

## The Nature and Function of Immunogenic Tumor Proteins That Characterize Pancreatic and Colorectal Cancer: A Review

Arlen M<sup>1,3\*</sup>, Arlen P<sup>3</sup>, Crawford J<sup>2</sup>, Coppola G<sup>1</sup>, Saric O<sup>3</sup>, Bandovic J<sup>2</sup>, Doubakovski A<sup>3</sup>, Conte C<sup>1</sup>, Wang X<sup>3</sup>, Molmenti E<sup>1</sup> and Hollinshead A<sup>3</sup>

<sup>1</sup>Department of Surgery, NSUH, Manhasset NY, USA

<sup>2</sup>Department of Pathology, the Hofstra University College of Medicine, Hempstead, NY, USA

<sup>3</sup>Precision Biologics, Great Neck NY, USA

\*Corresponding author: Myron Arlen, MD, Co ordinator Surgical Oncology, North Shore/LIJ Medical Center, Surgical Oncology, 450 Lakeville Rd., Lake Success, NY 11042, USA, Tel: 1-516-482-1200; E-mail: [marlenmd@msn.com](mailto:marlenmd@msn.com)

Received date: May 04, 2015; Accepted date: October 28, 2015; Published date: October 30, 2015

Copyright: © 2015 Arlen M, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

### Abstract

The use of immunotherapy as it relates to the treatment of solid tumor malignancies appears to be an effective approach for managing many patients who present with both recurrent and metastatic disease. This holds true, especially in those cases where patients have failed current chemotherapeutic protocols and evidence of tumor progression is noted. In such instances, the delivery of the proper immunotherapeutic agents may be effective either alone or in combination with chemotherapy. The ideal approach would be in the identification of an immunogenic protein that characterized and was specific for the tumor system to be treated. One of the first attempts to utilize specific active immunotherapy for treating cancer patients with advanced disease was by Ariel Hollinshead. She produced several vaccine preparations composed of tumor associated antigen (TAA) derived from pooled allogeneic tumor membrane preparations. These vaccines, obtained from operative specimens were shown to exhibit varying degrees of improvement in the overall survival of such patients. They were employed in treating patients with advanced malignancies including those having lung cancer, colon cancer and malignant melanoma. For most who received the vaccine following resection of an advanced disease process, survival results demonstrating 80-90% freedom of disease at 5 years could be achieved. These results were considered significant when compared to other therapeutic protocols available at the time. Further use in clinical trials were held back at the suggestion of the FDA due to possible viral contamination in the next set of vaccine preparations if any tumor specimen used contained the possible presence virulent strains of virus such as hepatitis, AIDS and HPV. At this point recombinant vaccines were felt to be essential if such vaccines were to be used in future clinical trials. Monoclonal antibodies were therefore developed against each of the pooled vaccine preparations and used for affinity purification and sequencing of the antigens. In reviewing our survival data results it became apparent that those who failed therapy were patients unable to mount an effective IgG1 response and not related to the presence of CD8 T cells. The mAbs were now produced in GMP format and clinical trials as such were initiated for patients with recurrent colon and pancreatic Ca having failed standard chemotherapy.

**Keywords:** Monoclonal antibody; Vaccines; Tumor immunity; Pancreatic cancer; Colon cancer

### Introduction

Much of the original work with the newly developed TAA vaccines of Hollinshead [1], utilized background information derived from Prehn's experiments with animal tumors [2,3]. He had clearly shown that for a tumor vaccine to be effective, it had to be delivered at the proper threshold level as well as be specific for the tumor system it was being employed for. Hollinshead was faced with defining which protein component from pooled membrane proteins was essential for inducing an immune response. In an attempt to resolve this issue, a preparation of solubilized membrane antigens were passed over a Sephadex G-200 gel column and group of proteins were then separated and fractionated by molecular weight. To determine which of these antigenic groups contained the proper immunogenic material for use in the vaccine preparation, patients with the disease of concern (colon cancer, lung cancer or melanoma) as well as other cancers were then skin tested with material obtained from the Sephadex preparations, for signs of delayed hypersensitivity reactions (DHR). Normal volunteers were also

skin tested to confirm the specificity of the antigens being separated. The tumor proteins defined by DHR, were further separated at a later time by discontinuous polyacrylamide gel electrophoresis. Regional bands obtained from these gels were skin tested again in order to narrow down the relatively purified preparation that would eventually be employed in the immunization process. It was important, that once a relatively purified preparation of antigen was obtained, that the level of TAA necessary to turn on the full immune response in patients be defined.

A series of quantitative antigen preparations were delivered intradermally, with cell and humoral responses being evaluated [4]. It was determined that a level of 1000 µg was the apparent dose to employ for immunization to achieve a therapeutic response. Because the antigen preparations were produced from solubilized material, it was found to dissipate quickly from the immunization site in 24 h. It was therefore necessary to employ an oil based adjuvant in which the tumor protein could be homogenized and remain at the immunization site for a prolonged period of time. After looking at all potential vehicles in which to deliver the antigen, complete Freund's adjuvant (CFA) was chosen and eventually approved by FDA for use. It was felt that since the antigen preparation represented tumor specific protein

with no evidence of cross reactivity to normal tissue, such a preparation would only target malignant cells and spare surrounding normal tissue. In none of the patients who received the vaccine plus adjuvant for lung or colon cancer were there any instances of pneumonitis or colitis that could be demonstrated [5-7].

On completion of the colon and lung immunotherapy trials in 1988, FDA was approached with the possibility of employing this form of therapy in more extensive trials with the goal of commercialization, considering the beneficial responses that had been achieved in the clinical studies. They suggested that for any further vaccine preparations or for new clinical trials to be initiated, one would have to provide the antigen in the form of recombinant protein. This was to obviate any problem that could arise from the use of pooled allogeneic membrane material, as noted above, due to the potential contamination of a donor tumor specimen with AIDS, Hepatitis C and HPV.

In order to further define the structure of the antigen to be used in the vaccine preparations as a means of developing a needed recombinant product, FDA requested development of monoclonal antibodies (mAbs) for affinity purification of the original membrane preparation. Following this, the necessary protein sequencing could then be accomplished [8]. Monoclonal antibodies were developed to provide the purification procedures. Hybridomas were produced utilizing the original tumor antigen preparations. The monoclonals so derived from these preparations were tested and found to be extremely specific to the neoplasm of interest. Three monoclonal antibodies (mAbs) were developed from the colon cancer antigen, each targeting colon cancer along with pancreatic cancer immunogens found within the tumor cells. There was no cross reactivity to the surrounding normal tissue by immunohistochemistry (IHC). These mAbs were termed NPC-1, 31.1 and 16C3. They were found to react with

corresponding tumor proteins which proved to be oncofetal in origin. In the tumors examined, these immunogenic molecules appeared to represent post translational modifications of the original proteins found in the fetal state following demethylation of the gene whose product was essential for fetal development.

