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Recently a new drug (Herceptin) has been shown to be effective in the treatment of malignant breast cancer, and has been approved for use. Herceptin is essentially a humanized mouse monoclonal antibody that binds to ErbB-2, a membrane growth factor receptor tyrosine kinase that is over expressed in 25-30% of patients with malignant breast cancer. Herceptin prevents targeted cells from proliferating. Our goal is to induce the body to produce its own antibodies to ErbB-2, which hopefully like Herceptin will target breast cancer cells and then prevent them from growing. The specific purpose of this proposal is to test the use of phage particle mimetopes of ErbB-2 to make a vaccine, which when injected into mice, will induce the animals to make antibody against ErbB-2. Progress so far has included the development of a phage selection protocol, and the subsequent collection of M13 phage clones that have high affinity for binding to Herceptin, and thus likely will constitute effective mimotopes to ErbB-2.

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Introduction

One of the major difficulties in using immunotherapy as an effective cancer treatment protocol has been to define suitable antigens to which immunotherapeutic protocols might be targeted. In the case of breast cancer, an excellent target for immunotherapy has been identified. The HER2/neu proto-oncogene codes for a membrane receptor tyrosine kinase (ErbB-2) of the ErbB growth factor receptor family. This protein is overexpressed in the tumor cells of 25-35% of breast cancer patients. Herceptin, a recombinant humanized mouse monoclonal antibody (4D5) with high affinity to ErbB-2, inhibits the growth of these tumor cells and is currently being used as a safe, effective reagent in combination with chemotherapy in women with HER-2-overexpressing metastatic breast cancer. This clinical application is thus an example of a passive immunotherapeutic approach. However, like in other passive immunotherapies, there are drawbacks. Some women do not achieve adequate serum levels of the reagent, while others in which Herceptin is initially effective, may develop an immunological defense reaction that neutralizes the drug. Clinical experience has shown that, although initial success is often achieved with passive immunotherapy, in the long run, active immunization is the superior treatment strategy. In this case, antibodies and T cells reactive to ErbB-2 have been isolated from breast cancer patients, indicating that ErbB-2 is a potential target for active immunization. The aim of this research project is to identify and evaluate the use of M13 phage particles displaying ErbB-2 (Herceptin reactive) mimotopes as immunization agents in mice. The goal is to stimulate the active production of antibodies which specifically recognize ErbB-2 at 4D5 (Herceptin) defined sites in BALB/c mice. Phage display peptides (called mimotopes) isolated by immunoaffinity selection (biopanning) from linear or circular phage display libraries have been shown to induce antigen-specific immune responses directed against the epitope recognized by the monoclonal antibody used for the biopanning of the phage clones. Mimotope immunization is therefore a way to induce epitope-specific antibody responses *in vivo* for cases where it might be difficult to elicit a response from the native antigen, such as would be the case for proteins where tolerance has been established.

Body

1. We have isolated several different M13 peptide display phage clones as preliminary ErbB-2 mimotope immunogens utilizing biopanning techniques.

The Ph.D.-7 Phage Display Peptide Library kit (New England BioLabs) was used in these affinity selection experiments. This is a combinatorial library of random peptide 7-mers fused to a minor coat protein (pIII) of M13 phage. The library consists of ~2.8 x 10^9 electroporated sequences, amplified once to yield ~70 copies of each sequence in a 10µl phage sample used in each panning experiment. A panning protocol using elements of those published in the Ph.D.-7 kit instruction manual (1), literature (2,3), plus additional modifications, was developed and used to select several peptide display phage clones specifically binding to Herceptin (humanized 4D5 antibody) coating the affinity selection plates. An example is shown in Figure 1.

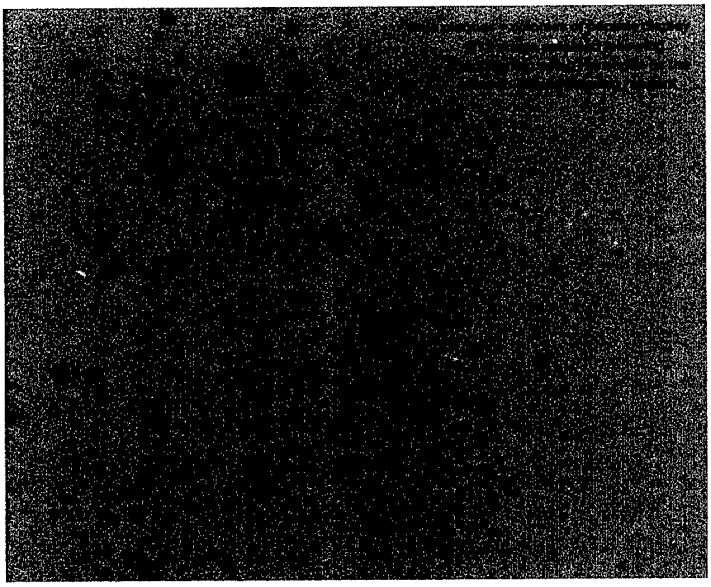

Figure 1. One of several display phage clones isolated following biopanning with Herceptin. M13 is a male specific coliphage. It is propagated on *E coli* host strain ER2738, a rapid growth F+, lacZa- strain. The phage carries the lacZa gene, so plaques appear blue when plated on this strain of *E coli* growing on media containing Xgal and IPTG.

2. We have developed an effective protocol to generate high-titer phage clone solutions to be used as immunization reagents in mice. Using this protocol, we have generated several high-titer, amplified panning output solutions that were stabilized in glycerol and stored at -23^{0}C.

