2008

Lack of Glycosylation Does not Affect the Antigenicity of Epithelial Mucin 1 in a Rare Case of Aglyconic Extramammary Paget's Disease

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LACK OF GLYCOSYLATION DOES NOT AFFECT THE ANTIGENICITY OF EPITHELIAL MUCIN 1 IN A RARE CASE OF AGLYCONIC EXTRAMAMMARY PAGET'S DISEASE

BY

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A THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

DEPARTMENT OF GRADUATE MEDICAL SCIENCES

MIAMI SHORES, FLORIDA

(FALL, 2008)

BARRY UNIVERSITY

FACULTY OF GRADUATE MEDICAL SCIENCES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Medical Sciences for acceptance, a thesis entitled Lack of glycosylation does not affect antigenicity of epithelial mucin 1 in a rare case of Extramammary Paget's disease submitted by Shakira Lita Ismay Henderson in partial fulfillment of the requirements for the degree of Master of Science in Anatomy

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DEGREE Master of Science in Anatomy **YEAR GRANTED** 2008

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ABSTRACT

It has been noted in the research literature that there is no systematic difference in affinity of monoclonal antibodies raised against the native glycoprotein (MabW) as compared to monoclonal antibodies raised against the aglyconic tandem polypeptide of the core protein (MabT) for the native glycosylated epithelial mucin 1. However, there is no research in the literature comparing the affinity of these two monoclonal antibodies for the aglyconic epithelial mucin 1 core protein. This project investigated the affinity of MabW and MabT for the aglyconic epithelial mucin 1 core protein in an atypical case of extramammary Paget's disease. The limiting dilution was used and results were analyzed using the Kolmogorov- Smirnov two sample test with an alpha of 0.05. There was no systematic difference in the affinity for the aglyconic epithelial mucin 1 between MabW and MabT. This suggests that the numerous oligosaccharide side chains of the human epithelial mucin 1 have little to do with antigenicity.

ACKNOWLEDGEMENTS

I would like to thank Barry University's School of Graduate Medical Sciences in Miami Shores, Florida for supporting this research project.

I thank Dr. Allen Smith for suggesting this research project and teaching me all the lab techniques. I am also gratefully indebted to him for all his constant support, guidance and wisdom throughout this entire project. I will always remember him for his concern, patience and kindness. He is a truly the embodiment of a dedicated professor.

I would like to acknowledge and thank the pathologists for providing the extramammary Paget's disease tissue for this study.

I thank the following persons for critiques of the thesis: Dr. Allen Smith, Dr. Sanjay Sesodia, Dr. Gilbert Ellis, Dr. Ramjeet Pemsingh, and Dr. Michael Siegel.

Many thanks to Dr. Sanjay Sesodia for his critical role in the preparation of this manuscript but mostly for always pushing me to attain excellence.

I am very thankful to Dr. Gilbert Ellis for making the research and all my presentations a fun experience. I will especially remember him for his optimism.

I gratefully acknowledge Dr. Ramjeet Pemsingh for all his support and guidance in helping me attain this goal.

I thank Dr. Michael Siegel for his constant support throughout this experience. I will remember him mostly for his understanding nature and encouragement.

Lastly, I thank my family for all their love and support.

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Introduction

Extramammary Paget's Disease

In 1874 Sir James Paget first described a malignant skin change of the areola that now bears his name (Paget, 1874). This case was unique in that there was also an underlying breast carcinoma. The origin of malignancy was of great interest. Paget proposed that the neoplastic cells came from the large lactiferous ducts and that the skin changes not only preceded the malignancy in the underlying breast tissue, but also induced it. Paget also suggested that such a phenomenon could be possible in other epithelial tissues in the body. Fifteen years later, Crocker (1889) described the first case of extramammary Paget's disease (EMPD) in the scrotum and penis with histological features similar to those described by Sir James Paget. Crocker believed that the neoplastic origin of this tumor was from the sweat and sebaceous glands or hair follicles. Later, EMPD was found in other areas of the body. As referenced by Bodnar, Miller, and Tyler (1999) perianal EMPD was first described four years after Crocker's report. Dubreuilh later described the first case of vulval EMPD in 1901 as reported by Lloyd and Flanagan (2000).

Controversy still exists over the cell of origin in EMPD. There is an explanation for this debate of uncertainty. Unlike mammary Paget's disease, in which an associated neoplasm can be identified, many cases of EMPD (over 75%) do not appear to have an associated tumor in underlying organs or in adnexal structures (Goldblum & Hart, 1998).

Goldblum and Hart (1997, 1998) argue that underlying neoplastic disease probably does occur, but it is so small that detection is not likely. Lloyd and Flanagan (2000) agree and add that this may also be due to inadequate histological sampling.

The current theory of cell origin in EMPD holds that this malignancy arises from a primary intraepidermal lesion in the majority of cases. The tumor cells are thought to originate from apocrine sweat ducts or from pluripotent keratinoctye stem cells (Jones, 1985; Willman, Golitz, & Fitzpatrick, 2005). To date, there are still cases of EMPD where the site of origin is still not clearly known (Lloyd & Flanagan, 2000). Regardless of location, Paget's cells are always adenocarcinoma cells.

Epidemiology

EMPD is a rare adenocarcinoma. The etiology is not known. The precise incidence is also not known, but reported and diagnosed cases are extremely low. EMPD of the vulva constitutes 1-2% of all vulval malignancies (Parker et al., 2000). Patients presenting with EMPD are usually between the ages of 50 and 80 years. The disease is most common in postmenopausal Caucasian women (Zollo & Zeitouni, 2000). Familial occurrence is rare, with only six reports in the Japanese literature and one in the British literature (Demitsu et al., 1999).

