Breast Cancer Immunodiagnosis and Immunotherapy

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Organized by the John Muir Cancer & Aging Research Institute, with the cooperation of the International Association for Breast Cancer Research.

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PREFACE

The convening of the 3rd International Workshop on Monoclonal Antibodies and Breast Cancer had the character of a self-search exercise. After almost a decade of research in the basic and applied aspects of the use of serological means to diagnose and possibly treat breast cancer several milestones have been reached. Among them a clear understanding of immunopathological use and limitations of monoclonal antibodies against breast epithelium, the complete development and clinical use of immunoassays for circulating breast epithelial antigens, the striking advances in the diagnostic use of monoclonal antibodies to estrogen and progesterone receptor proteins and the first communications on proposed immunotherapeutic use of different conjugates of anti-breast antibodies.

New areas of investigation have developed in our field, some which are reaching a full blossom while others are still facing obstacles and at times a re-definition of their goals and objectives. These meetings have acted in a way as a clearing house and have permitted their attendees to derive predictions that have helped shape future research and fine-tune objectives. But above all, the re-evaluation of past research at these Workshops and the renewed excitement brought to them by new information has helped generate a momentum and enthusiasm that assures for the future large scientific gains.

The sustainment of interest of scientists in these Workshops has allowed for the development of an ordered historical perspective of the growth and development of the field of immunology and breast cancer and how it relates to the use of serological approaches. The ingenuity of investigators has produced an inexhaustible amount of studies that have enlarged this area of research. As these reagents become used in other areas of breast cancer studies, such as immunotherapy, there will be a great need for interaction among those dedicated to fight breast cancer and for the facilitation provided by scientific dialogue such as the one sustained in these Workshops.

R.L. Ceriani

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THOMSEN FRIEDENREICH (TF) AND Tn ANTIGENS AS MARKERS FOR BREAST CANCER

The Thomsen Friedenreich (TF) antigen may be important for the detection and immunotherapy of a number of common cancers including breast cancer. Revealed on normal human erythrocytes by neuraminidase treatment, TF has been characterized as: ß-D-Gal-(1-3)-α-GalNAc, attached to glycophorin or other glycoproteins through O-serine or O-threonine linkages (1). Tn, the TF precursor, is reported to be α-GalNAc-O-serine/threonine. While TF is normally cryptic due to the presence of a terminal sialic acid residue, Tn is exposed in individuals with a recessive genetic disorder (2). Springer (1) has claimed expression of TF and Tn antigens on over 90% of cancers of the breast, lung and pancreas, although the nature of the molecules which bear these antigens and their exact structures has not been defined.

Our group was the first to generate monoclonal antibodies (MAbs) against TF-like antigens expressed on human cancer cells (3,4). Since then, however, we have been able to derive many different MAbs of predetermined specificity for TF-like epitopes utilizing our ability to generate synthetic tumor-associated glycoconjugates (S-TAGs) and MAbs against these. Different conformations of TF were synthesized and used as immunogens to derive MAbs which were selected for their reactivity with the S-TAGs. Several of these MAbs were also demonstrated to have in-vitro specificity for human adenocarcinomata in frozen tissue sections (5). We describe here the initial radioimmunoimaging studies with two of these radiolabelled anti-synthetic-TF MAbs, in patients with metastatic adenocarcinoma of the breast. Such studies are enabling us to probe the *in vivo* expression of TF on metastatic human adenocarcinomata, and to ask whether TF-like antigens can be used as targets for the detection of metastatic cancer.

The ease with which anti-TF MAbs could be generated demonstrated the immunogenicity of TF in mice. We thus wanted to know whether synthetic TF could be used (in an appropriate formulation) to actively induce a protective specific immune response against tumor cells? In a relevant murine mammary adenocarcinoma model (the tumor cells express TF-like structures) we recently showed that synthetic TF and Tn could be used to induce a T lymphocyte response which had protective anti-cancer reactivity (6). Further experiments, reported here, have probed strategies to optimize

both the cellular and humoral immune responses to synthetic TF, asking whether synthetic TF has potential for active specific immunotherapy.

Thus, our aim has been to identify human tumor-associated glycoconjugate antigens, synthesize them, and test whether these synthetic glycoconjugates have potential both as immunogens for active specific immunotherapy (ASI), and as immunogens to derive relevant MAbs for localizing (radioimmunoimaging) cancers. Successful radioimmunoimaging in humans would validate a particular synthetic glycoconjugate, while the animal model would be used to study the potential of the same antigen for ASI. We recently summarized this approach to cancer therapy and detection with TF S-TAGs and their corresponding MAbs (7).