### Monoclonal Antibody Development

The pooled allogeneic protein vaccines that were given to patients in the original trials as immunotherapeutics were also used to help in the development of those monoclonal antibodies necessary for protein identification and characterization. Tumor proteins were injected into BALB/c mice to initiate the immunization process. Approximately 100 µg of membrane protein were admixed with complete Freund's adjuvant and were then injected into the flanks of the female mice. This was followed by three booster shots of approximately 50 µg separated by 2-3 weeks. Three weeks after the final immunization, the mouse serum was tested by enzyme linked immunoabsorbent assay (ELISA) for an antibody response. Splenic B cells were removed and fused with the SP2/-Ag14 myeloma cell line to produce the hybridomas necessary to provide cells to develop in culture. Single cell clones were selected after approximately 2-3 weeks based on robust reactivity to the immunogen used. Figure 1 illustrates the protocol employed. Hybridoma culture media was tested for expression of the mouse IgG against both the immunizing antigen and cell line extracts such as COLO-205 and LS174T. The monoclonal antibody, most reactive to colorectal and pancreatic cell lines proved to be what we termed NPC-1. This monoclonal was specific for identifying tumor protein expressed in most colorectal and pancreatic cancers by IHC (immunohistochemistry). The protein could be defined in both cell lines as well as tissues removed at surgery from similar malignant lesions.

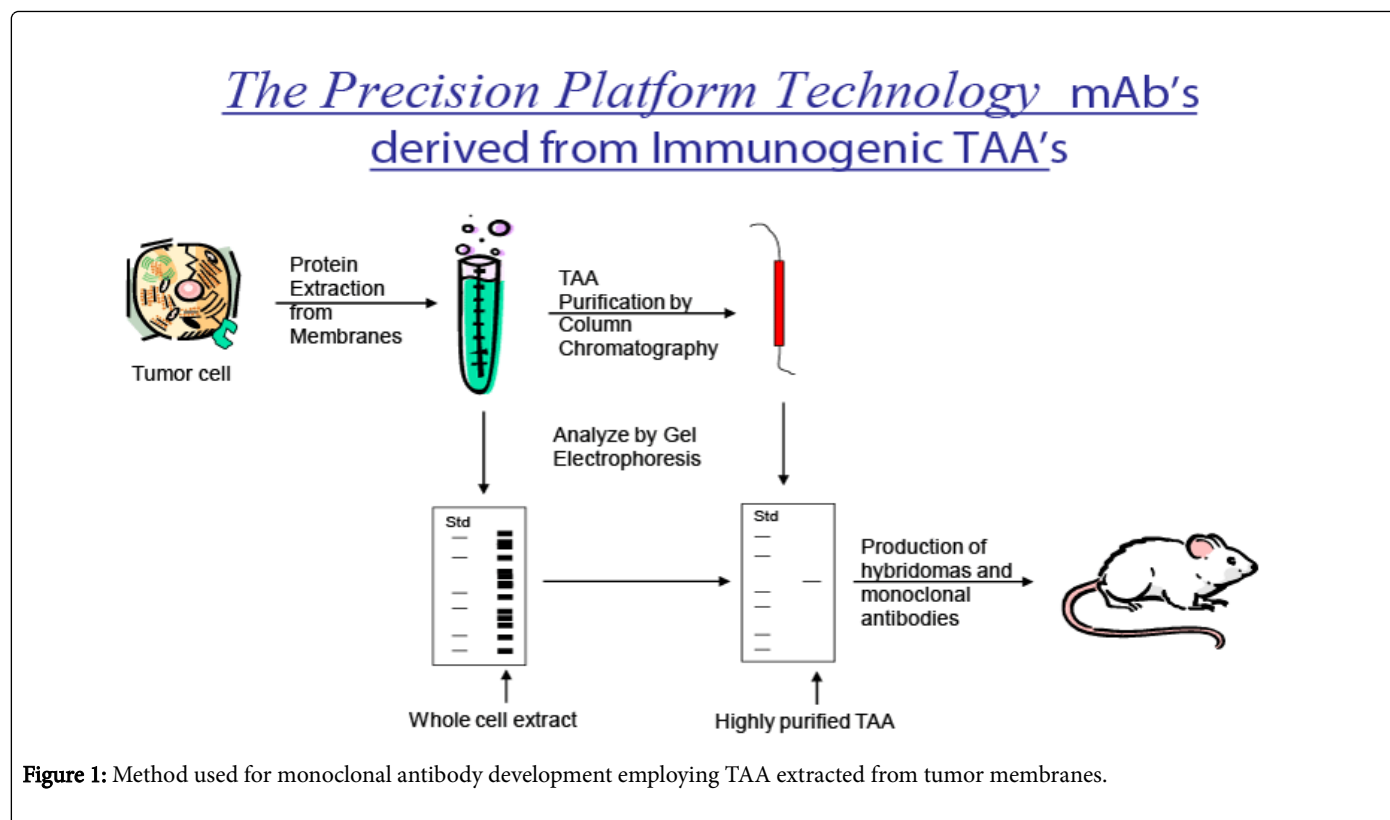
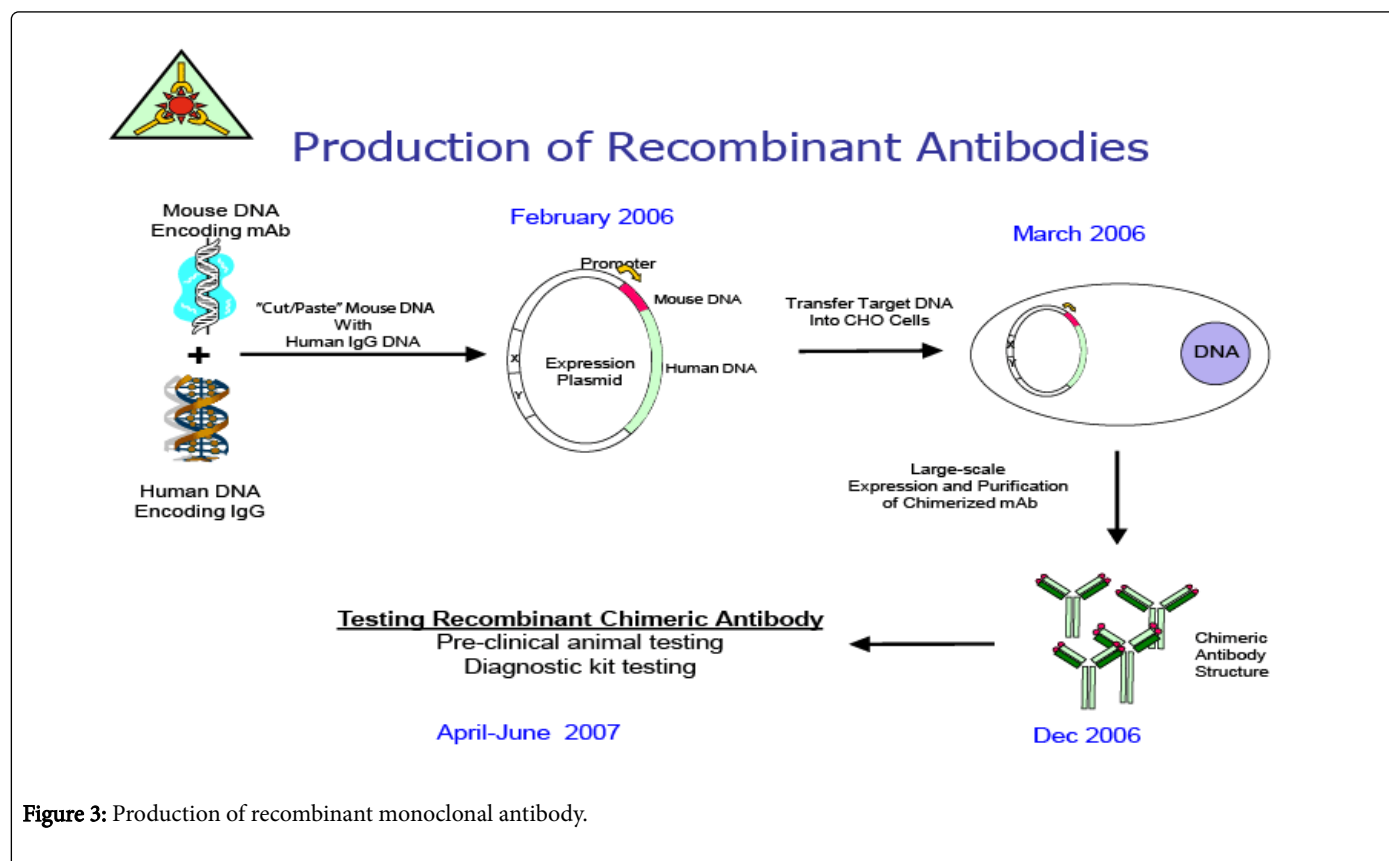
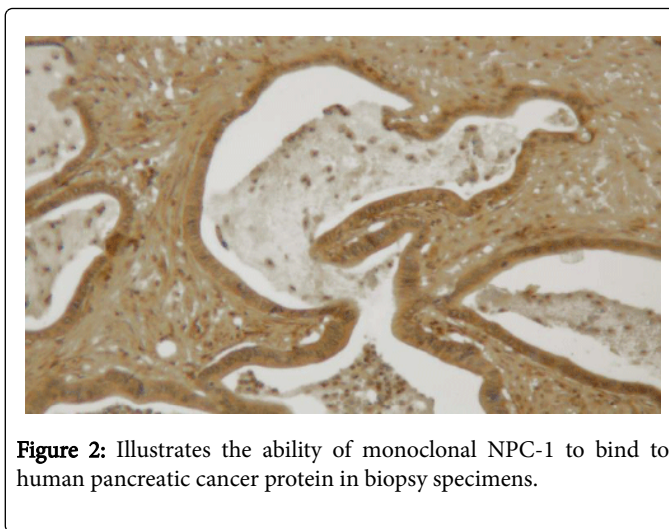