Clinical Features

The site most frequently affected by EMPD is the vulva. This is followed by the perineal, perianal, scrotal and penile skin. Areas such as the eyelids, buttocks, thighs, external auditory meatus, axilla, and thighs are rarely affected. EMPD arising in the male and female genitalia can also be secondary to bladder and urethral neoplasms or to prostate cancer in men. Perineal EMPD can be associated with a colorectal neoplasia. (Allen, McLaren, & Aldridge, 1998; Goldblum & Hart, 1998; Powell, Bjornsson, Doyle, & Cooper, 1985; Koga, Gotoh, & Suzuki, 1997; Shimizu et al., 1991; Stephenson & Cotton, 1987)

The most common presenting symptom occurring in over 70% of patients with EMPD is pruritus. Other symptoms include burning, irritation, pain, tenderness, bleeding, and swelling. About 10% of patients may be asymptomatic. There is usually a two year time interval between the onset of disease and diagnosis. (Parker et al., 2000; Tebes, Cardosi, & Hoffman, 2002; Zollo & Zeitouni, 2000)

Patients present with lesions that are well demarcated, erythematous or leukoplakic plaques. The latter have been reported to occur more frequently in the anogenital area. EMPD is a slow growing tumor, thus lesions are often traumatized, exhibit excoriation or superimposed infection. As a result, there may be crusting, scaling, bleeding, and ulceration. A palpable mass with or without lymphadenopathy may be present and raises suspicion of invasion. (Lloyd & Flanagan, 2000)

Treatment

EMPD has many treatment options but the success rate is dependent on early detection and depth of invasiveness. In fact, no treatment guarantees full recovery. Surgery is the most recommended modality of treatment. Nonetheless, even extensive resection has not been able to combat the complication of high local recurrence. This has been attributed to the complications of EMPD such as irregular margins and multicentricity. (Zollo & Zeitouni, 2000)

Mohs' micrographic surgery (MMS) is a special technique that has shown promise in reducing local recurrence in EMPD patients (Mohs & Blanchard, 1979). Zollo and Zeitouni (2000) reported higher recurrence rates in patients with vulval (43%) or perianal (50%) EMPD treated by wide local excision as compared to those treated with Mohs' surgery technique (27% and 28% respectively). Coldiron, Goldsmith, and Robinson (1991) also reported lower recurrence rates of EMPD for patients treated with MMS. They found a 23% recurrence rate in patients treated by MMS technique versus 33% for standard surgery.

Mohs' surgery technique is designed to remove cancerous tissue with precision. The aim is to spare most of the healthy surrounding tissue. It can be done as an outpatient procedure with local anesthesia. The operation is done in stages. The first stage involves the removal of the visible cancerous tissue along with a small section of surrounding healthy tissue. The removed tissue is processed into sections, stained for microscopic

examination and marked on a detailed diagram often referred to as a Mohs' map. The patient waits while this tissue processing is conducted. Stage two begins when the surgeon utilizes the Mohs' map to direct further removal of the cancer. In stage three, the process is repeated until all cancerous areas are located within tissue specimens, correlated to the area on the patient via Mohs' map, and subsequently removed. When no cancerous tissue is revealed by microscopic examination, surgical repair is commenced. This is stage four. (Mohs & Blanchard, 1979; Coldiron et al., 1991)

Surgery for EMPD can be very mutilating and extensive. Very frail patients are not recommended for undertaking such a drastic procedure. There have been a few reports of treatment using radiotherapy. Originally it was not considered suitable for use with EMPD because of higher than normal recurrence rates. However, a few studies showed that it was effective for a small number of patients with no underlying organ involvement (Burrows, Hudson, & Pye, 1995)

Chemotherapy has also been used as pre-operative as well as post-operative treatment. Topical 5-Fluorouracil has been used for symptomatic relief. Small EMPD lesions have also been successfully treated with only imiquimod (Zampogna, Flowers, Roth, & Hassenein, 2002) or in combination with 5-fluorouracil (Ye, Rhew, Yip, & Edelstein, 2006). The problem of unbearable discomfort is associated with the frequent use which is necessary to produce a therapeutic effect (Misery, 2007). Misery (2007) further reported that many patients are not compliant because of the resulting chemo-inflammation. Bleomycin is yet another chemotherapeutic agent employed but is

associated with many side effects including moderate to severe local pain, moist desquamation, allergic reaction and even death due to toxicity. (Watring et al., 1978)

Systemic chemotherapy is another treatment option. Chemotherapy drugs are injected into the veins, muscle, and spinal fluid or taken by mouth. The effect of the drugs is seen on the whole body. Thus, this option also has many undesirable side effects. The type of drug, dose of drug and length of administration, all play a role in the potency of the therapy. The most effective regime has not yet been formulated. There have been cases of complete responses to mitomycin C and 5-fluorouracil. However, total recovery using systemic chemotherapy has been rare. (Thirlby, Hammer, Galagan, Travaglini, & Picozzi, 1990)

The literature has a few promising reports of the use of photodynamic therapy (PDT). This entails the use of a tumor localizing photoreactive drug (e.g. 5-amino - levulinic acid) in combination with light of an appropriate wavelength to kill tumor cells. Shieh et al. (2002) reported complete responses in only three patients in a study using a combination of PDT and topical or systemic chemotherapy.

Laser therapy was recommended for use in treatment with EMPD (Becker-Wegerich et al., 1998). The hope was that it would prevent recurrence as well as save the anatomy of affected areas, especially the genital area. The majority of EMPD patients with genital involvement under go such severe mutilation of that part of their body, whether from the disease or treatment, that patient confidence is severely impacted.

Unfortunately, it was found that laser therapy alone did not reduce the recurrence rates (Becker-Wegerich et al., 1998). Becker-Wegerich, et al. (1998) theorized that this could be due to the multicentric nature of EMPD.

There is no one definitive treatment option for EMPD noted in the literature (Curtin, Rubin, Jones, Hoskins, & Lewis, 1990; Kodama et al., 1995). Regardless of the treatment choice utilized, the high recurrence rate is always a major possibility (Shepherd, Davidson, & Davies-Humphreys, 2005).

Prognosis

The most important prognostic factor for EMPD involves depth of invasion (Burrows, Hudson, & Pye, 1995; Goldblum & Hart, 1997, 1998; Shepherd et al., 2005). EMPD confined to the epidermis has an excellent prognosis. Early detection is essential. Invasion of underlying structures by EMPD worsens the prognosis (Goldblum & Hart, 1997, 1998). The prognosis is very poor if there is lymphovascular invasion. There is a 33% five year survival rate in the presence of inguinal lymph node metastases (Parker et al., 2000). The prognosis of EMPD resulting from an underlying carcinoma is dependent on the prognosis of that tumor. The establishment of an underlying carcinoma is generally worse than the prognosis of primary EMPD (Costello, Wang, Schnitt, Ritter, & Antonioli, 1988; Geisler et al., 1997; Lloyd, Evans, & Flanagan, 1999).

