Award Number:  DAMD17-99-1-9067

TITLE:  Vaccine Development Against Novel Breast Cancer Antigens

PRINCIPAL INVESTIGATOR:  Vasso Apostolopoulos, Ph.D.
Ian McKenzie, M.D., Ph.D.

CONTRACTING ORGANIZATION:  Austin Research Institute
Heidelberg, Victoria 3084 Australia

REPORT DATE:  November 2002

TYPE OF REPORT:  Final

PREPARED FOR:  U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:  Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are
those of the author(s) and should not be construed as an official
Department of the Army position, policy or decision unless so
designated by other documentation.
4. TITLE AND SUBTITLE
Vaccine Development Against Novel Breast Cancer Antigens

6. AUTHOR(S):
Vasso Apostolopoulos, Ph.D.
Ian McKenzie, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Austin Research Institute
Heidelberg, Victoria 3084 Australia
E-mail: i.mckenzie@ari.unimelb.edu.au

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. Abstract (Maximum 200 Words) *(abstract should contain no proprietary or confidential information)*
The goal of the proposal was to examine a number of antigens present in breast cancer to determine which could be a suitable target for immunotherapy. We also examined various delivery systems including mannan - all aimed at developing an effective T cell response against antigens expressed in human breast cancer. A number of antigens were identified and studied in detail e.g. MUC1, Cripto, Pim1, p53 - others were rejected because of their high distribution in normal tissues (nm23) and others as it was not possible to generate meaningful amounts of the material for studying. The mannan technology proved not to be feasible for direct injection in humans because of cross reactivity between MUC1 and a naturally expressed antibody in humans. However, we were able to demonstrate its effectiveness ex vivo - although outside the scope of the proposal - *this led directly to a clinical trial*. Other modes of delivery were examined - (i) prime boost, (ii) the use of beads to target dendritic cells in vivo and (iii) using a 16amino acid sequence from antennapedia to effectively deliver antigens to dendritic cells. The studies were successful, in that, several antigens were identified and characterised, and several modes of delivering antigens to dendritic cells were proven to be successful - all functioning as preclinical studies for planned clinical trials.

14. SUBJECT TERMS
breast cancer, mannose, HLA-A2, CTL's, MHC, class I molecules

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT
Unclassified

18. SECURITY CLASSIFICATION OF THIS PAGE
Unclassified

19. SECURITY CLASSIFICATION OF ABSTRACT
Unclassified

20. LIMITATION OF ABSTRACT
Unlimited

NSN 7540-01-280-5500
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cover</td>
<td>1</td>
</tr>
<tr>
<td>SF 298</td>
<td>2</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>3</td>
</tr>
<tr>
<td>Introduction</td>
<td>4</td>
</tr>
<tr>
<td>Body</td>
<td>5</td>
</tr>
<tr>
<td>Key Research Accomplishments</td>
<td>17</td>
</tr>
<tr>
<td>Reportable Outcomes</td>
<td>18</td>
</tr>
<tr>
<td>Conclusions</td>
<td>19</td>
</tr>
</tbody>
</table>
INTRODUCTION

Improvement in the treatments of breast cancer are required – at present, the foundations for therapy is still surgery, radiotherapy and cytotoxic drugs and added to this is hormonal manipulation and more recently the Herceptin antibody. However, the treatment is less than optimal with serious side effects occurring with chemotherapy and while the "cure" rate is steadily improving, it is appropriate to examine immunotherapy to give a major improvement and survival of patients with breast cancer. For immunotherapy to succeed two components are required a) antigens and b) delivery system. In this study, we identified a number of antigens which, at least initially, appeared to be over-expressed in breast cancer and which could be suitable targets – these included MUC1, Cripto, nm23, several oncogenes, ampheregulin, EGF receptor and Her2/neu. This was an ambitious project and along the way several of these were discarded as not being sufficiently cancer specific to use and also the failure to be able to produce adequate amounts for study hindered some of the proposed antigens to be discontinued. Delivery systems today mostly rely on antigen encountering dendritic cells by chance and other adjuvants have been described which non-specifically heighten the immune response all of which have side effects. Furthermore, the treatment with advanced disease, seeking an enhanced immune response, is almost doomed to fail because of the poor health (immune status) of the patients. With this mind, we not only examined the role of mannan to target the mannose receptor of dendritic cells, but variations of this using ex vivo cells (outside the patient to avoid cross reactivity and the suppressive environment in the patient). We compared the mannan technology with other modes of immunisation such as the prime boost technology, the use of antennapedia peptides for delivery and the use of the beads as carriers.