Figure 1: Method used for monoclonal antibody development employing TAA extracted from tumor membranes.

The nucleotide sequence for the heavy and light chains were then determined. Following this, RNA was extracted and the cDNA library was synthesized using reverse transcriptase. PCR was used to amplify the specific heavy and light chain variable regions using primers specific for mouse IgG1 KAPPA genes that hybridized to CH1 and CL domains. The amplified DNA fragments were then cloned into a TOPO vector. After determining the sequences of the variable regions, new primers were designed in order to generate the full length sequences of the HC (heavy chain) and LC (light chain). These sequences were found to be unique by employing a BLAST database search. For each of the heavy and light chains, molecular engineering was used for fusing the mouse variable regions in-frame with the human HC and LC IgG1 constant regions. A mammalian expression vector was used to transfect the genetic material into a Chinese Hamster Ovary (CHO) cell line. Two other monoclonals defining colorectal and pancreatic cancer, 16C3 and 31.1 were characterized in the same way and prepared for clinical use by GMP production. Figure 2 illustrates the immunohistochemistry response to this antibody reacting with the tumor antigen as seen in pancreatic cancer biopsies.



**Figure 3:** Production of recombinant monoclonal antibody.

We became cognizant of the fact that an important step in the production of monoclonals to be used for possible therapeutic purposes in humans, should they prove to have antitumor activity, was to have functional molecules capable of tumor destruction at least by antibody dependent cell cytotoxicity (ADCC). Here because of the need of the antibody to attract CD16 cells, binding them to receptors on the human Fc, of a chimeric molecule became the first step in confirming efficacy followed by initiating clinical therapeutic trials. This structural improvement in the murine monoclonal was then followed by chimerization and eventual complete humanization of the antibodies minimizing any potential for HAMA. We as such needed to

fuse the DNA from the mouse Fab with the human Fc for production of the initial chimeric format for further evaluation (Figure 3). The V regions for both chains of the mAbs were cloned by inverse polymerase chain reaction (PCR) with primers matching the known constant region sequences of the mAb. After sequencing, PCR fragments corresponding to the V regions of both chains were inserted in-frame into appropriate expression vectors leading to mAbs with unaltered N-terminal sequences after expression in CHO. Transfection of the cDNA into CHO cells with the proper high expression vector was found to be capable of yielding the needed levels of antibody required for clinical trials.

Our initial system produced approximately 140 mg/L of antibody Neo-101, before purification. Using a new vector system obtained from the Selexis SA, Zurich, we obtained an enhanced yield of approximately 2000 mg/L of bioreactor fluid for monoclonal NPC-1 now termed Neo-102. The development of the Selexis SUREtechnology Platform™ proved that it was possible to generate stable and high performing manufacturing cell lines in approximately 3 months with productivity levels around 2-5 g/L; thereby expediting entry into CMO manufacturing while reducing the need for larger bioreactor capacity.

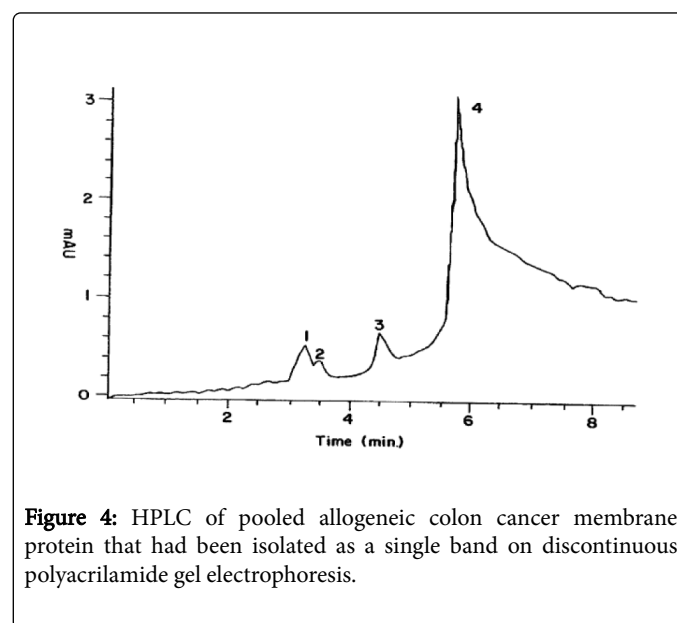
CHO cells were used for transfection in order to produce stability for antibody production. We found that if antibody development was left in the hybridoma state, even for diagnostics where the murine version functioned best, that many of the IgG's so derived were eventually found to have mutated with loss of their activity. Murine antibodies as such are now produced in CHO cell lines.