Nonetheless, all EMPD patients require long term follow up (Shepherd et al., 2005). There have been instances of recurrences more than 15 years after initial treatment (Zollo & Zeitouni, 2000). The focus of the follow up is to detect not only local recurrence but the possible development of internal malignancies (Shepherd et al., 2005).

Histopathological Description

EMPD has a distinctive histological appearance. Neoplastic cells showing glandular differentiation are seen infiltrating the epithelium (Sitakalin & Ackerman, 1985). Paget's cells are large round cells with abundant cytoplasm [Figure1]. The cytoplasm appears pale. It is basophilic or amphophilic and finely granular. The nucleus is vesicular, may be centrally or laterally located, and show considerable nuclear atypia and pleomorphisms. Paget's cells stand out clearly in contrast to the surrounding epithelial cells. In greater than 90% of EMPD cases, the tumor cells also contain cytoplasmic mucin, staining positively with mucicarmine and the periodic acid – Schiff (PAS) reaction. (Jones, Austin, & Ackerman, 1979)

Paget's cells may be dispersed singly or they may be grouped forming glandular structures, clusters or solid nests within the epidermis and epithelia of adnexal structures. There are frequently mitotic figures and signet cells present. Most of the Paget's cells are generally located in the lower strata of the epidermis. Cells may be observed in sweat glands. This could lead to confusion as to whether the Paget's cells arose from the sweat

glands or invaded from the epidermis. Paget's cells in the upper dermis are rare. (Sitakalin & Ackerman, 1985)

Typically, the upper dermis is penetrated with a dense array of inflammatory cells macrophages, lymphocytes, plasmocytes, and eosinophilic polymorphonuclear cells. There may be areas exhibiting hyperkeratosis, acanthosis, and parakeratosis. (Jones, Austin, & Ackerman, 1979; Sitakalin & Ackerman, 1985)

Differential Histopathological Diagnosis

The unique pattern of spread of Paget's cells within the epidermis, either singly or in clusters has resulted in the use of the term "pagetoid spread". Any condition that exhibits this arrangement has been histologically described as having a "pagetoid spread". Many skin malignancies, to include and not be limited to superficially spreading malignant melanoma, Bowen's disease, mycosis fungoides, Langerhans cell histiocytosis, and Spitz naevus, exhibit "pagetoid spread". All these pathological conditions also affect areas of the skin in which EMPD has been reported. (Lloyd & Flanagan, 2000)

Conventional histochemical stains differentiate primarily on a morphological level. Often, EMPD may be misdiagnosed as one of the other aforementioned diseases. Delayed diagnosis with wrong treatment can have adverse effects on patients with EMPD. With the advent of immunohistochemistry, Paget's cells can be differentiated by unique immunohistochemical markers to include Cam 5.2, EMA, and CEA. Cam 5.2 is a

combination of antigens that are low molecular weight cytokeratins. EMA is epithelial membrane antigen. CEA is the carcinoembryonic antigen which is a glycoprotein. Immunohistochemistry, though comparatively more expensive, significantly reduces the possibility of misdiagnosis. (Lloyd & Flanagan, 2000; Shepherd et al., 2005)

Differential Immunohistochemical Diagnosis

Misdiagnosis of EMPD can be very detrimental. For the very reason that treatment options for the different neoplastic diseases can be quite different, the wrong choice of treatment can be life – threatening. Early detection, and by extension prognosis, becomes a matter of proper diagnosis. (Shepherd et al., 2005)

The use of conventional histological staining techniques, such as hemotoxylin and eosin, is not adequate to differentiate between Paget's cells and pagetoid cells, since both are morphologically similar. Histochemical techniques such as periodic acid – Schiff, alcian blue, or Zirconyl hematoxylin offer some improvement in the diagnosis (Jones, Austin, & Ackerman, 1979; Sitakalin & Ackerman, 1985). Vanstapel et al. (1984) reported the feasibility of immunohistochemical staining for the differential diagnosis of Paget's disease. Today, immunohistochemistry is widely used in diagnosis.

There are number of immunohistochemical markers that have been used in staining for Paget's cells that include cytokeratins, gross cystic disease fluid protein, growth factor receptor and mucins (Guarner, Cohen, & DeRose, 1989; Jones, Spaull, &

Gusterson, 1989; Liegl et al., 2007). Kondo, Kashima, Daa, Nakayama, and Yokohama (2002) noted that monoclonal antibodies against epithelial mucin 1 (MUC1) are useful in the diagnosis of EMPD. Yoshii et al. (2002) also confirmed that the use of monoclonal anti-MUC1 as a potent diagnostic tool in identifying Paget's cells. Liegl et al. (2007) concluded after a study of over eleven immunohistochemical markers, that MUC1 was the most useful diagnostic marker and was expressed in almost all 23 cases of EMPD tested.

MUC -1 is a transmembrane glycoprotein that is expressed in normal ductal epithelial cells as well as adenocarcinomas of various tissues. MUC-1 has several glycosylation sites. It has a unique extracellular domain that consists of a variable number of tandem repeats of 20 amino acids. The degree of glycosylation varies between normal and malignant cells. However, the latter express underglycosylated epitopes. As a result, the epitope profile of MUC-1 on a malignant cell is different than that on a normal cell. (Brugger et al., 1999)