The significant progress was made with a number of potential targets being identified and several optimal delivery systems – the success of the proposal is measured by the considerations currently being given for the preclinical studies described herein to be extended into Phase I clinical trials.
Summary of work undertaken for Task 1

Task 1. Produce recombinant proteins to different antigens for linkage to mannan

Antigens studied:

MUC1
- Peptides
- Glycosylated peptide
- HMFG, whole MUC1
- Peptides from outside the VNTR region
- N-terminal fusion protein
- Mutants
- Mimics
- Immunisation studies were done on all these

Cripto
- Peptides 17mer, 37mer
- Production of Whole Protein – in progress
- Characterisation of Cripto antigen
- Successful production of inhibitory monoclonal antibodies
- Immunisation studies for T cell induction

Pim-1
- Whole protein
- Successful production of inhibitory antibodies
- Immunisation studies for T cell induction

nm23
- Protein
- Monoclonal Antibody production for characterisation
- Immunisation studies

p53
- Peptides

Her2/neu
- Peptides

Antigens not studied:
1. Bax & Bcl2
2. Cathepsin
3. Ampheregulin
4. EGF-R

The reason why we did not study these:
1. Recombinant material could not be produced in sufficient amounts for studies.
2. Further study indicated tissue distribution not sufficiently specific for breast cancer to warrant continuing.
3. Insufficient time to include this in detail with a concentration on the above

Task 2. Study of different delivery systems:
In vivo immune response to mannan and non-mannan conjugated materials in mice and ex vivo studies.

- Mannan, in vivo
- Ex vivo
- Prime Boost with vaccinia
- Beads
- Antennapedia peptide

Task 3. In vivo challenges
Inbred mice and HLA-A2 mice
- CTL/gIFN Elispot responses
- Tumour challenge

Further details of work undertaken for Task 1

MUC1: - The initial studies with MUC1 involved the use of the repeat region of 20 amino acids (VNTR), but later studies extended this to include glycosylated peptides from the VNTR, peptides of the VNTR, mutated peptides from this region, mimics, but studies were also performed outside the VNTR using whole MUC1 as HMFG (Human milk fat globule) and the N-terminal region of MUC1 as a fusion protein. The point of doing this was that MUC1 has numerous epitopes – not only those within the VNTR region but outside this region too. Since the whole MUC1 molecule is over-expressed in breast cancer it was reasonable to study other regions as these could also serve as targets for immunotherapy.

These reagents were produced either as recombinant proteins or as synthetic peptides and used to immunise mice to determine their immunogenicity.

- HMFG, whole MUC1

HMFG was isolated from human milk and coupled to mannan. Mice (BALB/c, C57BL/6 and HLA-A2 transgenic mice) were immunised. Strong CTL and CTLp were induced to regions outside that of the VNTR (as well as within the VNTR). Epitopes for H-2d, H-2b and HLA-A2 were mapped.

The figures show CTL responses in BALB/c and C57BL/6 mice specifically to peptides within the VNTR and outside the VNTR region of MUC1. High CTL precursor
frequencies were detected in these mice as well as in HLA-A2 transgenic mice (see table below). This work shows promise that CTL can be generated to outside that of the VNTR and we are starting a Phase I clinical trial (January 2003) to include all of the MUC1 rather than the VNTR region.