At this point in time, and of considerable interest to us, was a talk delivered by Lee Hartwell (Fred Hutchinson Cancer Center) at ASCO in 2006. He presented data to support the concept of the role that the ideal antibody could play in the diagnosis and management of a malignant lesion. He presented data that his group had obtained in a review of treated cancer patient over a 10 year period time. It regarded the survival of patients with metastatic solid tumor malignancies who had undergone treatment with various chemotherapeutic agents in an attempt to improve survival. This review suggested that forgetting toxicity, there were few if any cures that could be identified and attributed to the use of chemotherapy. He suggested that a more reasonable approach would relate to early histochemical analysis of transforming cells associated with the malignancy. This approach might then result in the needed improvement sought after in a manner that was achieved following use of the Pap test in cervix cancer [8,9]. He stressed the need for isolating and identifying specific tumor proteins that could characterize each of the major malignancies in their earliest stages of development. By using monoclonal antibodies to identify these proteins, earlier intervention in treatment could be achieved. The result would be seen in improvement in survival that he hoped for. The use of proper antitumor monoclonal antibodies in a diagnostic Immunohistochemical (IHC) assay for solid tumor malignancies would in effect accomplish what the Pap smear did to achieve improvement in cervical cancer survival.

In looking at the specificity of our monoclonal antibodies that were derived from and as such targeted tumor immunogenic proteins, it was felt that they fit into the class of tumor monoclonals that Hartwell had alluded to. We considered that with the proper studies that we could anticipate early detection of many malignancies, even in their premalignant state. This would then have the potential to improve patient survival outcomes as Hartwell had predicted could happen. Norton (Sloan Kettering) at the same meeting, suggested that if the cancer proteins that Hartwell was seeking to define were also immunogenic, then antibodies targeting these proteins could serve both to diagnose the presence of the cancer cell early in its development, and when the antigen was identified, delivery of the monoclonal intravenously could hunt, seek and destroy the neoplasm. As such we have learned that the ideal monoclonal is one that can both diagnose the presence of a tumor even in its earliest developmental phase and then go on to hunt, seek and destroy the tumor when delivered intravenously.

## Tumor Antigen Characterization

It appeared important at this point to define the nature of the tumor antigens derived from the pooled allogeneic material of Hollinshead, especially since we had produced monoclonals necessary for protein characterization and identification by affinity purification and mass spectroscopy. It was obvious that even though a single antigenic band was obtained by discontinuous polyacrylamide gel electrophoresis of a sonicated tumor membrane preparation, that several antigens had probably migrated to the site defined on the gel. HPLC of this material showed at least 4 peaks with a dominant protein comprising more than 50% of the antigen along with at least 3 other sub peaks (Figure 4).



**Figure 4:** HPLC of pooled allogeneic colon cancer membrane protein that had been isolated as a single band on discontinuous polyacrylamide gel electrophoresis.

In order to define the composition of this antigenic material and which peak comprised the primary immunogenic component, the monoclonal antibodies that had been developed, were used to characterize these antigens. Peak 4 seen in Figure 4 reacted with monoclonal NPC-1 (now termed Neo-102); peak 3 with monoclonal 31.1 and the first 2 peaks inter reacted with the antibodies defining 16C3 antigen. With further purification, these proteins were isolated, and characterized. When analyzing the frequency of expression of the different antigens within tumors examined, monoclonal NPC-1 in particular was found to detect tumor specific antigen in more than 70% of the colon and pancreatic Ca's studied. mAb 31.1 was expressed in less than half of the cases examined, but 16C3 protein which represented a CEA derivative, was present in most of the cases but at low levels. In Figure 5, a gradient polyacrylamide gel study indicated that the antigen targeted by monoclonal NPC-1 was approximately 600 kd in size which was confirmed by a similar band identified by Coomassie blue.

Each of the antigens that were identified and analyzed, first by IHC and then by affinity purification, appeared to be oncofetal in origin, functioning in the fetus to allow maturation of the organ in which the protein functioned. In the case where antigen was defined by the inter reaction with the NPC-1 monoclonal antibody, the active protein expressed in the fetal state proved to be the MUC5ac protein which served to induce production of needed mucin in the developing GI tract. Just prior to the fetus completing its prenatal period of development, the gene producing this oncofetal protein appears to be



remethylated, ending the need for the function of that gene. Should the gene for MUC5ac not be properly remethylated, the infant is then born with cystic fibrosis. Later on in life, it appears that there is a mutation in the MUC5ac gene which becomes reactivated in the cancer cell resulting in the expression of a post translational modification of the immunogenic oncofetal protein. In the malignant state, this mutated protein serves as an immunogen, helping the tumor to function in its malignant capacity. When our monoclonal is tested against the fetal MUC5ac, no interaction can be demonstrated and similarly when the commercial preparations of mAb used to study cystic fibrosis are used to stain the post translational form of antigen, no inter reaction can be demonstrated. This appears to hold true for our other monoclonals defining their oncofetal components.

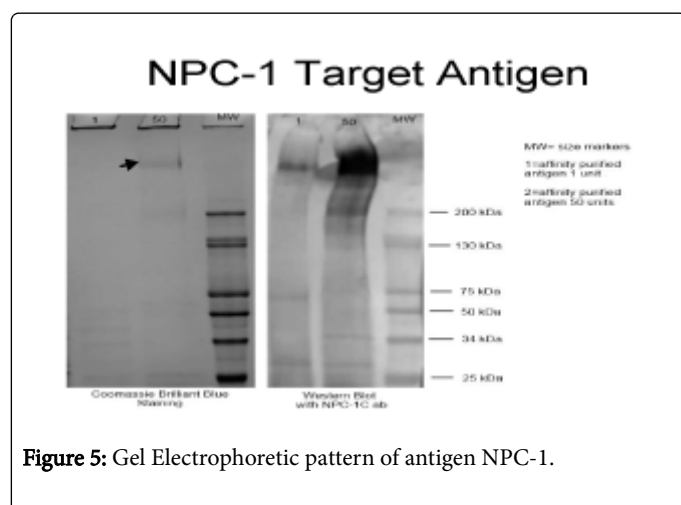


Figure 5: Gel Electrophoretic pattern of antigen NPC-1.

Unfortunately the expression of this new modified tumor antigen and its associated tumor proteins in the tumor cell milieu is considered too low to allow the host to mount an effective immune response. For most malignancies, among the many surface proteins expressed by the lesion, the tumor immunogen is rarely present at more than 25-50  $\mu$ g as measured in the original antigen preparation derived from pooled colon tumor membranes, a level far below what the host immune system needs for host identification. This same concept was supported with the animal vaccine preparations of Prehn as noted previously and where tumor surveillance existed. To see an effective immune response, a threshold level of antigen is required in the form of a vaccine. This has proven to be 1000  $\mu$ g delivered in 3 divided doses monthly. Within 3-4 months we have determined that titers of specific IgG1 are produced that appear to hold the host tumor in check or essentially prevent recurrence when a high risk malignancy has been resected. We have measured the presence of the IgG at up to 20 yrs. post immunization.

We had the opportunity to test the other monoclonals that we had developed against several of the protein bands identified on HPLC. They were similarly found to be oncofetal in origin and represented post translational modifications of those proteins represented by A33 as defined by mAb 31.1 and CEAcam 5,6 as defined by 16C3. Each of the antibodies developed from the tumor immunogens comprising the original TAA vaccine were found to have excellent diagnostic capabilities as well as anti-tumor responses as defined by ADCC.