The use of monoclonal anti-MUC1 along with other immunohistochemical tumor markers can help differentiate a case of EMPD from Bowen's disease (Cohen, Guarner, & DeRose, 1993; Guarner, Cohen, & DeRose, 1989; Guldhammer & Norgaard, 1986; Helm, Goellner, & Peters, 1992; Hitchcock et al., 1992; Jones, Spaull, & Guterson, 1989; Nakamura et al., 1995; Ordonez, Awalt, & Mackay, 1987; Reed, Oppedal, & Eeg Larsen, 1990; Roth, Lee, & Ehrlich, 1977). Bowen's disease, like EMPD, is a neoplastic skin condition. The cancer cells are atypical and invade only the epidermis. These cells are

referred to as pagetoid because of their large size with glass-like cytoplasm. The tumor cells can also be multinucleated similar to Paget's cells in EMPD. If the atypical cells are seen as squamous instead of glandular, EMPD can be misdiagnosed as Bowen's disease. Also like EMPD, the epidermis in cases of Bowen's disease exhibits hyperkeratosis, parakeratosis, and acanthosis. A misdiagnosis can have detrimental side effects for the patient (Quinn, Sienko, Basrawala, & Campbell, 2004). Superficially spreading malignant melanoma is another disease that can be differentiated from EMPD by use of monoclonal antibodies raised against MUC1. Melanoctyes found in superficially spreading malignant melanoma cannot be labeled with anti-MUC1 (Bacchi, Goldfogel, Greer, & Gown, 1992)

Histochemical staining of superficially spreading malignant melanoma, Bowen's disease and EMPD, all exhibit the pagetoid spread, and thus can appear morphologically similar. In view of that, the importance of immunohistochemical staining of tumor markers, adds a beneficial differentiating advantage - an advantage that avoids the patient the personal and financial expense of misdiagnosis. (Cohen, Guarner, & DeRose, 1993; Guarner, Cohen, & DeRose, 1989; Guldhammer & Norgaard, 1986; Helm, Goellner, & Peters, 1992; Hitchcock et al., 1992; Jones, Austin, & Ackerman, 1979; Jones, Spaull, & Guterson, 1989; Nakamura et al., 1995; Ordonez, Awalt, & Mackay, 1987; Reed, Oppedal, & Eeg Larsen, 1990; Roth, Lee, & Ehrlich, 1977)

Epithelial Mucin One (MUC1)

Mucins have been studied for many decades as a large part of the field of glycobiology. In the 1970s, mucins were described as a group of glycoproteins found in secreted mucus lining the surfaces of glandular epithelia. The structural model that exemplified mucins was that from ovine and bovine submaxillary glands. These glycoproteins were proposed to be very high in carbohydrate (over 50% of total weight) with a threonine/serine rich peptide core. (Hanisch & Muller, 2000)

Mucins were described as having a "rod-like" confirmation. They were also high in buoyant density. On this basic structure, variations in mucins could be formed by addition of different oligosaccharides. According to Hanisch and Muller (2000), the hydrophilic coating of O – linked negatively charged gylcans was the ideal structural model which supported the lubrication and protective function of mucins on epithelial surfaces.

With the advent of better research instrumentation and methodology in structural chemistry, several groups were able to demonstrate that mucin glycosylation played a major role in cancer biology. The normal polarized epithelium expressed mucins solely on the apical side, toward the lumen of the organ. Soluble mucins were also secreted in the lumen of the organ. With malignancy of the epithelium, mucins were expressed on all cell surfaces and the soluble mucins were able to enter the extracellular spaces and body fluids. (Burchell et al., 1987; Hanisch, Egge, Peter-Katalinic, & Uhlenbruck, 1986;

Hanisch et al., 1985; Hilkens et al., 1984; Hounsell et al., 1985; Lamblin et al., 1984; Magnani, Steplewski, Koprowski, & Ginsburg, 1983; Mutsaers, van Halbeek, Vliegenthardt, Wu, & Kabat, 1986)

More importantly, mucins from cancerous cells were found to have aberrant glycosylation (Brugger et al., 1999). In many instances, aberrant glycosylation in carcinomas were seen in mainly the mucins. Glycosylation was usually incomplete or in extreme cases, non –existant. The most significant breakthrough however, was the identification of tumor- related epitopes on mucins as immunotargets on malignant epithelial cells and their secretions. Monoclonal antibodies were generated to detect mucins from carcinoma. (Finn et al., 1995; Price et al., 1998)

The first human mucin gene was sequenced at the DNA level in 1990. It was first identified in human milk. It was named MUC1, an abbreviation for epithelial mucin one. It is also known as polymorphic epithelial mucin (PEM), the polymorphic urinary mucin (PUM), episialin, epithelial membrane antigen, and DF3 antigen. (Gendler et al., 1990; Lan, Batra, Qui, Metzgar, & Hollingsworth, 1990; Ligtenberg, Vos, Gennissen, & Hilkens, 1990; Wreschner et al., 1990)

Structurally, MUC1 is a high weight transmembrane molecule with a polymorphic protein core containing a large domain of a variable number of highly conserved tandem repeats composed of 20 amino acids (AHGVTSAPDTRPAPGSTAPP) that are normally heavily O – glycosylated. It is proposed that this glycosylation process

is done sequentially resulting in elongated carbohydrate chains. Glycosylation of the core protein shows organ and differentiation. (Gendler et al., 1990)

MUC1 has a widespread organ distribution – mammary glands and acini, salivary gland ducts and serous acini, squamous cell epithelia of the esophagus, canaliculi and peptic cells of the stomach, acini and ducts of the pancreas, bile ducts of the liver, enterocytes of the duodenum, parietal cells, respiratory and ciliated epithelium of the lungs, serous bronchial glands, distal tubules of the kidney but not the proximal tubules, bladder urothelium, prostate gland epithelium, resting endometrium of the uterus, rete testis and activated mesothelium. Skin epithelia, mesenchymal tissue, and normal epidermis, including the non – keratinizing epidermis of the genitalia never contain MUC1. (Hanisch & Muller, 2000)

The MUC1 glycoprotein is an epithelial luminal surface glycan. There it serves as lubricant for the epithelial surfaces and as a physical protective barrier for entraping particulate or cellular debris, qaa function that is typical of mucins (Hanisch & Muller, 2000). The cytoplasmic tail of MUC1 has been proposed to be involved in cell signaling (Pandey, Kharbanda, & Kufe, 1995). This function is not typical to mucins. While the actual mechanism of MUC1 cell signaling is still not clear, there is some evidence that it may be particularly involved with cell adhesion (Yamamoto, Bharti, Li, & Kufe, 1997).

