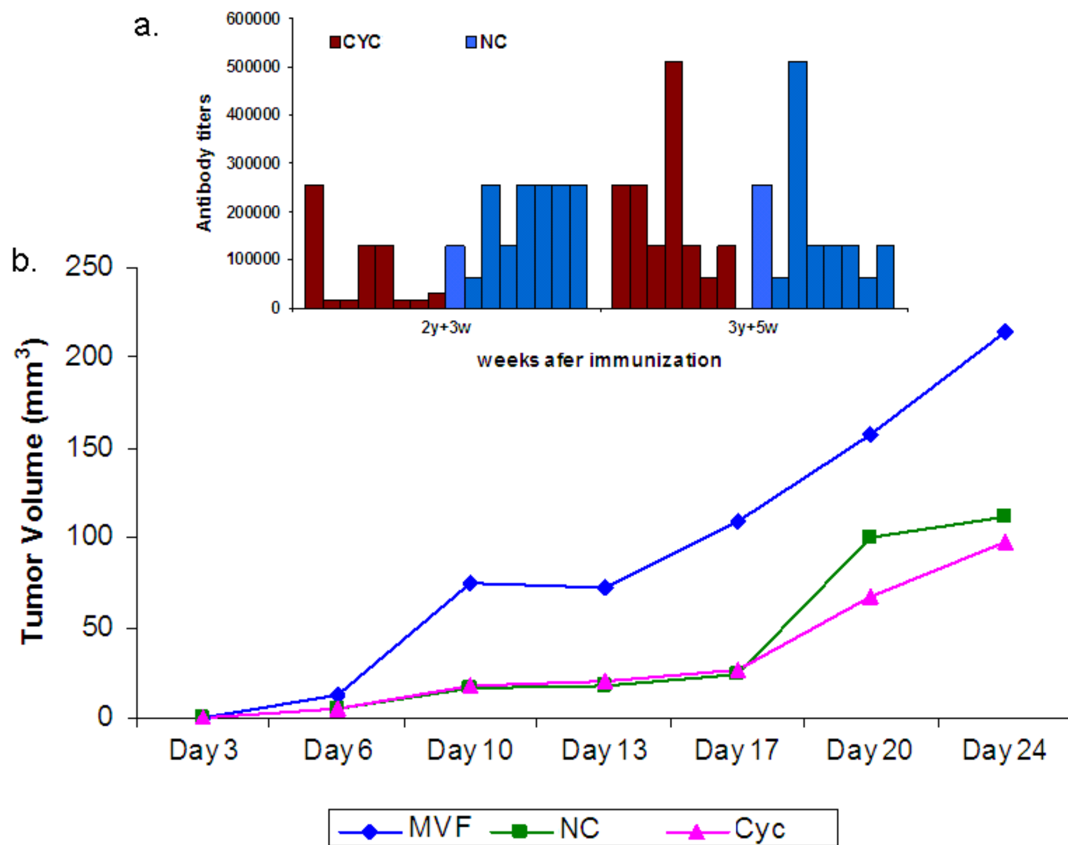


Fig.3.14 NT2.5 tumor cell challenge in FVB/n mice. a. Antibody response to synthetic vaccines in mice prior to tumor challenge. b. NT2.5-tumor challenge results.

An additional tumor challenge experiment with TUBO cells was performed. Mice immunized with either peptide construct showed high antibody titers prior to the tumor challenge (Fig.3.15a) Balb/c mice were challenged with *neu* overexpressing TUBO cells from the same mouse strain 14 days after the last immunization (Fig. 3.15b). The mice immunized with the MVFHER-2 (pertuzumab loop) Cyc or MVFHER-2 (pertuzumab loop) NC peptide showed a statistically significant reduction in tumor

growth compared to MVF immunized mice (p=0.0007 and p= 0.0002 respectively). In this study the synthetic NC peptide vaccine demonstrates higher tumor inhibitory effects.