## Therapeutic Efficacy

After developing those antibodies needed for immunopurification of the tumor antigen preparations, and in anticipation of moving ahead with the development a recombinant tumor vaccine, we decided to return to examine the data obtained from the original Hollinshead clinical trials wherein significant improvement in survival was noted over surgery alone. In lung cancer patients, those receiving surgery without immune enhancement yielded a 20% survival at 7 y. whereas among 130 patients undergoing surgery and immunization, between 80 and 90% survival was noted [10] depending on the institution in which the trial was carried out. It appeared that in the group having failed the stated immunotherapy protocol, that an IgG1 humoral response could not be achieved. We had already produced these mAbs, in the IgG1 format and were now able to test their antitumor response as a way of confirming their role in the process of vaccination. In anticipation of the role these mAbs could play in future immunization trials, they were also humanized for future therapeutic use.

## Animal Studies to Confirm Efficacy

Monoclonal NPC-1 was tested in nude mice injected first with human pancreatic and then colon cancer cell lines. At this point we had chimerized our first group of monoclonals and planned to evaluate their therapeutic effectiveness. When delivering them to the mice intraperitoneally, our hope was to observe resulting tumor destruction. Because we anticipated that the response was a result of probable antibody dependent cell cytotoxicity (ADCC), human effector cells were also given intraperitoneally to assist in tumor destruction since the mechanism behind any ADCC response was related to the monoclonal Fc having receptors binding to circulating CD16 cells and then delivering these cells to the neoplastic growth.

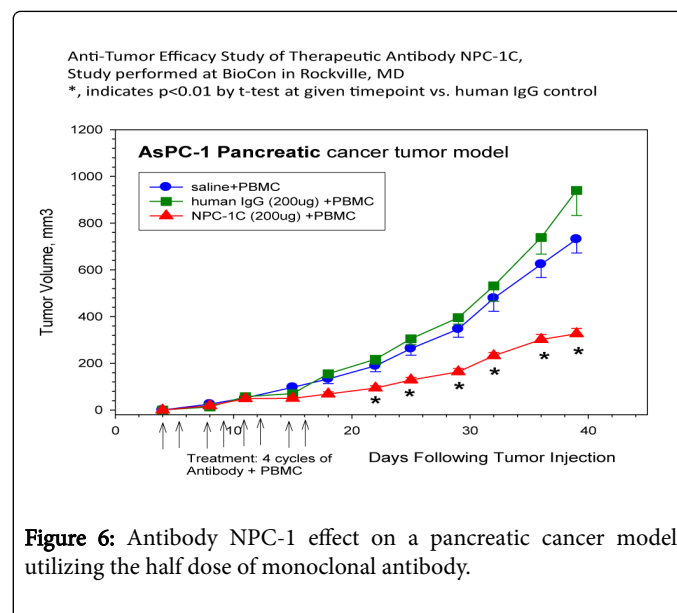


Figure 6: Antibody NPC-1 effect on a pancreatic cancer model utilizing the half dose of monoclonal antibody.

In our initial studies we were able to demonstrate that when the inoculation of human tumor cells (pancreatic as well as colon cancer) resulted in a neoplastic growth reaching 3-4 cm in size, that intraperitoneal administration of 400  $\mu$ g of the chimeric monoclonal antibody with human effector cells would cause approximately 90% of the established tumor to regress. In planned human trials addressing patients with advanced recurrent malignancies, it was felt that an

equivalent dose to be delivered would be in the range of 4-5 mg/kg of body weight if a therapeutic response were to be achieved.

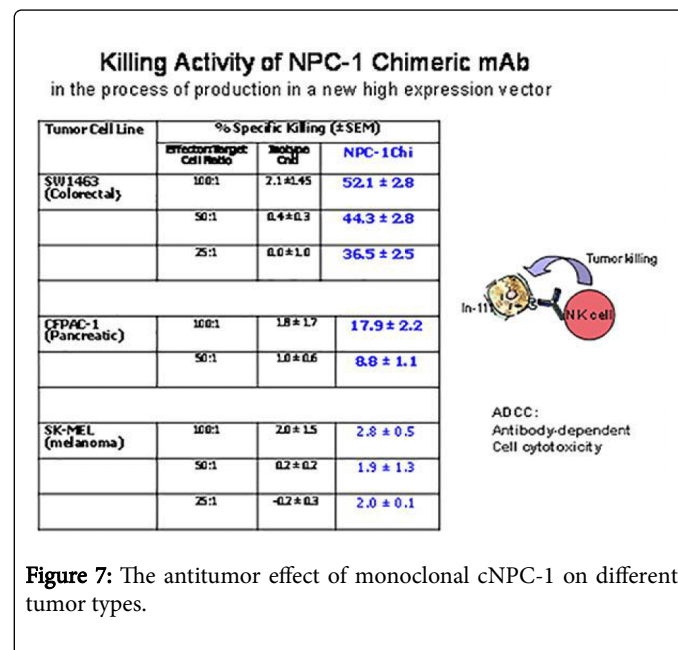
In further antibody studies to confirm antitumor activity, where the antibody was administered at a half dose of 200 µg, following administration of pancreatic cancer cells on day 1 of the experiment, one could compare resulting sizes of tumor as a response to immunotherapy by comparing the effect of saline vs. IgG vs. antitumor NPC-1 (Figure 6).

## Role of the Tumor Immunogen and Humoral Tumor Immunity

From data obtained from the original pooled allogeneic vaccine trial, it was determined that an ideal immunotherapeutic response could be achieved by employing a specific recombinant tumor vaccine. In such clinical trials a decision has to be made as to whether active or passive immunization should be employed. Vaccination when utilized, does result in the enhancement in both cell mediated as well as a humoral response with the appearance of high levels of serum IgG1 which can last for many years; active immunization does however takes 4 or more months to become effective in producing the levels of the IgG1 needed to control metastatic tumor. When treating patients with metastatic disease, especially when all chemotherapeutic protocols have failed, passive immunization appears to be the most plausible approach. Here, the use of one of the specific monoclonal antibody that we have developed, when delivered intravenously, would begin to attack the tumor within 6-8 h after administration. The primary mechanism for the antitumor effect was shown to be through ADCC, where the antibody passing through the circulation is able to attract the CD16-NK cells which bind to receptors on the Fc component of the antibody [11]. When the antibody targets the tumor cell via its Fab, the NK cells delivered to the tumor begin the process of tumor destruction. When considering those patients needing therapy post-surgery such as with pancreas cancer, those undergoing a Whipple procedure have the potential of at least 90-95% for recurrence within 1-2 years post-surgery. Use of monoclonal therapy would not achieve the needed goal. Rather vaccines, inducing the appearance of therapeutic levels of the necessary IgG1 would be needed. We have looked at the molecular structure of the immunogenic proteins and find them too complicated to synthesize except in a linear format. Here we approached the problem using phage display to define epitope binding sites on the molecule. Our antibodies and in particular Neo 102 bind to a 12 mer peptide which when synthesized, is capable of inducing the necessary IgG1 response. In the future we will be treating patients post-surgery with a high level of recurrence with peptide vaccines, leaving the monoclonal passive immunization to patients with active recurrent disease needing a relatively quick therapeutic response.

These monoclonals and in particular the ones targeting the NPC-1 antigen, that represent the mutated or post translational modification of MUC5ac have a high ADCC response especially when targeting the immunogenic proteins expressed in metastatic pancreatic and colorectal cancer. In order for this mechanism to produce an effective result, we have found that the ADCC response should achieve at least a 40% or better tumor kill (Figure 7). CFPAC-1 levels here were noted to be low even at the 100:1 E:T (effector to target cell ratio), but were found to be enhanced in several samples of patients tumor cell lines grown after biopsy and culture. While NPC-1 (mutated MUC5ac) antigen is a common target for treatment, mAb 31.1 which interacts with a mutated form of A33 may not be as frequently expressed, but

has better than a 70% ADCC. When defining the antigens or target proteins present in the tumor, we have noted that several lesions examined by IHC express two targets and as such may be candidates in the future for receiving 2 mAbs during a course of therapy, in a manner similar to employing several chemotherapeutic agents in combination when attempting to bring a malignancy under control.