MUC1 has become increasingly important in cancer biology. A major change in the MUC1 molecule in carcinomas is the varying degrees of glycosylation (Brugger et

al., 1999). Commonly, varying degrees of glycosylation can occur in one type of cancer. Generally, glycosylation is reduced or completely non-existent in malignant cells (Finn et al., 1995). This results in the demasking of normally cryptic peptide and carbohydrate structures. This is a physical alteration that raises questions as to the potential immunohistochemical consequences of such a change. For example, in breast cancer the chain lengths of the O-linked glycans on MUC1 are restricted to the core-type level and sialylated glycans predominate over neutral ones. (Hanisch & Muller, 2000)

Moreover, the topological change of mucins, like MUC1, in carcinoma is claimed to be a facilitator of tumor invasion and metastasis. The aberrant MUC1 is believed to sterically interfere with the functions of adhesion molecules such as integrins and cadherins. It is suggested that due to this interference, mucins such as MUC1, may play a role in the initial displacement of cells that result in metastasis. It has also been proposed that mucins also block adhesion between blood-borne carcinoma cells and the host cytolytic cells such as natural killer cells. In addition, mucins may mask expression of antigenic peptides by major histocompatibility complex molecules. (Hilkens, Ligtenberg, Vos, & Litvinov, 1992; Sherblom & Moody, 1986; Wesseling, van der Valk, Sonnenberg, & Hilkens, 1995; Wesseling, van der Valk, & Hilkens,1996)

In light of all these observations, the overexpression of MUC1 by carcinomas is a primary target for tumor defense strategies (Hanisch & Muller 2000). Yoshii et al. (2002) reported a strong correlation between the expression of mucin antigens and patient survival. Their studies of carcinomas of Vater's ampulla (junction of the common bile

duct and main pancreatic duct) and stomach patients expressing MUC1 had significantly worse survival rates than those not expressing MUC1. Croce, Rabassa, Price, and Segal-Eiras (2001) noted that MUC1 has been a widely demonstrated immuhistochemical tumor marker and thus a likely candidate for anti-cancer vaccine development.

Currently immunohistochemical identification of MUC1 is done using monoclonal antibodies (Croce, Rabassa, Price, & Segal-Eiras, 2001; Liegl et al., 2007). As previously mentioned, MUC1 is usually underglycosylated in malignant cells (Brugger, et al., 1999). Futhermore, many variations of glycosylated forms of MUC1 antigens can be present in one type of cancer (Finn et al., 1995). Therefore, there is the question whether these aberrant forms of underglycosylated MUC1 in tumor cells cause the variation in binding affinity to available monoclonal antibodies.

Monoclonal Antibodies to MUC1

By the mid 1990s, a plethora of monoclonal antibodies to the new tumor marker MUC1 was available. Comparison and standardization of these new antibodies were needed. The International Society for Oncodevelopmental Biology and Medicine (ISOBM) TD-4 International Workshop on Monoclonal Antibodies against MUC1 in 1996 became a landmark in the field. The workshop was able to compare fifty-six supposedly MUC1-specific monoclonal antibodies and to map their epitopes. (Price et al., 1998)

Commercially, two main types of monoclonal antibodies to MUC1 are available for diagnostic use. MabT are monoclonal antibodies raised against the 20 amino acid tandem repeat of the core protein. MabW are monoclonal antibodies raised against the native whole glycoprotein.

Thus with the multitude of available monoclonal antibodies to MUC1 and the characterization of these antibodies by the ISOBM TD-4 International Workshop, study groups began investigating binding affinity in tumor conditions and to synthetic and recombinant peptide antigens of MUC1. As can be imagined, the number of different tumors expressing MUC1 is vast. On top of that, MUC1 may not be expressed in the same form in the same tumor (Finn et al., 1995). Much work is needed in this field to study the binding affinity of MUC1 antibodies in as many neoplasmic systems as possible.

Price et al. (1998) reported, after comparing the 56 monoclonal antibodies against the MUC1 in the ISOBM TD-4 International Workshop, that MabT and MabW did not show any significant binding differences to the native fully glycosylated MUC1 mucin. The group did note however, that a few MabT bound poorly to the complete glycoprotein. This was explained as possibly due to masking of some peptide epitopes by the lengthy carbohydrate chains of the mucin.

Karsten and Cao (2001) studied the binding affinities of 51 monoclonal antibodies to peptide and carbohydrate epitopes of the epithelial mucin (MUC1) on tissue sections

of Warthin's tumors - benign tumors of the salivary glands. They reported that gylcosylation had profound impact on immunohistochemistry. Different staining patterns were observed by the monoclonal antibodies and this was again explained by eptiope masking by gylcan chains. This demonstrated that the choice of monoclonal antibody could give various staining patterns based on glycosylation of the tumor invovled.

Three years later, Karsten, Serttas, Paulsen, Danielczyk and Goletz (2004) published results involving binding patterns of eleven MabT and eleven MabW against a synthetically derived form of a glycosylated and non-glycosylated peptidic epitope of MUC1. They hypothesized that there would be no difference between MabT and MabW in binding to either the glycosylated epitope or the non-glycosylated one. Their results showed otherwise. The presence or absence of glycosylation splits the antibody population into two groups. All MabW bound better to the glycosylated epitope than to the naked peptide. On the other hand almost all MabT were glycosylation-independent.

Croce, Rabassa, Pereyra and Segal-Eiras (2005) compared *in vitro* the binding affinity of three monoclonal antibodies in enzymatically desialylated and native sialylated samples. One monoclonal antibody was raised against desialylated MUC1. The second type of monoclonal antibody was raised against the native sialylated MUC1. The third type of monoclonal antibody was raised against a four amino acid epitope of MUC1. The results revealed that desialylation improved affinity of monoclonal antibodies against desialylated MUC1 for tumor mucin. However, desialylation had little effect on affinity

of monoclonal antibodies for the sialylated mucin or on the affinity of monoclonal antibodies against a naked four amino acid epitope.

In light of these studies, it is clear that there is much controversy over the binding affinity of monoclonal antibodies to the MUC1 antigen. Critically, all three studies were done in vitro and it is not clear whether or not these results can be replicated on human tissue. Is the core protein the sole epitope recognized by monoclonal antibodies raised against MUC1? In other words, would there be a difference in binding affinity of MabW to an aglyconic form of MUC1 as compared to MabT. Karsten et al. (2004) suggest that there would be a significant difference. Their results propose that the MabT would bind better than the MabW. At the other end of the spectrum, Price et al. (1998) indicate that there should be no significant difference.