In addition to the potential of TF as a target antigen of breast cancer, there is evidence that TF and Tn antigens may be functional markers of malignancy. Investigators studying the expression of TF in human bladder cancers have shown a relationship between TF expression and likelihood of invasive recurrence (8,9). Springer and colleagues have extensively studied TF and Tn antigen expression on human breast carcinomas (1,10) and claim that the ratio of expression of Tn to TF correlates with the aggressiveness (and degree of dedifferentiation) of the cancer. Howard and Batsakis (11) analysed 22 breast carcinomas, and found that the 17 well differentiated tumors expressed TF antigen, while the remaining 5 undifferentiated tumors lacked TF antigen.

Yuan and coworkers (12) used one of our TF MAbs (3) to study the early expression of TF antigens on premalignant polyps and colon adenocarcinomas (normal adult mucosa was not marked by the MAb) and showed that this MAb marked pre-malignant polyps as well as adenocarcinoma. We have also confirmed this. This MAb also marked fetal colon tissue, suggesting that TF may be an oncodevelopmental antigen in human colon cancer.

Perhaps the best evidence for a functional role of the TF system in malignancy is provided by a genetic disorder which results in a selective loss of 3-ß-D-galactosyl-transferase activity and the appearance of Tn positive RBCs and hematopoietic stem cells (the Tn syndrome). This disorder is associated with a high incidence of leukemia and other hematopoietic disorders. This suggests that Tn expression might be indicative of deregulation of pleuripotent stem cells, with a proliferative advantage for Tn^+ cells (2).

The first question to be answered then was whether the TF S-TAGs were identical to the TF-like structures expressed on human cancers *in vivo*.

SUCCESSFUL *IN VIVO* IMAGING OF METASTATIC HUMAN BREAST CANCER, USING MAbs 155H.7 AND 170H.82 GENERATED USING SYNTHETIC TF GLYCOCONJUGATES

Two monoclonal antibodies (MAbs), designated 155H.7 and 170H.82, were generated against synthetic TFß conjugated to HSA, and were shown to react strongly *in vitro* with most adenocarcinomata with little or no obvious tumor heterogeneity (very few negative cancer cells) (5). Of relevance here is that 27/27 adenocarcinomata of the breast reacted with the MAb 155H.7. Reactivity with normal tissues and other malignancies was limited, so it was proposed to study the reactivity of these two MAbs *in vivo*, in clinical radioimmunoimaging.

It proved possible to radiolabel each of these without loss of immunoreactivity. Phase 1 radioimmunoimaging studies were instituted, prior to the development of Phase 2 and 3 imaging trials. Because of the unique development of these MAbs - being derived against S-TAGs - the pilot studies have been designed to answer the following questions:

1) Are the radiolabeled anti-S-TAGs toxic?
2) Are the compounds stable *in vivo*? Do they bind to known sites of adenocarcinomata *in vivo* ?
3) Can a dose response be demonstrated, with improved imaging efficacy at increasing doses of antibody?

The underlying questions were:

a) Can MAbs derived against synthetic glycoconjugates bind to human cancers *in vivo* ? and
b) Are the S-TAGs being developed for specific immunotherapy (and which were used to derive the MAbs for imaging) relevant to human cancers?

The first clinical imaging study used MAb 155H.7. This pilot study included a comparison between the MAb radiolabeled with Iodine-131 and with Indium-111. Iodination was performed carefully over no more than two minutes (to avoid loss of immunoreactivity) using an Iodogen method. Indium labeling was accomplished by pre-chelating the MAb with 4-6 chelate groups via the DTPA anhydride reaction, with free chelate removal by diafiltration. The addition of Indium-111 in citrate buffer resulted in >98% binding to the chelated MAb 155H.7 after 30 minutes. All radioimmunoconjugates for clinical imaging are tested for immunoreactivity, sterility and apyrogenicity prior to clinical use.

As the MAb had shown a broad range of reactivity with adenocarcinomata *in vitro*, patients with a similar range of adenocarcinomata were entered into the study, including patients with adenocarcinomata of the breast, endometrium, ovary, colon and lung. As this was a pilot study, only sites of known tumor were evaluated, and the results of the immunoimaging study were compared with the results of conventional clinical and imaging investigations. No attempt has been made to define sensitivity and specificity in this phase 1 protocol. Included in this study were six patients with metastatic breast cancer evaluated with the Iodine-131 label, and two evaluated with the Indium-111 label.