<table>
<thead>
<tr>
<th>Restimulating Antigen</th>
<th>Target cell</th>
<th>A2&lt;sup&gt;+&lt;/sup&gt;EBV Bcells+peptide</th>
<th>MCF7 (human breast cancer line)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CTLp frequency</td>
<td>CTLp frequency</td>
</tr>
<tr>
<td>HMFG</td>
<td></td>
<td>1/39,000</td>
<td>1/2,000</td>
</tr>
<tr>
<td>VNTR peptide (30mer)</td>
<td></td>
<td>1/33,000</td>
<td>1/8,000</td>
</tr>
<tr>
<td>Outside the VNTR region</td>
<td></td>
<td>1/40,000</td>
<td>1/2,000</td>
</tr>
<tr>
<td>p31-55</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p344-364</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p471-493</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*This work was published:*

- **N-terminal fusion protein**
  We generated an N-terminal region of MUC1 fusion protein successfully. A monoclonal antibody was generated which was used to further characterise MUC1 expression. Of most interest, was that conjugation of this fusion protein to mannan successfully generated CTL in C57BL/6 and BALB/c mice. This data gives further reason on using other regions within the MUC1 for tumour immunotherapy. Whole MUC1 is been prepared for clinical trial.

- **MUC1 VNTR peptides**
  H-2d, H-2b and HLA-A2 CTL peptide epitopes were identified after mannan-MUC1 fusion protein (of the VNTR region) immunisation. These are summarised below:

  Protein epitopes detected by anti-MUC1 CTLs

<table>
<thead>
<tr>
<th>Restriction</th>
<th>MUC1 VNTR</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-2K&lt;sup&gt;b&lt;/sup&gt;</td>
<td>SAPDTRPAP</td>
</tr>
<tr>
<td>H-2D&lt;sup&gt;b&lt;/sup&gt;</td>
<td>APGSTAPPA</td>
</tr>
<tr>
<td>H-2L&lt;sup&gt;d&lt;/sup&gt;</td>
<td>APDTRPAPG</td>
</tr>
<tr>
<td>H-2D&lt;sup&gt;d&lt;/sup&gt;</td>
<td>SAPDTRPAP</td>
</tr>
<tr>
<td>H-2K&lt;sup&gt;k&lt;/sup&gt;</td>
<td>PDRPAPGS</td>
</tr>
<tr>
<td>HLA-A2</td>
<td>SAPDTRPAP</td>
</tr>
<tr>
<td>HLA-A2</td>
<td>STAPPAHGV</td>
</tr>
</tbody>
</table>

*Note: Other Responder mice = H-2<sup>a</sup> and H-2<sup>z</sup>*

HLA-A2 mice were also immunised with the HLA-A2 9-mer peptides conjugated to mannan. Specific CTL responses were generated (above right), indicating that peptide based immunotherapy is feasible when conjugate to mannan.
Mutant peptides

Mutant peptides were based on the HLA-A2 epitopes (SAPDTRPAP and STAPPAHGV). These peptides were identified to bind to HLA-A2 with low and intermediate affinities respectively. Based on known anchor residues for tight binding of peptides to HLA-A2, mutations were made to STAPPAHGV to make this peptide of higher affinity. STAPPAHGV was mutated to TTAPPVHGL. Mice were immunised with the mutated peptide conjugated to mannan and CTL were generated which specifically lysed MCF7 human breast cancer cell line.

Mimics

We previously demonstrated that a peptide mimic DAHWESWL (H-2d) in mice, mimicked that of MUC1 VNTR peptide, SAPDTRPAP. This work was taken further and mutations were made to DAHWESWL peptide, to make it compatible with HLA-A2 (of relevance to human studies). Successful mutations were made and in particular the peptide DLHWASWV when conjugated to mannan, induced specific CTL responses which lysed MCF7 cells (figure above).

Both mutant peptides and mimic peptides will be used in a clinical trial to begin January 2003 together with VNTR fusion protein and whole MUC1.

Glycosylated peptide

Previous studies from our Institute and elsewhere have used peptides within the MUC1 VNTR region. This region contains highly immunogenic peptides. However, on analysing the data from patients who received mannan VNTR it was clear that large amounts of antibody could be made to the peptides, but not to glycosylated MUC1, not to the patients own tumor. We thought that tolerance was being broken, however, MUC1 peptides do not occur in isolation, they are heavily glycosylated and therefore it is sensible to immunise with glycopeptides. We received a glycopeptide (MUC1-8, SAPDTRPA with the Thr glycosylated with GalNAc) from Dr Henrik Clausen in Copenhagen, Denmark. In addition, According to the crystal structure that Dr Vasso Apostolopoulos determined (published in J Mol Med, 2002) of the 8-mer peptide from MUC1 VNTR which binds to H-2b (peptide SAPDTRPA), it was clear that the Threonine (P5) points down into the peptide binding groove (C pocket), however, a large cavity is formed in the pocket (below: crystal structure of MUC1-8 peptide, SAPDTRPA) in complex with H-2Kb.