A rare case of EMPD whose Paget's cells containing aglyconic MUC1 created the exceptional opportunity to qualitatively study binding affinity of the two commercially available types of monoclonal antibodies, MabT and MabW. Though the experiment is novel, this study design is not unique in comparative antibody research. Literature review has revealed that several studies have employed this design. Reis, David, Seixas, Burchell, & Sobrinho-Simoes, 1998 investigated the immunohistochemical staining by one MabT and one MabW in 73 cases of gastric carcinoma and 180 cases of gastric carcinoma respectively. Croce et al. (2001) performed immunohistochemical staining on sections from twenty-nine patients representing six cancer types to compare the binding patterns of one monoclonal antibody to MUC1.

Baldus, Goergen, Hanisch, and Dienes (2001) compared the binding affinity of eight monoclonal antibodies raised against MUC1 for sections from ten blocks representing four different cancer types. Some of the cancer types studied by Croce (2001) and Baldus et al. (2001) were represented by a single block. This study, however, is the first to use significant numbers of MabTs and MabWs to study their affinities for aglyconic MUC1 in tissue sections.

There are many forms of MUC1 antigens in one type of cancer. Often, the MUC1 antigen exhibits varying degrees of glycosylation within the same malignancy (Finn et al., 1995). Thus, comparative studies of binding affinity of MabT and MabW to a specific MUC 1 antigen in a carcinoma, has not been explored. This study is unique in that the form of the MUC1 antigen in the rare case of EMPD is the aglycone. There is no glycosylation present. Thus, a comparative study between MabT and MabW binding affinity to one type of MUC 1 antigen from a carcinoma can be done. The investigation of the binding affinity of MabT and MabW for specific MUC1 antigen has significant implications on the use of monoclonal antibodies as diagnostic tools. If there is a significant difference in the binding affinity of the two types of monoclonal antibodies for a specific MUC1 antigen, then the choice of monoclonal antibody used for making a diagnosis must be done with care to avoid misdiagnosis.

This study, therefore, attempts to examine the effect of glycosylation on antigenicity. It adds to the increasing knowledge of the MUC1 antigen which has already

been associated with tumor progression, metastasis rate, and patient survival in all tumors (Hanisch & Muller 2000).

Hypotheses

There is no difference between the binding affinity of MabW and MabT to the aglycone of MUC1.

There is a difference between the binding affinity of MabW and MabT to the aglycone of MUC1.

Materials

Tumor Sample

One paraffin-embedded - formalin-fixed block of EMPD tissue, containing an aglyconic form of the MUC1 antigen was used. EMPD has a very low incidence and so having a rare form of a rare disease creates a unique opportunity for comparative antibody research (Parker et al., 2000).

Monoclonal Antibodies

The four pairs of mouse monoclonal antibodies to MUC1 were purchased from commercial manufacturers. Each pair (MabT and MabW) was bought from the same company. The first pair, SM3 and GP1.4, was bought from Abcam (Cambridge, MA, USA). The immunogen used in SM3 production was the hydrogen fluoride deglycosylated milk mucin. Its concentration was 1.0 mg/ml. The immunogen used in GP1.4 production was human milk fat globule membranes. Its concentration was 0.3mg/ml.

The second pair of monoclonal antibodies, ZCE113 and VU4H5, was bought from Zymed Laboratories (Carlsbad, CA, USA). The immunogen used in ZCE113 production was cream from human milk. Its concentration was 0.012 mg/ml. The immunogen used in the production of VU4H5 was non-glycosylated 60-mer MUC1 triple tandem repeat peptide. Its concentration was 5.5 mg/ml.

The third pair and fourth pair of monoclonal antibodies came from US Biological (Swampscott, MA, USA). The third pair was clones 3G145 and 3G196. The immunogen used in the production of 3G145 was delipidated human milk fat globules. Its concentration was 5.0 mg/ml. The immunogen used in the production of 3G196 was non-glycosylated 60-mer MUC1 triple tandem repeat peptide. Its concentration was 3.4 mg/ml.

The fourth pair of clones was 0.N.272 and 0.N.273. The immunogen used in the production of 0.N.272 was a 60-mer tandem repeat. Its concentration was 0.2 mg/ml. The immunogen used in the production of 0.N.273 was human fat globule membranes. Its concentration was 0.1 mg/ml.

The monoclonal antibodies were all stored at 4°C and used within a week of arrival. This small sample size of monoclonal antibodies raised against MUC1 antigen was due to both limited commercial availability and high cost.

Controls

The control slides were kidney sections obtained from B.B.C. Biochemical (Stanwood, WA, USA). The tissue had already been fixed in 10 % neutral buffered formalin. The sections were paraffin embedded and had been cut at 5 μm and dried for 60 minutes at 60°C. The sections were then placed on aminosilane slide with no cover slip.

These served as both negative and positive controls. The distal tubules of the kidneys contain MUC1 antigens. The proximal tubules do not contain MUC1 antigens.

Immunohistochemical Kits

The Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA) was used. This contains Avidin DH and biotinylated horseradish peroxidase H reagents which have been specifically prepared to form an ideal complex (ABC complex) for immunoperoxidase activity. Avidin binds the peroxidase to the biotin conjugated secondary (horse anti-mouse) antibody. The kit also contains the biotinylated, affinity-purified anti-mouse immunoglobulin that this complex attaches to. The biotinylated, affinity-purified anti-mouse immunoglobulin attaches to the anti-MUC1. This is the standard avidin-biotin complex (ABC) method.

The Vector Nova Red Substrate Kit, for Peroxidase (Vector Laboratories, Burlingame, CA, USA) was used to stain the tissue sections once the ABC complex had been added. The Nova Red serves as a substrate for the peroxidase. Vector Nova Red produces a red-brown reaction product [Figure 2].

Method

Tissue preparation

Paraffin sections from the block of aglyconic EMPD tissue were cut using a standard rotary microtome. The sections were cut at 7 μm. These tissue sections were

then placed on pre-albumenized slides, and then heated on a heating plate at 40 °C for 48 hours to allow for drying and adhesion. The control slides were purchased already prepared and dried.