**Figure 7:** The antitumor effect of monoclonal cNPC-1 on different tumor types.

When those monoclonals derived from the original vaccine preparation were delivered intravenously in a number of patients with metastatic pancreatic cancer having failed chemotherapy, they were found to demonstrate a marked anti-tumor effect on the existing metastatic malignancies with no cross reactivity to adjacent normal tissue. This is important to note since it stands in contrast to epidermal and other growth factor mAbs which target equivalent factors expressed on normal cells such as those found on the skin and in the intestinal tract. A number of patients having failed Gemzar or the combination with Abraxane were shown to have responded for better than 30 weeks post chemo failure where Abraxane presented approximately 8 week enhanced survival following Gemzar failure.

In FDA meetings to discuss the nature of the planned trials, one question raised was when the tumor antigen being targeted first appeared. In a detailed immunohistochemical study it could be demonstrated that such immunogenic antigen was first noted to be expressed by the cell approximately 4-6 months before phenotypic features of malignancy were noted [11,12]. As a result, in a retrospective study of anastomotic recurrences following colectomy, normal colonocytes were examined at the margins of resection employing H&E. Here, we were able to demonstrate that such cells were already expressing tumor antigen using a rapid immunohistochemical procedure. The same antigen was noted to have been present in the primary lesion for which the colectomy had been performed and appeared in the malignant cells comprising the anastomotic recurrence. An ongoing study as such is in place to evaluate the extent of the so called anticipated field effect, wherein these premalignant cells can be found to be present. Gene mutations resulting in this process of transformation will also be defined. Figure 8 illustrates normal appearing colonocytes adjacent to the tumor stained by H&E, and in contrast the same area is duplicated with the

colonocytes now stained by immunohistochemistry utilizing those mAbs that targeted the tumor antigen in the primary lesion.

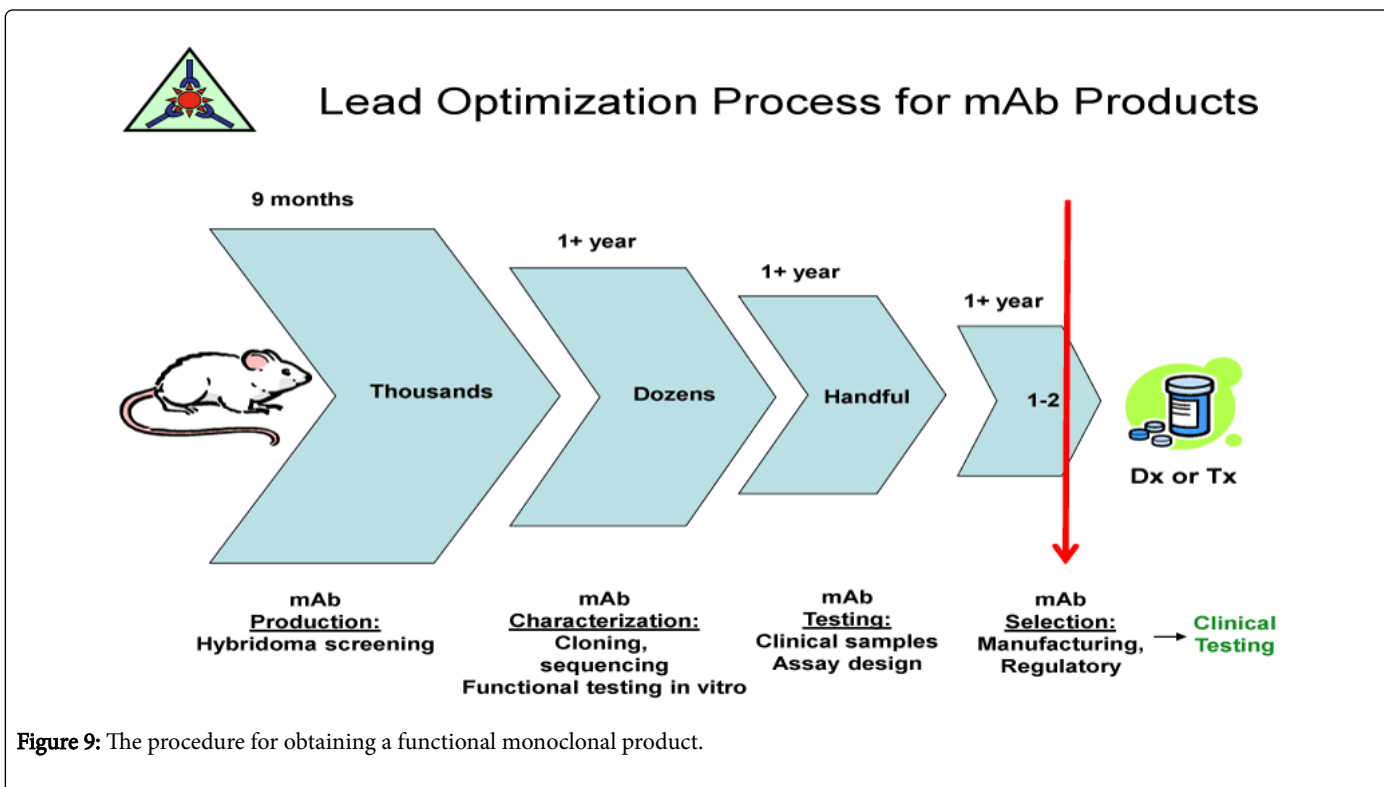
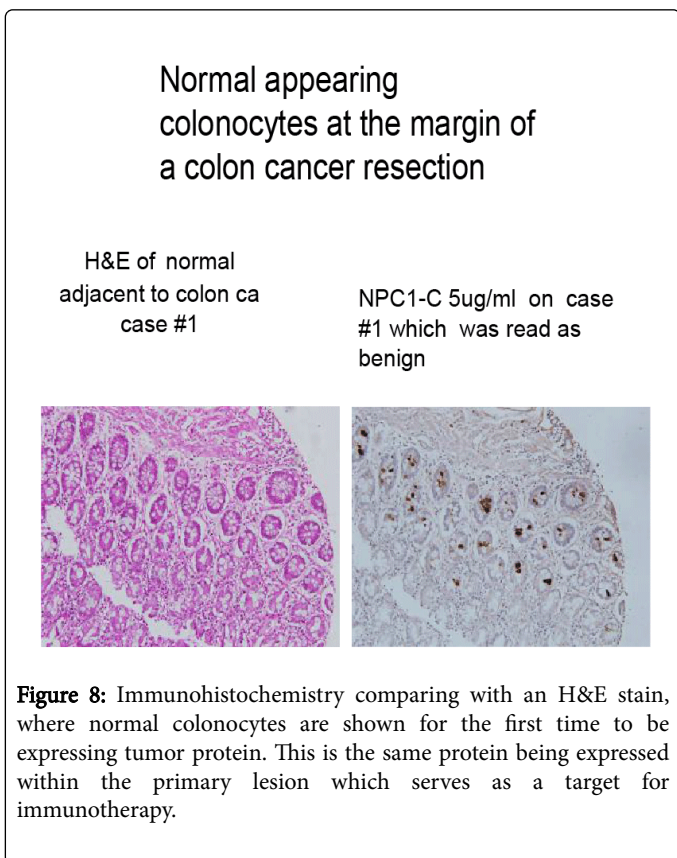
### Nature of Antibody Production

When employing the original NPC-1 (Neo 101) monoclonal antibody to initiate the FDA Phase II trials, Phase I showing no evidence of toxicity, we had run out of the originally produced antibody. By filing a new IND with FDA we were given approval to use the newer production batches of monoclonal Neo-102 derived from the use of a newer expression vector system that yielded 2000 mg/L bioreactor fluid [12].