Large cavity is formed in the C-pocket, allowing a sugar to fit in this region
We demonstrated both in vitro and in vivo that the MUC1-8 peptide with GalNAc at the P5 Threonine, induce specific CTL responses to the glycopeptide. This is of interest as MUC1 is highly glycosylated on cancer cells and glycosylated MUC1 peptides could be presented by MHC. We are currently expressing whole MUC1 glycosylated, and this will be used in a clinical trial.

**CRIPTO**: Cripto had some appeal as the literature indicated this was expressed in cancers, but largely absent from normal tissue. The strategy involved here was to make a monoclonal antibody to determine the tissue distribution of Cripto and then to use Cripto peptides and recombinant protein to determine its immunogenicity. The results were as follows:

- **Monoclonal Antibodies**
  Although outside the scope of this original proposal, the antibodies were used to examine Cripto expression in breast cancer when it was present in 15/17, and 0/13 normal breast cancers. It was of interest that the antibody was able to inhibit cell growth in vitro and in vivo and is now becoming the subject of a separate study. However for the point of this proposal, the clear definition of high distribution on tumours and virtual absence from normal tissue indicated this is an appropriate target.

There were initial difficulties in producing recombinant protein by transfecting with the whole Cripto molecule, thus, Cripto peptides (17mer and 37mer) were produced and gave the following results (we are continuing with the whole CRIPTO protein production).

Interesting findings have emerged recently with the use of monoclonal antibodies (Mabs) such as Herceptin (anti-HER2/neu humanized Mab.), which is now in clinical trial. This Mab does not act primarily as cytotoxic or by ADCC, but rather bind antigen (HER2/neu) and blocks the binding of a growth factor, leading to interference in cell signalling, and apoptosis. There are some limitations, as Herceptin only reacts with HER2/neu positive breast cancer patients (30%), and in only a portion of those is life extended for few months. We have generated Mabs to CRIPTO, which were initially selected for their growth inhibitory capability - a novel method of screening. CRIPTO is "more" tumour-specific than other growth factors (EGF, Amphiregulin, TGFβ). **We have produced 3 unique Mabs to CRIPTO** [C3 (IgG), C4 (IgM) and C13 (IgM) to a 17 amino acid peptide CPPSFYGRNCEHDVRKE, - residues 97-113 of the human CRIPTO protein]. The Mabs were generated by using an INITIAL screen of GROWTH INHIBITION, and demonstrated that the Mabs activated a JNK/SAPK pathway and induced cancer cell apoptosis, and had significant inhibitory effects in both in vitro and in vivo studies. The Mab specifically bound to breast cancer not to normal breast tissue.

*The inhibitory effect of the Mabs*: The Mabs inhibited by 60-90% growth of the breast cancer cell line MCF7 in tissue culture.
Figure 1. Enhanced sensitivity of MCF7 cells to Cisplatin (Cis) by C4 following 72 hours incubation as measured with [3H]-thymidine incorporation. Points, mean of triplicate experiments, bars, SD.

Figure 2. Inhibitory effect of Mabs C3, C4, C13 and control Mab BCP7 (anti-MUC1 Mab). MCF7 cells (1X10^6) were cultured with the Mabs for 7 days. Viable cells were counted. Columns, mean of triplicate experiments, bars, SD.

Figure 3. Western blot tested by Mab C13 using cell lysates of prostate (DU145 and LNCap), breast (MCF-7), colon (LS174T) cancer cell lines and embryonal kidney cell line 293, showing that C13 specifically detected CR-1.

Figure 4. Inhibitory effects of anti-CR-1 Mabs in SCID mice. SCID mice were inoculated with: (A) 2X10^6 MCF7 cells subcutaneously and treated with C4 (arrows), and (B) with 2.5X10^6 LS174T cancer cells, subcutaneously and treated with C13 (arrows). The tumour sizes (lines) were measured and removed at Day 24 and 25 respectively and the weights were showed (columns). The points, means of tumour sizes; bars, SD.