Limited imaging efficacy was seen at the lower dose levels utilized (4 and 8 mg). However at increasing doses of antibody (16 and 32 mg) increasing imaging sensitivity was demonstrated, with 32 mg offering the most effective images.

It was clear from the study that Indium-111 was the more effective imaging label at all MAb dose levels used. This isotope also offers the opportunity of SPECT (single photon emission computed tomography) imaging which greatly increases diagnostic efficacy, image quality, resolution and the ability to detect presence of cancer in nodes defined as "normal" on C.T. SPECT imaging was performed using a GE 400 AT Tomographic camera interfaced to a Picker PCS 512 computer, with data acquisition on a 128*128 matrix and stored for later reconstruction analysis.

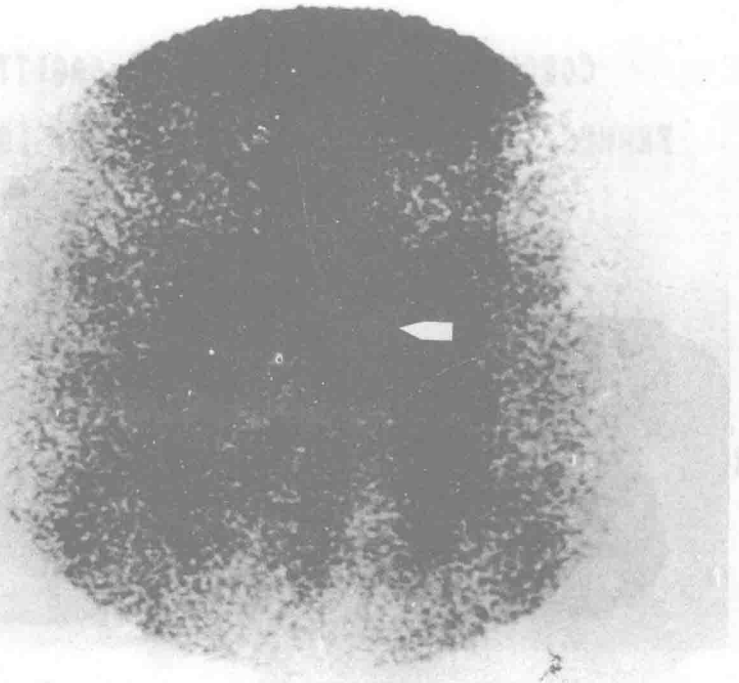

Figure 1. ^{131}I.155H.7: Uptake in metastases to bone.

A wide range of known metastatic sites in patients with breast cancer were successfully imaged, including lymph nodes, lung and bone. Figure 1 is a planar posterior abdominal image of a patient with metastatic breast cancer, using 32 mg MAb 155H.7 labeled with 2 mCi Iodine-131. Tracer uptake is seen in the known bone metastases in the right sacro-iliac joint and in the lumbar vertebrae. Figure 2 shows selected chest SPECT images of a patient with metastatic breast cancer, imaged 6 days after the infusion of 32 mg MAb 155H.7 labeled with 5 mCi Indium-111. Uptake can be clearly seen in the metastatic mass in the mediastinum.

While clinical accuracy was not the primary purpose of this study, particularly without surgical and histological confirmation of positive tracer uptake, there was a high concordance demonstrated between the findings on radioimmunoscintigraphy and the findings on CT scanning, ultrasonography and clinical examination.

A current study is investigating the diagnostic potential of radioimmunolymphoscintigraphy using ^{111}In.155H.7 injected into the finger web spaces of patients with breast cancer, to detect regional lymph node metastases. Tracer activity is seen in supraclavicular nodes within one hour, with retention of activity in nodes which are definitely abnormal clinically (enlarged, hard, and irregular). The next phase in this study is to biopsy these nodes for surgical/histological confirmation of the specificity of the binding of the immunoconjugate in these "involved lymph nodes".

A second pilot study has now commenced to evaluate the other anti-TF MAb, 170H.82. Although the immunogen used to derive this MAb is identical to that used to derive the MAb 155H.7, the immunoreactivity of the two MAbs with both synthetic glycoconjugates and tissue sections is different. The next study is therefore designed to compare the imaging efficacy of the two MAbs.