Tissue Staining

Paraffin was removed from the tissue in two consecutive xylene solutions for 10 minutes each. Xylene was washed out in two changes of 100% ethanol and one change of 95% ethanol for 5 minutes each. The slide was then immersed in 0.3% hydrogen peroxide in methanol (Appendix A) for 30 minutes to block any endogenous peroxidase activity. Thereafter, the slide was washed twice with phosphate buffered saline also known as PBS (Appendix A), at pH 7.4 for five minutes. The slide was placed in a hydration chamber [Figure 3] where the tissue was covered with two drops (100 µl) of antibody diluting solution (Appendix A) from the Vectastain ABC kit. The hydration chamber was made up of a closed Petri dish with damp filter paper (Whatman Ltd, England, UK) fitted in the bottom.

The slide was left in the hydration chamber to incubate with the diluted normal serum for 30 minutes. Application of normal serum in a relatively high concentration is a blocking mechanism to prevent non-specific binding. All the steps mentioned above were carried out at room temperature (approximately 25°C).

It was at this time that desired dilutions of the primary antibodies were carried out. Each antibody would be diluted initially to a 1:100 concentration of antibody diluting solution. The diluted solutions of the monoclonal antibody were stored at 5°C until needed. It was imperative that dilutions of antibodies and the concentrated antibodies were not out of 5°C for extended periods of time. Solutions containing antibodies were not left at higher than 5°C temperature for more than two minutes.

At the end of the non-specific antibody blocking step, the primary antibody at the predetermined dilution (initially 1:100) was added. The tissue in the hydration chamber was covered with 50 µl (1 drop) of diluted antibody. The hydration chamber was then quickly placed in a refrigerator to incubate for 12 hours at 5°C.

At the end of the 12-hour 5°C incubation period, the hydration chamber was placed at room temperature again. The slide was washed with PBS at pH 7.4 three times for five minutes each. The slide was placed back in the hydration chamber and two drops (100 µl) biotinylated horse anti-mouse antibody from the Vectastain ABC kit was applied. This was allowed to incubate for 30 minutes in the hydration chamber at room temperature.

At the end of the 30 minute incubation, the slide was washed three times for 5 minutes with PBS at pH 7.4 for five minutes. Two drops (100 µl) of the Vectastain ABC complex were applied to the tissue in the hydration chamber and left to incubate for 30

minutes at room temperature. Then, the slide was washed with PBS at pH 7.4 for five minutes.

About two minutes before the end of this five minute period, the Nova Red substrate is prepared as per instructions of the kit. The Nova Red substrate was made as close as possible to use, because the substrate is oxidized on prolonged exposure to air.

50 µl of the Nova Red substrate was added and the slide was incubated for 15 minutes in the hydration chamber. It was then washed three times with distilled water for a total of five minutes. The slide was counterstained with Ehrlich's hematoxylin for two minutes. This gives a blue contrast.

The slide was washed in distilled water for five minutes after staining with hematoxylin. It was dipped in lithium carbonate solution for one minute. This enhances the blue contrast coloration. The slide was then washed in tap water for three minutes. The slide was dehydrated again through a graded series of ethanol [70 % (once); 95% (once); 100% (twice)] and then three changes of xylene for five minutes each. The slide was then mounted in Permount and cover-slipped. The slide was then heated on heating plate at 40°C for two days.

Control slides were used to test the specificity of all monoclonal antibodies used. Usually a 1: 100 primary antibody dilution was used depending on staining intensity.

Only the control slide for VU4H5 showed staining at a 1:10 dilution. Again, only the distal tubules of the kidney should stain red.

A negative control was already present in the kidney slide since only the distal tubules should stain positively. Two additional negative control slides were (i) the elimination of the addition of the primary monoclonal antibody step in the protocol and (ii) the elimination the secondary biotinylated horse anti-mouse serum step. There should be no detectable staining in either situation.

Limiting Dilution Method

After immunohistochemical staining of each the EMPD specimen by one of the eight antibodies with the initial 1:100 dilution, the entire tissue on the slide was then examined for red staining of the Paget's cells at X4 objective. If there was any visible staining, one layer of a two sided yellow beige plastic Slant binder pocket from Better Office Products Inc. (Canoga Park, CA, USA) was placed over the slide as in Figure 4. This binder was visibly orange though the manufacturer labeled it as yellow beige. The slide was then examined to see if staining was still visible through the one layer of orange plastic at X X4 objective. If staining was still visible, the procedure was repeated with increasing dilutions (1/200, 1/300, 1/400 etc.) until no staining was seen with one layer of the orange plastic. The affinity dilution at which staining was no longer visible was recorded for each antibody. Based on intensity of staining, dilutions were chosen out of sequence on some occasions. Only one investigator examined all slides.

If no staining was visible at the initial 1:100 dilution with an antibody, the procedure was repeated with decreasing dilutions (1/90, 1/80, 1/70 etc) until staining was visible in the Paget's cells. The slide was then examined with the one layer of orange plastic over the slide at X4 objective. The dilution result, at which visual extinction of the red staining occurred, was recorded. Fresh paraffin slides of EMPD tissue sections were used for each dilution. Based on intensity of staining, dilutions were chosen out of sequence on some occasions. Also all slides were examined by one investigator.

Final limiting dilution values for the eight antibodies were not repeated due to limited tissue availability for additional testing.

Statistical Analysis

Once all the limiting dilutions were found for all 8 monoclonal antibodies used, the actual concentrations at the limiting dilutions were calculated from the original company packaging concentrations.

Due to the fact that each pair of monoclonal antibodies were obtained from the same company, the Kruskal-Wallis nonparametric analysis of variance of ranks was used to test independence of the samples (Conover, 1980).

Since the data for MabW and MabT had different distributions, neither the t-test (Glass, Peckham, & Sanders, 1972; Thompson, Green, Chen, Stockford, & Lo, 2005) nor

the Mann- Whitney test (Conover, 1980) could be used. The Kolmogorov-Smirnov nonparametric test of agreement between two groups (MabW and MabT) was used. This is a nonparametric alternative to the t test for independent groups. The minimum group size required is three. Group one had 4 MabW and Group two had 4 MabT. This test is sensitive to all kinds of differences in the location and shape of empirical cumulative distribution functions between two independent randomly selected samples. Unlike the t test, the Kolmogorov-Smirnov test assumes neither a normal distribution of the sampled population nor an interval scale of measurement (Conover, 1980).