Employment of, or potential modification in a procedure that could cause the slightest alteration in shape or structure of a biologic product frequently requires reevaluation by the FDA. The major concern here is the question of bioequivalency, where it has been illustrated in many instances, that changing the type of bioreactor or culture media used, could modify the activity of the product. In the case of Neo-102 originally derived from Neo-101, by changing the expression vector we noted not only improved output, but enhanced ADCC as well as clarity of staining by IHC was noted. Any of the minor hemolytic responses that we had previously seen with Neo 101 were also resolved with the production of Neo-102. The optimization process for developing the proper monoclonal is seen in Figure 9.

### Clinical Trials

The ability to initiate new clinical therapeutic studies was important because of the failure of other existing protocols to result in significant enhancement in survival of patients so treated. This held true for both the newer chemotherapy protocols as well as the immunochemotherapy trials that were currently available for clinical use.





To date we have indications of improved responses obtained from initial Phase I-IIa trials [13,14]. Where the introduction of Abraxane to Gemzar added 8 weeks to survival of recurrent pancreatic Ca patients, employing one half of the therapeutic dose of Neo-101/102 antibody indicated that the average pancreatic cancer patient having failed Gemzar had better than a 26 week improvement in survival [15]. The FDA has now approved use of Neo-102 at doses of 3-4 mg/kg, which is double the dose of antibody used in Phase I-II. In addition, a subset of patients will be receiving chemotherapy along with the mAb in a randomized two arm trial of chemotherapy vs. immunochemotherapy. The introduction of chemotherapy should help reduce levels of inhibitory substances in the serum that function to suppress an immune response following the introduction of antibody therapy. There is little question that employing antibody targeting immunogenic tumor proteins, when given in combination with chemotherapy should result in a better response.

It is also important during clinical studies, that a means for diagnosing as well as monitoring a tumor response be made available. We have developed what appears to be an effective approach utilizing a serum ELISA to define the presence of shed tumor antigen in the serum. The early results suggest that the procedure we have developed offers better than 90% sensitivity and specificity. When this ELISA protocol is fully implemented, see below and employed in monitoring patients, it will be the first step in introducing these newer monoclonals as part of an overall approach in cancer management, that is the use of a single immunogenic monoclonal antibody for both diagnose and then for treating and monitoring the tumor [16].

### **Sandwich ELISA Assay Exploiting Murine NPC-1C Antibody to Detect Neo101 Antigen**

1. Coat plate (NUNC Maxisorp) with 100 µl of murine NPC1 (5 µg/ml) in carbonate coating buffer (pH 9.5) at 4°C overnight;
2. Wash once with TBS-T (Tween 20 - 0.05%; TBS recipe from "Bio-Rad" : 20 mM Tris-HCl, 0.5M NaCl, pH 7.5);
3. Block 20-30 minutes with blocking buffer (1% milk-5mMEDTA-TBS-T);
4. Wash once (see p.2);
5. Add 50 µl ELISA Diluent, ED (blocking buffer: TBS=1:10) into each well;
6. Prepare the standard, control and sera dilutions in ED buffer in dilution plate as shown below;
7. Add 50 µl of pre-diluted standards (0-100 ng/ml BSM in ED), controls and tested serum samples (1/25 in ED) in duplicate from dilution PP to experimental plate;
8. Incubate at room temperature on orbital shaker (40 rpm) for 1h;
9. Wash 3 times (see p.2);
10. Add 100 µl chiNPC1-HRP at 1µg/ml in ED;
11. Incubate at room temperature on the table for 1h;
12. Wash 3 times (see p.2);
13. Add 100 µl 1 step TMB substrate (BioFX) for 20minutes;
14. Add 50 µl stop solution (1M H<sub>2</sub>SO<sub>4</sub>);

15. Clean bottom with alcohol soaked paper. Measure immediately OD450

### **Preparation dilution polypropylene plate:**

Add 120 µl ED to 6 columns in dilution plate

Add 120 µl of BSM (4 µg/ml in ED) to A1. Make dilutions (1/2) by transferring 120 µl down until H1

Add 5 µl of 36 tested sera and controls (pos.1-3; negative)

In looking for an early tumor marker for diagnosing pancreatic and colorectal cancer, it appears that the same monoclonal antibody that is used to monitor the shedding of antigen into the serum from metastasis, can also be used for early detection and diagnosis. In the earliest phases of tumor development including *in-situ* lesions, antigen is shed from such cells. In the case of cells arising intraluminally in the pancreatic duct system as well cells transforming within the lumen of the bowel, antigen is shed and can be detected by an ELISA. For early colon cancer, this antigen appears in the stool where it can be detected by a stool ELISA. The development of pancreatic cancer similarly occurs with the initial appearance of intraductal atypical cells developing over a 15-20 year period of time and that eventually transform to the invasive lesion [17]. Here, at the time of pancreatic duct brushings used to screen for pancreatic cancer samples of fluid used to irrigate the duct can be used for a diagnostic ELISA. The ideal monoclonal antibody as such, one that can be used for overall tumor management, is the one defined by Hartwell. It is an antibody that functions through its capability of having excellent diagnostic as well as therapeutic functions [18,19]. And as for value in diagnostics, the earlier, a lesion can be detected, the greater the possibility for cure.

There has always been the question that as a tumor progresses from its earliest stages to that of metastasis that there is an increasing numbers of oncogene mutations. As such, the target antigens expressed by the tumor may be altered, necessitating new therapeutic agents. We have on the other hand noted, that in the earliest phases of clinical disease, with regard to tumor immunogens, that the same tumor associated antigen is expressed throughout all stages of tumor development that is from the earliest premalignant lesion to that of the most advanced metastatic tumor. The therapeutic antigen (vaccine) or monoclonal used to target the primary lesion therefore remains the same whether one is treating the earliest or late phase of tumor growth [20].

As to the pathway which leads to the development of a malignant lesion, pancreatic tumors arise over a long period of time resulting from the progression of intraductal pathology to the highly invasive malignant parenchymal growth. In the case of the bowel lesion, it appears that transformation arises within a mucosal field effect. Polypoid growths that do transform to bowel malignancy probably represent a small population of the growths that arise in the area encompassed by the field effect. Many of the polypoid growths that have been stained for tumor protein have shown no evidence of antigen expression suggesting that not all undergo malignant transformation. In addition, in those instances where anastomotic recurrences were noted, the premalignant cells transformed to the malignant format without the appearance of polypoid lesions suggesting that that the long held concept that polypoid transformation was the primary pathway for bowel cancer development did not hold true [21]. Whether there is an early transforming polypoid lesion or a small malignancy, the antigen expressed by the cancer cell does shed into the bowel lumen and can be



detected in the stool. Obviously if a stool ELISA proves to be fully effective in a planned for clinical trial then only those patients showing evidence of tumor antigen in the stool will need to undergo colonoscopy in order to define the lesion and establish what treatment will be necessary for cure.