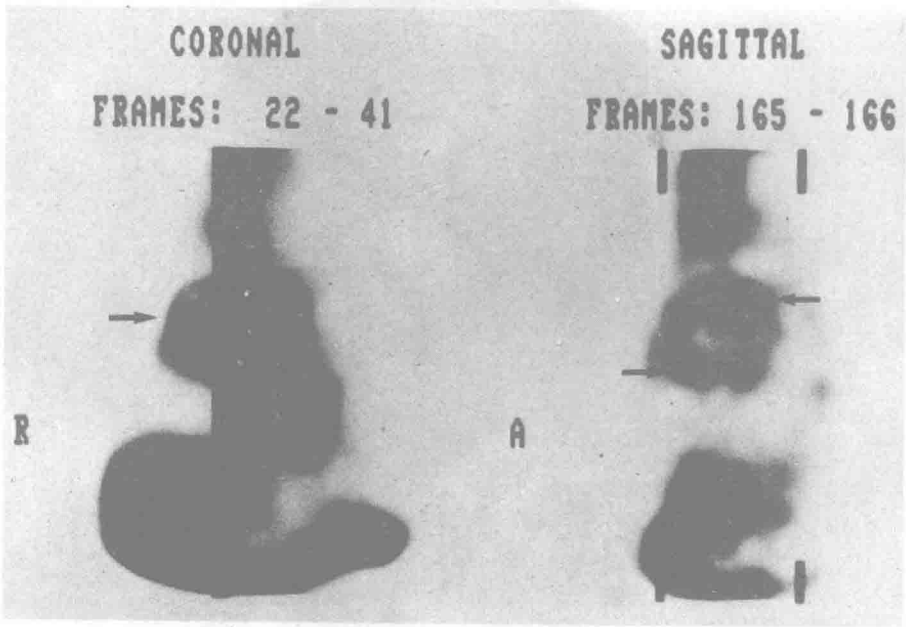

Figure 2. ^{111}In.155H.7; SPECT: Uptake in metastasis to mediastinum.

In this protocol Indium-111 has been used as the radiolabel, and SPECT imaging has been performed in all patients. Of the seven patients imaged to date with ^{111}In.170H.82, two patients have metastatic breast cancer. Figure 3 shows chest SPECT of a patient with breast cancer, imaged using 8 mg MAb and 3 mCi Indium-111. Disease is detected within the left hemi-thorax, left supraclavicular fossa and right axilla.

The data on imaging metastatic breast cancer are limited, being extracted from two pilot studies in patients with a wide variety of metastatic adenocarcinomata. However, the evidence from all the data is that these murine MAbs of predetermined specificity, derived using synthetic glycoconjugates as immunogens, have clinical potential for detection of metastatic adenocarcinoma including breast cancer. Larger prospective studies are being planned.

Based on our wider experience in patients with metastatic adenocarcinomata arising from the female genital organs (ovary, fallopian tube, uterus and cervix) we predict an expanding clinical role for radioimmunoscintigraphy in:

(1) screening for metastatic disease;
(2) *in vivo* analysis of radiologically detected "lesions";
(3) *in vivo* prediction of tumor response to treatment;
(4) intra-operative localization of metastatic disease.

If the initial, encouraging results are validated, the next phase of development will be the evaluation of a dose response protocol for targetted therapy in patients with breast and gynecologic cancers.

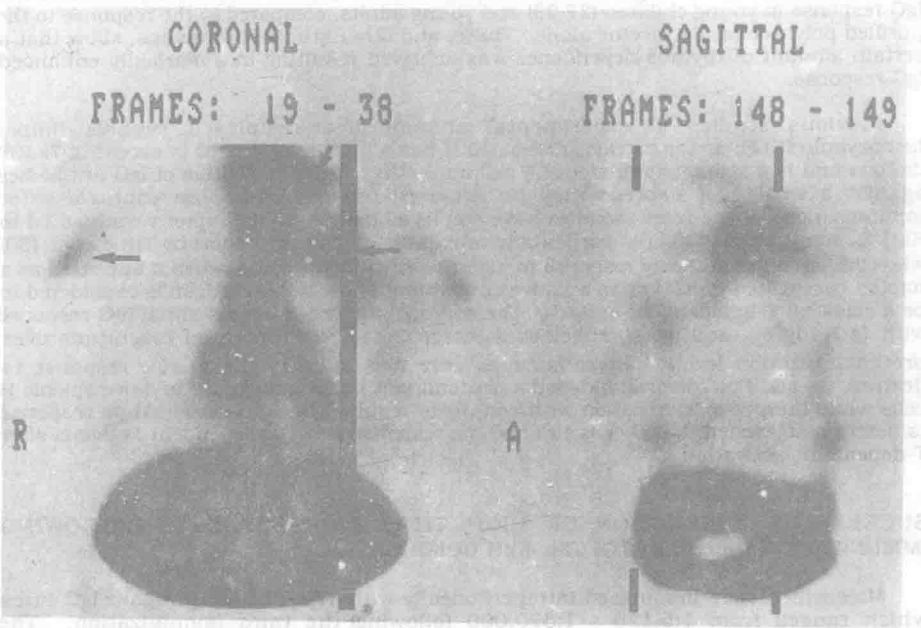

Figure 3. ^{111}In.170H.82; SPECT: Uptake in metastases in nodes and lung.