This is the first time that Mabs to CRIPTO have been shown to inhibit cancer cell growth in vitro and in vivo. This study strongly suggests that CRIPTO is a novel marker and that the anti-CRIPTO Mabs may of value for the cancer therapy in humans, perhaps better than Herceptin.

This work is currently being written for publication

- CRIPTO cellular assays

CRPTO was examined in BALB/c and in HLA-A2 transgenic mice for the generation of specific INF-gamma secreting T cells. Mice were immunised with dendritic cells pulsed with a 17mer CRIPTO peptide or mannan conjugated to peptide. ELISPOT assays were performed for IFN-gamma secreting T cells and specific T cells were generated to CRIPTO-17mer peptide; Con A was used as a positive control stimulus, VNTR was from MUC1 as a negative control. IL-4 secretion by T cells was negative (not shown).

This work is currently being written for publication
**Pim-1**: This antigen had only recently been demonstrated to be present in breast cancer and intracellularly. To confirm this, we produced 3 Monoclonal antibodies to Pim-1 (P3, P4, P9) and confirmed the presence of this antigen inside breast cancer cells (see below for immunoperoxidase staining data). We are currently producing whole protein. We are also in the progress to examine any cellular immunity that can be induced to Pim-1.

![Mab P3](image1.png) ![Mab P4](image2.png) ![Mab P9](image3.png)

**Immuno peroxidase stain on MCF7 cells using Mab generated against Pim-1**

**Nm23**: While this antigen was reported to be overexpressed in breast cancer, we had some concerns about this because of its distribution on normal tissues. We initially opted to make antibodies to determine the tissue distribution. We produce a recombinant fusion protein for nm23 and rats were immunised and polyclonal antibodies were generated against nm23. It became apparent that not only was nm23 present on breast cancer cells but on normal breast tissue too. At the same time however, for the cellular immunity part of the project, demonstrated that specific T cells secreting IFN-gamma could be generated in 3 different mice (B1, B2, B3) after immunisation of nm23 conjugated to beads, (below left) (see below for further details on beads). In addition, mice immunised once with nm23 beads generated high antibody titers (>1x10^6) specific for nm23 (below right).

![IFNg Fresh ELISPOT after one immunization with beads-nm23](image4.png) ![IgG ELISA on beads-nm23 immunized mice](image5.png)

Despite encouraging preclinical data, generation of specific T cells and antibodies in mice immunised with beads-nm23, we decided not to continue with the use of nm23 for a target for immunotherapy for clinical use, due to its widespread distribution on normal breast tissue.

**p53**: We generated peptides which had been identified as HLA-A2 binding epitopes from p53 protein. The peptides were: STPPPGTRV (149-157) and LLGRLSFEV (264-272). Peptides were conjugated to mannan and specific CTL were generated. These peptides will be included in a clinical trial to begin early 2003.
**Her2/neu:** - We generated peptides which had been identified as HLA-A2 binding epitopes from Her2/neu. The peptides were: KIFGSLAFL (369-377) and VMAGVGSPYV (773-782). Peptides were conjugated to mannan and specific CTL were generated. These peptides will be included in a clinical trial to begin early 2003.

**Antigens not studied further:** - As indicated earlier, a number of antigens were not studied further as the clones did not produce recombinant material eg. bax and bcl. Secondly, we were not completely convinced of the over-expression in breast cancer as compared to normal tissue eg. FR – even though this is a target for monoclonal antibodies C225 at present – the EGFR was also extensively expressed on normal tissue and the same applied to Ampherregulin. Cathepsin is still on the list, but essentially ran out of time studying MUC1, Cripto, Pim-1 and nm23 and the other MUC1 antigens listed above.

In conclusion, a number of antigens were originally identified as their reported over-expression in breast cancer compared to normal tissues. Further studies we conducted with antibodies indicated this was not the case for many of the antigens e.g. nm23 and technical difficulties were encountered in producing certain proteins. However, sufficient amounts of antigens (MUC1 - a variety, Cripto, Pim-1, nm23, p53, Her2/neu) so that 13 different antigens were examined (see below) – while not all were taken successfully to completion, our preclinical data indicates that MUC1 and the many variants would be suitable targets as would be Cripto for inducing T cell responses. We noted as we did these studies that a number of antigens were identified through gene array and other studies which included Claudin, telomerase and survivin, all of which we are currently studying.