Biopanning is a relatively easy, although time-consuming procedure. However, amplification of selected phage particles and production of a final reagent solution that had a high viral titer, yet no live *E coli* host bacteria (which would prove fatal to the test mice), proved to be more problem-prone than expected. Published procedures were modified and improved, resulting in a reproducible, 1 day phage amplification protocol that routinely yielded about 10^{18} phage particles per milliliter of final solution. This final panning output solution generally has a total protein amount of 1 - 2 mg/ml and is almost always bacteria-free (all viable *E coli* viral host bacteria are removed from the final solution).

3. We have confirmed the Herceptin binding specificity of the generated phage solutions by direct immunoblotting.

Although biopanning has a reputation in the literature of being highly selective for the specific mimic peptides one is panning for, we decided to attempt to directly gauge the specificity in our phage output solutions. Accordingly, 1 µl aliquots of phage preparations, and serial dilutions of these solutions were directly blotted to nitrocellulose in a series of experiments. After blocking with 1% BSA (Sigma), blots were probed with Herceptin as the primary detection antibody. Herceptin binding was determined by probing with a secondary antibody, goat anti-human Ig with HRP conjugate (SouthernBiotech). HRP on immunoblots was visualized with SuperSignal

West Pico chemiluminescent substrate (Pierce). An example of one of these experiments is shown in Figure 2.

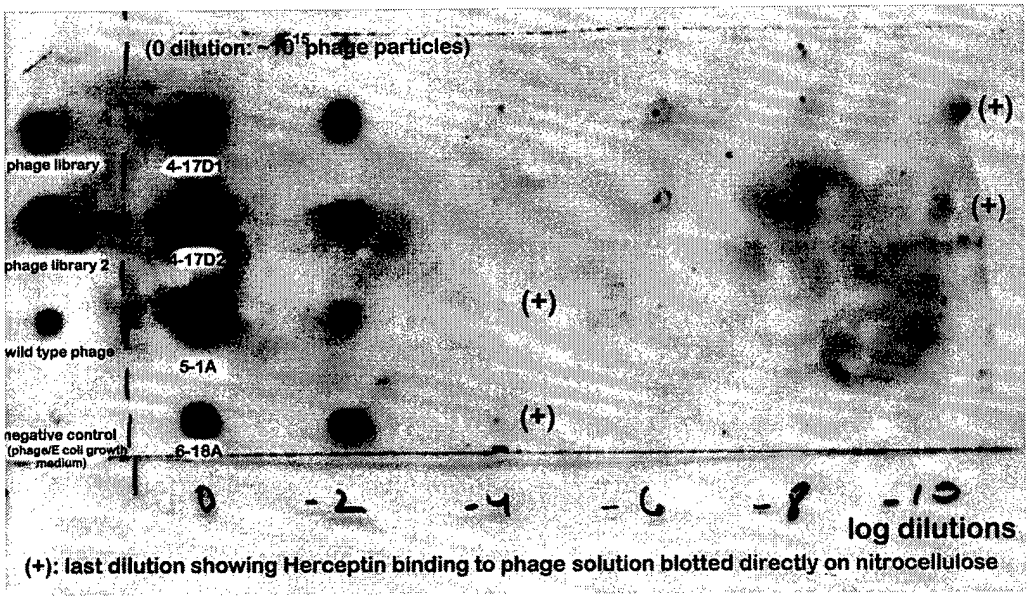

Figure 2. Direct immunoblots of 4 panning output phage solutions. Herceptin is the affinity antibody used to probe the phage blots. Phage dilutions blotted run from the dotted line to the far right, while control blots are at the far left of the dotted line. Note that Herceptin binds nonspecifically to M13 phage, to both wild type and the Ph.D. 7-peptide mimotope phage libraries used in the earlier biopanning experiments.

These experiments confirmed our assumption that there might be a fair amount of nonspecific phage binding to Herceptin, as some phage solutions bind only at low dilution levels. (Phage solutions 5-1A, and 6-18A in Figure 2.) However, we also found that many of the panned phage solutions collected have detectable Herceptin specific affinity at high dilutions, indicating that at least some of the phage in these panned solutions may carry mimotopes to the Herceptin antigen binding site. In Figure 2 phage solutions 4-17D1 and 4-17D2 yield detectable Herceptin binding activity out to dilutions of $1/10^{10}$.

Key Research Accomplishments

1. Isolated several different high affinity M13 peptide display phage clones by biopanning using Herceptin as the affinity selection reagent.

2. Developed an effective protocol to generate high-titer, *E coli*-free phage clone solutions to be used as immunization reagents in mice.

3. Gauged the Herceptin binding specificity of the phage solutions generated by direct immunoblotting.

Reportable Outcomes

None

Conclusions

We have generated several very high viral titer, M13 peptide phage solutions using a biopanning protocol and Herceptin as the immuno-affinity selection agent. Additionally, we have developed an effective amplification protocol to produce high yield, high-titer (to Herceptin), bacteria-free immunogens from the selected phage solutions. However, as Herceptin is an ErbB-2 binding, humanized mouse monoclonal antibody, immunization has been delayed until the number of human IgG, but non ErbB-2 mimotopes within the selected phage solutions could be reduced. We will solve this problem by incorporating an additional negative selection immunoabsorption procedure against human IgG-agarose into our selection protocol after the biopanning procedure. This should remove most M13 clones with mimotopes that are not specific to the Herceptin ErbB-2 binding site. Biopanning for Herceptin-specific mimotope phage will then be repeated using the immunoabsorbed phage libraries. This should produce highly specific, ErbB-2 mimotope phage solutions that should generate a much more specific immune response to ErbB-2 in vaccinated mice.

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