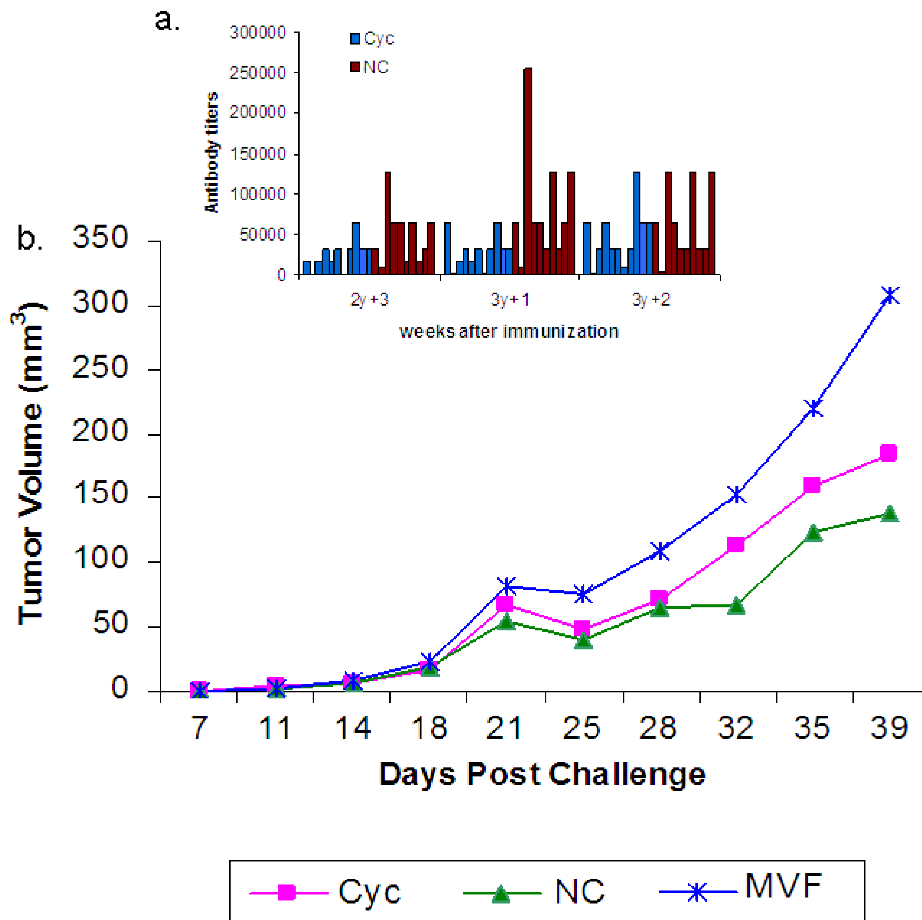


Fig 3.15 TUBO tumor challenge in Balb/c mice. a. Antibody response in mice to synthetic vaccines prior to challenge. 2y+3 indicates blood drawn 3 weeks after second boost. b. TUBO challenge results.

Both tumor challenge studies confirm the ability of each synthetic peptide vaccine to decrease tumor growth in HER-2/*neu* overexpressing tumor cells *in vivo*.

Chapter 4

Discussions

The overexpression of HER-2 in many cancers, its low levels of expression in normal adult tissues, and the surface exposed extracellular domain make it an ideal target for immunotherapy. Since the crystal structure of HER-2 complexed with the anti-HER-2 monoclonal antibody pertuzumab has been solved (18), it is now possible to directly asses antigenic sites on HER-2 based on this crystal structure, such as, the pertuzumab loop region. It is imperative that peptides are modeled like the native conformation of the target protein in order for their antibodies to bind the target antigen with a high enough affinity to be biologically significant. In this study we systematically assessed the biological effects of two synthetic peptide vaccines that contained an antigenic HER-2 B-cell epitope, in the native cyclized structural form and in the linear form. The focus of this thesis is the design of peptide vaccines that against the HER-2/neu dimerization loop. We hypothesize that developing a vaccine that incorporates residues pertuzumab loop from the HER-2 dimerization loop will inhibit tumor growth by eliciting antibodies that sterically hinder the dimerization loop of the HER-2 protein.

Our approach involved the *de novo* design of peptide vaccines that focused on maintaining the native protein structure while introducing a minute structural change, removal of the disulfide bond, to analyze the biological effect. Previous work in different model systems demonstrated that the incorporation of promiscuous T-helper (T_H) epitope derived from non-human molecules can be used to help elicit high-titered antibodies that recognize the native protein in an outbred population (24). The synthetic vaccine design incorporated a promiscuous T_H epitope, a GPSL linker and the HER-2 B-cell epitope

(pertuzumab loop). The chimeric peptides were synthesized co-linearly using Fmoc-/t-But chemistry. An intramolecular disulfide bond between two cysteine residues of the B-cell epitope was formed in the cyclized construct.

An important characteristic of vaccines is the ability to generate protection in outbred populations. Therefore, we analyzed the immunogenicity of each chimeric peptide in outbred rabbits and eventually in inbred FVB/n and Balb/c mice as tumor challenge models. The MVFHER-2(pertuzumab loop) NC vaccine elicited slightly higher antibody titer responses than the cyclic vaccine. However, this didn't correlate to superior anti-tumor inhibitor effects of the linear construct in comparison the cyclic construct. A successful peptide vaccine is able to generate antibodies that recognize and bind the native protein. We used flow cytometry to assess the ability of the antibodies generated from our synthetic peptide vaccines to recognize the native protein. Flow cytometry indicated that the antibodies to the cyclized peptide construct showed better binding to the HER-2 overexpressing BT474 cells than the linear construct. The HER-2 ELISA showed similar results of the Cyc antibodies binding better to the native HER-2 protein. Even though the Cyc antibodies showed better binding to the native structure of HER-2, this did not correlate to consistently better indirect and direct anti-tumor effects.