**Task 2.** In vivo immune response to mannan and non-mannan conjugated materials in mice and ex vivo studies.

**Further details of work undertaken for Task 2**

Mannosylation Vs other methods of immunisation. While the original program used oxidised mannan, analysis of our clinical results gave a disappointing lack of responsiveness of tumors. Admittedly, the patients had advanced disease, but they did make antibody and CTL responses - albeit the latter were fairly weak. We were concerned that the mode of immunisation while superb in mice was not appropriate in humans and we are moved in 4 different directions (see below).

For the MUC1 studies, satisfactory immune responses were found in vivo with most of the MUC1 variants. However, mannan was not highly satisfactory with some of the other antigens eg: listeriolysin, wherein antibody responses seemed to be preferentially induced. Several studies were performed to determine ways around this: i) examining the use of mannan ex vivo and ii) comparing the mannan results with that obtained with other novel methods in the laboratory such as prime boost or polystyrene beads or antennapedia.
i) The ex vivo studies: - Dendritic cells and macrophages express high levels of mannose receptor. Thus, it would be appropriate to use mannan conjugates to load dendritic cells or macrophages, and then use to immunise mice. In fact, 1 immunisation of ex-vivo pulsed mannann-MUC1 generates the same CTLp frequency to 3 direct mannann-MUC1 immunisations. **This work led on to a Phase I clinical trial** wherein dendritic cells were isolated from patients with breast cancer, they were pulsed with mannann-MUC1 and injected back into patients. Patients developed strong INF-gamma secreting T cells specific for MUC1, little antibody responses were generated. We are going to start a clinical trial in January 2003, which will include MUC1 variants, whole MUC1, MUC1 with VNTR region, mimic peptides, mutant peptides, CRIPTO 37mer peptide, p53 peptides, Her2/neu peptides using this ex-vivo approach. *This work is being written for publication*

![IFCN 1 Patient M-6 207](image)

![IFCN 3 Patient B-T 208](image)

ELISPOT assay: Two patient responses are shown. Patients were immunised with dendritic cells pulsed with mannann-MUC1 fusion protein (from VNTR region). Samples X-1 are prebleeds, X-2 after 1st injection; X-3 after 2nd injection; X-6 3 months after the 3rd injection. Specific MUC1 VNTR responses are generated.

ii) The prime boost studies: - We compared the immune responses generated to mannann-MUC1 as to prime boost studies. Mice were immunised with mannann-MUC1 and either boosted with mannann-MUC1 or vaccinia virus MUC1. Cellular immune responses to MUC1 peptides were generated, although this method was not better than ex-vivo use of dendritic cells with mannann-MUC1.

![IFN gamma](image)

Mice immunised with mannann-MUC1 (MFP) and boosted with vaccinia virus MUC1. INFgamma responses are lower to those generated with DC pulsed MFP. Peptide 1+2 gave statistically significant results, peptide was SAPDTRPAP from VNTR.

iii) The beads technology: - We also determined the role of beads in the immune response. Beads were conjugated to MUC1 fusion protein by covalent linkage and used to immunise mice. One immunisation was strong enough to generate a strong and specific immune response (INFgamma secreting T cells). This work has led onto other studies, whereby, strong cellular and humoral immunity is generated in mice against a variety of antigens, such as MUC1, malaria, ovalbumin, etc. This work led to a patent and a publication which has been

![One dose](image)

Mice were immunised with bead-MUC1 once. Strong INFgamma producing T cells are generated (red column). The other two are controls. B1, B2, B3 represents 3 different
submitted to Nature Medicine;  
This work has been submitted for publication

iv) The antenapedia studies: Another method we developed to obtain strong cellular responses in vitro and in vivo to the model antigen ovalbumin is the use of peptides with cell membrane translocation activity. The Drosophila Antennapedia DNA binding domain contains 60 amino acids and consists of 3 α-helices, the region responsible for internalisation having been mapped to a 16 amino acid peptide (RQIKIWFQNRRMKWKK) within the third. Antennapedia protein/peptides have been used in studies in drug delivery but not cancer vaccines. We have utilised a tandem peptide based approach. We initially immunised mice with a synthetic peptide incorporating the CTL epitope peptide SIINFEKL of the model antigen ovalbumin (OVA) linked to the antennapedia sequence (Published). These mice produced strong CTL to OVA and protected the mice from a lethal dose of OVA+ EL4 tumour cells.