In addition to demonstrating the imaging potential of these MAbs, these studies have also demonstrated that TF-like antigens are expressed *in vivo* on metastases of breast cancer, that these antigens are accessible to the immune system, and that the epitopes of the synthetic glycoconjugates are identical to the epitopes expressed on cancers. Can these same S-TAGs then be used to induce a protective immune response - for immunotherapy?

IMMUNE RESPONSE TO CARBOHYDRATE ANTIGENS

In planning active specific immunotherapy we should consider and seek to measure the likely humoral and cellular immune responses to the specific antigens. Antibodies to carbohydrates (CHOs) generally have a peculiar isotype distribution and are usually of relatively low affinity. The major isotype response is IgM in both humans and mice (13). In our experience in the generation of several thousand MAbs to TF, Tn and sialated Lewis^a haptens conjugated to HSA, greater than 90% of the clones produced IgM antibodies despite prolonged immunization. In general, the IgG anti-CHO response is predominantly IgG_2 in humans (14,15) and IgG_3 in mice (16-18). However, exceptions to these rules have been published, suggesting that the variation depends on the particular carbohydrate used for immunization or the age of the responder (17,19).

Immune responses to most polysaccharides have been classically referred to as "T-independent" since they can apparently trigger B cells to produce antibody in the absence of T-helper cell activity (20-22) and T-suppressor cells may even inhibit responses to these antigens (23-25). Immune responses to T-independent antigens are usually restricted to IgM and immunization with these antigens often fails to stimulate the production of memory cells (26).

Proteins are typical T cell-dependent antigens capable of producing a booster response and of causing a progressive switch of IgM to IgG antibodies of increasing affinities after repeated injections. Many attempts to generate IgG anti-carbohydrate antibodies have involved the conjugation of the polysaccharide to a protein carrier. This conjugation is meant to convert the polysaccharide from a T-independent to a T-dependent antigen. Such polysaccharide-protein conjugates have been used in the development of vaccines against bacterial diseases and found to markedly enhance the IgG response in young children (27,28) and young adults, compared to the response to the purified polysaccharide vaccine alone. These, and other studies in humans, show that a certain amount of thymus-dependence was achieved resulting in a markedly enhanced IgG response.

Previous studies in experimental animals have employed keyhole limpet hemocyanin (KLH) as the carrier protein. KLH has a molecular weight in excess of 7×10^6 daltons and is a strong stimulator of T cell immunity. Thus high titres of IgG antibodies against a variety of haptens may be achieved using KLH-hapten conjugates for immunization. Some recent studies have employed carbohydrate haptens conjugated to KLH as immunogens (29,30). Particularly revealing was a recent report by Tittle et al. (30) who compared the antibody response to nigerose [α(1,3)diglucoside] when it appeared as a hapten conjugated to KLH or as a native constituent of dextran. Dextran is considered to be a classical T-independent antigen. The conjugate induced a substantial IgG response with IgG_1, IgG_{2a} and IgG_{2b} subclasses increasing by 2-3 orders of magnitude over pre-immunization levels. These isotypes were not found in the *in vivo* response to dextran. In addition, dextran induced a predominant lambda response in naive splenic B cells while the pre-immunization with conjugate resulted in a dramatic kappa response to dextran, suggested that B cells can become responsive to T-independent antigens after T-dependent activation.

SUCCESSFUL GENERATION OF HIGH TITRE IgG RESPONSES FOLLOWING IMMUNIZATION OF MICE WITH TFα–KLH CONJUGATES

Mice which were immunized intraperitoneally with TFα–KLH in CFA gave IgG titres which ranged from 1:5,120 - 1:320,000 following the third immunization. The substitution of CFA by Ribi adjuvant composed of trehalose dimycolate (TDM) and monophosphyl Lipid A (MPLA) gave equivalently high IgG titres (see Table 1).