The cross reactivity ELISA established the ability of both peptide induced antibodies to cross react with the cyclic and linear HER-2 B-cell epitope constructs and immunogen peptides. These results confirm the conservation of the antibody binding region in the cyclic B-cell epitope and the specificity of the antibodies to the B-cell epitope and not other components of the vaccine.

The ability of the peptide induced antibodies to inhibit HER-2/*neu* dimerization by steric hindrance of the dimerization loop and indirectly limit cellular growth was assessed by the HER-2 phosphorylation assay. The Cyc antibodies showed a 17% inhibition of phosphorylation of the HER-2 protein, whereas the NC antibodies showed an 18% inhibition. Therefore, these antibodies are capable of directly affecting the phosphorylation of HER-2 via blockage of the dimerization loop. This displays the potential of these antibodies to disrupt downstream signaling of the HER-2 homo- or heterodimer that is responsible for cell proliferation. Direct inhibition of cell growth was studied by an *in vitro* cell proliferation assay. The cell proliferation assay demonstrated the ability of both peptide induced antibodies to limit growth of human cancer cells *in vitro* compared to cells treated with only normal rabbit IgG antibodies. Indirect anti-tumor effects of the peptide antibodies were shown in ADCC. The NC antibodies showed an increase in cytotoxicity against HER-2 overexpressing cells compared to the cyclized construct as measured by ADCC. These findings show the ability of both antibodies to kill tumor cells by mediating cytotoxic effects in natural killer cells.

In the transgenic mouse models, direct tumor growth inhibition was slightly better in mice passively treated with the purified cyclized IgG peptide antibodies or actively treated with the cyclized peptide itself as opposed to ones treated with the linear antibodies or peptide. Statistical significance was obtained in these experiments. The p-value is a statistic term that indicates the probability that the difference between groups during an experiment happened by chance. The lower the p-value the more likely the difference in tumor size was due to treatment. Mice passively immunized with Cyc or NC antibodies showed a statistically significant smaller average tumor size compared to

pre antibody treated mice (p=0.0001 and p=0.0008). The tumor challenge mice treated with the synthetic peptide vaccine have displayed lower tumor volumes that are statistically significant. In the NT2.5 challenge, mice immunized with MVFHER-2 (pertuzumab loop) Cyc or MVFHER-2(pertuzumab loop) NC showed a statistically significant reduction in tumor volume compared to mice treated with MVF alone (p= <0.001 and p= 0.002). The TUBO challenge showed that mice immunized with the MVFHER-2 (pertuzumab loop) Cyc or MVFHER-2 (pertuzumab loop) NC showed a statistically significant reduction in tumor growth compared to MVF immunized mice (p=0.0007 and p= 0.0002 respectively). This shows the ability of both peptide vaccines to successfully limit tumor cell development *in vivo*.

This study has shown that subunit peptide vaccines can target immune responses to biologically active epitopes. The ability to focus the immune response is particularly relevant to HER-2, where antibody interaction with specific sites has the potential to sterically hinder dimerization and thus limit cellular proliferation. In this thesis the peptide vaccines tested were capable of eliciting HER-2 specific antibodies in an outbred population with the ability to inhibit development of HER-2 overexpressing cancers by blocking homo- and heterodimerization.

Chapter 5

Conclusions

The revolutionary approach of using peptide vaccines to treat cancer proves promising for future endeavors. The findings of this thesis show potential for designing vaccines that target the HER-2 oncoprotein. Further testing of the synthetic peptide vaccine's direct and indirect anti-tumor effects is necessary to establish clinical significance in the data. Future work includes the goal of obtaining FDA approval to move into clinical trial phase. Continual evaluation and testing of different antigenic sites on the HER-2 protein for potential synthetic peptide vaccines proves useful to developing an effective cancer treatment. Other studies could include a multi-epitope approach to vaccine design. The incorporation of two or three B-cell epitopes may prove to be a better more effective vaccine design. For example, a vaccine that targets a HER-2 antigenic site and incorporates the VEGF epitope can create an immune response that causes inhibition of tumor development two ways. First, seeking to prevent dimerization of HER-2 that leads to signaling and cell growth, and secondly, creating antibodies that effectively bind the VEGF protein and cut off blood supply to the tumor, which is vital for tumor growth. The use of multiepitope vaccines incorporating both Herceptin and pertuzumab epitopes in combination with a HER-2 vaccine that prevents dimerization could suppress tumor development completely. In this thesis the design of a synthetic peptide vaccine targeting the HER-2/*neu* dimerization loop and incorporating both T and B-cell epitopes proves useful in inhibiting the growth of HER-2/neu overexpressing tumor cells.

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