We further extended these studies to make similar peptides utilising identified and predicted CTL epitopes of the MUC1 tumour antigen. Several H2 and HLA-A2 CTL restricted epitopes have been characterised for MUC1 and we have fused these in tandem to the antennapedia peptide. These peptides were used to immunise HLA-A2K×xMUC1 transgenic mice. Splenocytes from the mice immunised with antennapedia tandem peptides containing STAPPVHNV (non-VNTR epitope) and STAPPAHGV (VNTR epitope) gave high levels of IFN-γ (see figure). The amount of IFNγ was far greater than that obtained in C57BL/6 mice immunised with MFP (15 dots / 0.4 million spleen cells for 1 injection and 39 for 3 injections, not shown). Moreover, these levels of IFNγ were obtained in transgenic mice expressing MUC1 using only a single injection of 100 μg (c.f 3 injections for MFP).

This work was published: Pietersz, G.A., Li, W. and Apostolopoulos, V. A 16-mer peptide (RQIKIWFQNRRMKWKK) from antennapedia preferentially targets the Class I pathway. Vaccine, 19:1397-1405, 2001.

We are also in the process of writing a second paper on the findings with antennapedia peptides.

Measurement of immune responses: While our initial proposal planned to measure antibodies and cellular responses by way of proliferation and CTLs, we have developed a far more suitable method for measuring cellular responses: ELISPOT assays detecting IFN-gamma production by either CD4 or CD8 cells. These tests can be done immediately, rather than after days of in vitro culture, and give an answer on the status of total cellular immunity, as opposed to CTLs which often takes weeks to obtain an answer, and are limited to one effector mechanism. Thus, we did ELISPOTs to determine specific T cell responses to the immunising antigen. ELISPOT assays are shown above.
Task 3. In vivo challenges and tumor protection

Further details of work undertaken for Task 3

As we only have mouse tumor models only for MUC1 or CRIPTO positive cells, we did tumor protective challenges in mice immunised with either MUC1 or CRIPTO antigens. We have MUC1 mouse tumor lines (P815, RMA or 3T3 transfected with human MUC1); as we do not have an equivalent for CRIPTO, as only human cell lines were positive for CRIPTO, SCID mice were used for tumor experiments whereby, tumor bearing mice were injected with anti-CRIPTO inhibitory antibodies (see above, CRIPTO results). We have demonstrated that CTLp or INF-gamma secreting cells correlate with tumor protection, thus, Elispot assays were done to measure immunity for all antigens and antigen formulations (Representative elispot assay results are shown above under each section).

Ex-vivo studies: - Ex vivo pulsing of mouse dendritic cells pulsed with mannan-MUC1 and injected into tumor bearing RMA-MUC1 cells, effectively reduces the tumors, whereas mice given DC alone as control, the tumors grew (below left). In addition, we were able to generate specific MUC1 T cells in HLA-A2 transgenic mice after immunisation with dendritic cells pulsed with mannan-MUC1. The T cells were able to specifically lyse MUC1+ tumor cells MCF7 (below right); HT20 which are MUC1+ve cells but HLA-A2 negative was not lysed by the CTL.

The antennapedia studies: - Splenocytes from the mice immunised with antennapedia tandem peptides containing STAPVHNV (non-MUC1VNTR epitope) and STAPAHGV (VNTR epitope) gave high levels of IFN-γ (see figure above - task 2). In addition mice were immunised with antennapedia tandem peptide linked to SAPDTRPAP peptide (a H-2b epitope) from MUC1 VNTR region. Strong CTL responses were generated specific for RMA-MUC1 target cells; RMA cells were negative (see below left). Mice were challenged with RMA-MUC1 cells after being immunised. Immunised mice were protected, whereas, non immunised mice were not protected (below right).
The beads studies: - After one injection of beads conjugated to MUC1 fusion protein in C57BL/6 mice, strong IFN-gamma secreting T cells were generated (shown in task 2 above). In addition, mice were protected against a MUC1 tumor challenge (RMA-MUC1).