In a preliminary study, evaluation of the stage at which a bowel lesion has developed and the correlation of antigen shedding by a potential lesion into stool, was examined in 50 patients utilizing the simplified office stool ELISA. We were able to easily detect an array of lesions starting with a malignant polyp and progressing to a small adenocarcinoma and further to a fully malignant lesion. In 25 of the patients where no antigen was detected, the bowel was found to be free of any detectable pathology following colonoscopic exam.

## Conclusion

The use of monoclonal antibodies targeting immunogenic proteins expressed in malignant lesions has now become an important aspect in planning for the treatment of the advanced cancer patient and in particular pancreatic and colorectal cancer having failed standard forms of therapy. For therapeutic purposes, it does require that the antibody be specific for an immunogenic target in the tumor and in the overall scheme, that such an antibody fit into Hartwells description of a product that can both detect tumor with a high degree of accuracy *in vitro* and when confirmed, be used as a therapeutic agent to target and destroy the malignancy. Antibodies for a large array of neoplasms are now in development, all based on having been able to define the immunogenic protein expressed in the tumor. Because of the size of the immunogenic proteins derived from the tumor, synthesis for vaccine development has been difficult since the final product produced is linear and non-functional. Phage display has been used to define the nature of the epitope binding site of the antibody. This proved to be a short chain (12 mer) linear peptide which when sequenced and constructed, was found to be capable of inducing the antibody response needed for treatment. Clinical trials with the peptides that we are developing will be tested for activity in various forms including that of MAP (multiple antigenic peptide). If studies confirm the efficacy of this type of vaccine, then we will consider initiating clinical trials aimed at preventing the high incidence of recurrent tumors post-surgery such as is seen with pancreatic cancer post Whipple.

## References

- Hollinshead AC, Stewart T, Yonemoto R, Arlen M, Takita H (1981) Immunotherapy of Advanced Disease, Proceedings VI Chicago Cancer Symposium Tumor Progression. Cresspin (Ed) Elsevier Press.
- Prehn RT, Main JM (1957) Immunity to methylcholanthrene-induced sarcomas. *J Natl Cancer Inst* 18: 769-778.
- Smyth MJ, Godfrey DI, Trapani JA (2001) A fresh look at tumor immunosurveillance and immunotherapy. *Nat Immunol* 2: 293-299.
- Hollinshead A, Arlen M, Yonemoto R, Cohen M, Tanner K, et al. (1982) Pilot Studies Using Melanoma Tumor Associated Antigens (TAA) in Specific-Active Immunochemotherapy of Malignant Melanoma. *Cancer* 49: 1387-1404.
- Hollinshead A, Elias G, Arlen M, Buda B, Scherrer J, et al. (1984) Surgery Plus Specific Active Immunotherapy in the Treatment of Adenocarcinoma of the Colon. *Am Assoc Cancer Research Proceedings* 704: 178.
- Hollinshead A, Elias G, Arlen M, Mosely MS, Buda B, et al. (1984) Immune Monitoring of a Specific Active Colon Immunotherapy Trial. *Proceedings Conf. Immunity to Cancer, Williamsburg*, pp 29.
- Hollinshead A, Takita H, Stewart T, Raman S (1988) Specific active lung cancer immunotherapy. Immune correlates of clinical responses and an update of immunotherapy trials evaluations. *Cancer* 62: 1662-1671.
- Hartwell L, Mankoff D, Paulovich A, Ramsey S, Swisher E (2006) Cancer Biomarkers: a systems approach. *Nature Biotechnology* 24: 905-908.
- Raffle AE, Alden B, Quinn M, Babb PJ, Brett MT (2003) Outcomes of screening to prevent cancer: analysis of cumulative incidence of cervical abnormality and modelling of cases and deaths prevented. *BMJ* 326: 901-906.
- Hollinshead A, Stewart TH, Takita H, Dalbow M, Concannon J (1987) Adjuvant specific active lung cancer immunotherapy trials. Tumor-associated antigens. *Cancer* 60: 1249-1262.
- Arlen M, Tsang KY, Bartal A, Wolf J, Saric O (1991) Monoclonal Antibodies to Immunoreactive Tumor Associated Antigen (TAA) from Human Colon Carcinoma. *J Antibody Immunoconjugates and Radiopharmaceuticals* 4: 895-905.
- Arlen M, Tsang KY (1990) The Nature of the Monoclonal Antibodies Derived from Immunogenic Membrane Antigen of Human Colon Carcinoma Origin. *J Tumor Marker Oncology* 5: 313-319.
- Morse M, Patel SP, Beatson MA, Arlen PM (2013) A phase II study of NPC-1C: A novel therapeutic monoclonal antibody (mab) to treat pancreatic (P) and colorectal (CR) cancers. *Proceedings of the 104th Annual Meeting of the American Association for Cancer Research*.
- Patel SP, Morse MA, Diaz LA, Azad NS, Haley S, et al. (2013) A phase I safety study of NPC-1C, a novel, therapeutic antibody to treat pancreas and colorectal cancers. *Proceedings of the 104th Annual Meeting of the American Association for Cancer Research*.
- Arlen M, Saric O, Wang X, Dubeykovskiy A, Arlen P (2013) Nanocytology vs. Immunohistochemistry of Intestinal Colonocytes to Assess the Risk of Colon Cancer based on Field Cancerization - A Preliminary Report. *J Cancer* 4: 165-169.
- Arlen M, Wang X, Luka J, Gupta R, Saric O, et al. (2012) The Use of Specific Monoclonal Antibodies to Target Immunogenic Tumor Proteins in Patients with Recurrent Pancreatic and Colorectal Cancer. *Curr Drug Deliv* 9: 52-56.
- Ottenhof NA, de Wilde RF, Maitra A, Hruban RH, Offerha GJA (2011) *Molecular Characteristics of Pancreatic Ductal Adenocarcinoma*. Pathology Research International.
- Patel SP, Bristol A, Saric O, Wang XP, Dubeykovskiy A, et al. (2013) Anti-tumor activity of a novel monoclonal antibody, NPC-1C, optimized for recognition of tumor antigen MUC5AC variant in preclinical models. *Cancer Immunol Immunother* 62: 1011-1019.
- Arlen M, Arlen P, Wang X, Saric O, Martin DA, et al. (2013) The Clinical Detection of Pancreatic Carcinoma: A Comparison of the Standard Biomarkers to that of a Newer Class of Biomarkers used for both Diagnosis and Therapy. *Pancreatic Dis Ther* S4: 001.
- Arlen M, Arlen P, Coppa E, Crawford J, Wang X, et al. (2013) Identification of the Oncofetal Tumor Antigens that Characterize Colorectal Carcinoma for use in Diagnosis and Therapy *J Gastroint Oncol* 10: 156-169.
- Arlen M, Crawford J, Coppa G, Saric O, Bandovic J, et al. (2014) The Cause and Prevention of Anastomotic Recurrence following Colectomy: An Immuno-histochemical Approach for Detecting Transforming Colonocytes. *J Cancer* 5: 784-789.