In progress: For *in vivo* studies using HLA-A2 restricted peptides, HLA-A2/Kb transgenic mice will be immunised and the splenocytes adoptively transferred into SCID mice bearing subcutaneous human MCF-7 tumour xenografts in order to monitor effects on tumour growth. At the end of the experiments, the lymphocytes will be assayed *in vitro* for antigen specific CTL activity and cytokine release.

**Future work:**
Considerable progress was made in identifying different antigens and examining the mannan and other delivery systems. The current work has served as preclinical studies for targeting dendritic cells *ex vivo* and current and future work includes:

- Ex vivo trial in patients exposed to mannan MUC1
- Ex vivo trial in patients exposed to mannan-other antigens will begin
- Examination of the bead technology as a potential for clinical trial
- Examination of the antennapedia technology as a potential for clinical trial
- Determining whether multiple antigens used together have any deleterious effects or whether they have additive or synergistic value
- Inhibitory Mabs to CRIPTO, a clinical trial will begin to determine its therapeutic benefit
KEY RESEARCH ACCOMPLISHMENTS

- The use of ex vivo pulsed dendritic cells pulsed with mannan MUC1
- Ex vivo trial in patients exposed to mannan MUC1
- Examination of the bead technology
- Examination of the antennapedia technology
- Examination of the prime-boost technology
- Note: The ex-vivo dendritic cells, the bead and the antennapedia technology are unique findings in this grant
- Production of inhibitory Mabs to CRIPTO
- The use of a new antigen expressed in breast cancer, Pim-1
- The determination that nm23 is not tumor specific as previously reported

The key research findings

- Ex vivo use of mannan MUC1 to target dendritic cells, in clinical trial.
- Identification of other modes of delivery including beads, the prime boost strategy and the use of antennapedia peptides for delivering
- Production of monoclonal antibodies to Cripto, and Pim-1 (to aid in studies of tissue distribution) – which in themselves appear to be beneficial (inhibitory) and will be the basis for a future clinical trial
- Identification of other MUC1 antigens other than the VNTR which engender potent immune responses in mice and are currently being organised for clinical trial
REPORTABLE OUTCOMES

- The production of Mabs to CRIPTO which appear to be inhibitory have been patented.
- The production of Mabs to Pim-1 which appear to be inhibitory have been patented.
- The bead technology has now been patented
- The ex-vivo technology has been patented
- The bead technology manuscript has been submitted to Nature Medicine
- The CRIPTO Mabs are being written for publication
- The clinical trial using the dendritic cells pulsed with mannan-MUC1 is being written for publication
- The antennapedia technology has been published (see above) and another paper is being written for publication
- The first identification that epitopes for MUC1 exist outside the VNTR region was published (see above).
- The cellular immune responses generated to CRIPTO is being written for publication
CONCLUSION.

A number of different antigens were examined for their potential to be targets for immunotherapy and several identified including MUC1 and its variants; Cripto and possibly others. The studies were extensive and need to be proven in clinical trial. However, many other antigens remain to be tested. An unanswered question is whether 2 antigens are better than one – one would expect this to be the case, but there is the potential for some cross reactivity and cross inhibition which has been reported in other systems. Our own feeling is that more antigens are better as it would cover more cells in each tumour and more tumour cell types – irrespective of the HLA phenotype and pathological features of the tumor.

Although many antigens have now been identified, one of the key problems is how to deliver these to the immune system to generate an effective immune response. Indeed, this is the major problem in immunotherapy at present and this is moreso in patients with cancer wherein their disease leads to immunosuppressive environments. On the basis of this, we also examined the ex vivo use of mannan antigens and this was so effective, we are now going on to clinical trial in this area with other antigens (other than MUC1). In addition, targeting dendritic cells was shown to be very important and a strategy devised to do this directly. The findings of the study were such that patents have been lodged, a commercial company in Australia has licenced some of the technology and clinical trial either in progress or soon to commence.