Using the calculated concentration data, cumulative empirical distribution function graphs were done for each group – MabT and MabW- on the same graph. The largest absolute difference (Dmax) between the two distribution graphs was found.

Let F(x) represent the empirical cumulative distribution function for MabW.

Let G(x) represent the empirical cumulative distribution function for MabT.

Therefore the null hypothesis would be represented as: H (null): $F(x) = G(x)$ This would mean that there is no difference between MabW and MabT binding affinity for the aglycone.

Results

Controls

The control slides for all the monoclonal antibodies stained only the distal tubules red. Figure 5 demonstrates the typical staining of a control slide by the monoclonal antibodies, using MabW ZCE113 at 1:100 as the model. The two negative control slides eliminating the primary antibody for one and the secondary antibody for the other did not show any red staining.

Dilution Results

The limiting dilution is the dilution at which the monoclonal antibody just barely stains the MUC1 aglycone in the Paget's cells. This was demonstrated by visual extinction by one layer of two-sided yellow plastic. Figure 6 illustrates the typical pale staining intensity obtained by the limiting dilution method, again using MabW ZCE113 as an example. All antibodies exhibited this pale staining at their respective limiting dilution concentration. Table 1 and 2 show the limiting dilution results for the monoclonal antibodies.

Kruskal- Wallis Test Result

An H statistic of 4.108 was found. This indicated that there was a 25% probability that the difference between the companies was due to chance, i.e. p = 0.25 Thus, the null hypothesis that the samples are unrelated cannot be rejected and the samples must be considered independent.

Kolmogorov-Smirnov Two Sample Test Result

As shown in Figure 7, the largest absolute difference (Dmax) between the two distributions was found to be 2/4. The null hypothesis is rejected if Dmax exceeds the 1-alpha quantile as given by a standard table. From the table of quantiles of the Smirnov test statistic for the two samples of equal size, the 0.95 quantile (of Dmax) for both sample sizes of 4 is given as 3/4. Therefore the null hypothesis is accepted at the 0.05 level, i.e. the difference between the affinity of MabT and the affinity of MabW for the aglycone of MUC1 is not statistically significant.

Discussion

This study presents the results of a comparative study of the binding of two types of monoclonal antibodies to an aglyconic form of epithelial MUC1 mucin in a rare case of extramammary Paget's disease (EMPD). The study attempted to determine if the

binding affinity of monoclonal antibodies raised against a tandem repeat polypeptide (MabT) of MUC1 differed from the binding affinity of monoclonal antibodies raised against the native glycoprotein (MabW).The purpose of the research culminating in this study is to present data to determine whether glycosylation affects antigenicity.

There is much controversy on the issue of glycosylation and antigenicity in the literature. Price et al. (1998) reported that there is no difference in the binding affinity for the native glycosylated MUC1 between the two types of monoclonal antibodies raised against the MUC1 antigen. More recent research, however, has refuted these findings. Karsten et al. (2004) found that glycosylation did affect the binding affinity of MabW but had no affect on binding affinity of MabT. The issue is debatable because Price et al. performed tests against the native fully glycosylated MUC1 antigen while Karsten et al. worked with recombinant and synthetic tandem repeats of the core protein. Price et al. and Karsten et al. worked with two different test systems.

MUC1 antigens exhibit such varying degrees of glycosylation that a study to determine whether glycosylation affects antigenicity would need a well characterized MUC1 antigen. The varying degrees of glycosylation of the MUC1 antigen that often occurs within one type of cancer make comparative antibody research complex and arduous. To add, binding affinity may vary between neoplasms. Thus, research done on binding affinity in one neoplasm may not correlate to similar results in another type of neoplasm. This is yet another challenge of comparative antibody research as there are numerous neoplastic conditions.

The opportunity presented by this study lies in the rare case of EMPD exhibiting an aglycone of the MUC1 antigen. This special situation allows for a comparative study of the two types of antibodies to be done on a well-characterized antigen in a known neoplasm. Although aglyconic EMPD is rare, the work done in this study can contribute to future work on the controversial topic of glycosylation and antigenicity.

Previous studies of antigen affinity for MUC1 have been dichotomous staining versus no staining studies at a single dilution. Thus, low affinity binding was missed altogether. The method employed in this study was use of the limiting dilution method to obtain a dilution measurement where the monoclonal antibody in question barely stained the tissue. The higher the dilution measurement, the more affinity the monoclonal antibody would have for the MUC1 antigen.

The choice of this visual assessment of binding affinity by staining was chosen because in diagnostic settings, the microscope is the diagnostic tool. So while quantitative methods may give a more accurate measurement, it serves as an informative device rather than a practical one. Additionally, the choice of using commercially available monoclonal antibodies was based on the same premise. Diagnosis will be made using commercially available antibodies.

This decision also created limitations on availability of monoclonal antibodies that were raised against the required immunogens. Commercially available monoclonal

antibodies are also quite expensive. Ultimately, a sample size of only four pairs of monoclonal antibodies was obtained.

Based on the use of four pairs of commercially available monoclonal antibodies consisting of monoclonal antibodies to the native glycoprotein of MUC1 and monoclonal antibodies raised against a tandem repeat polypeptide of MUC1, no significant difference was noted in binding affinity to an aglycone found in a rare case of EMPD. These results are consistent with Price et al. (1998).

However, the result of this study is not conclusive. Though the number of antibodies per group (MabW or MabT) was sufficient for significance, future research with a larger number of antibodies would be more conclusive. The study could also be extended to include more tumor tissue samples from different patients with the aglyconic MUC1 antigen. More tissue samples would not only increase the validity of the study but also facilitate repetition of limiting dilution result for each antibody.

The effect of glycosylation on antigenicity continues to be a complex issue. The work presented in this study attempts to help decipher this mystery of cancer biology. In the end, the hypothesis that there may be a difference between binding affinities of MabW and MabT for the aglyconic MUC1 was not supported. This implies that glycosylation does not affect antigenicity in a rare case of extramammary Paget's disease.

FIGURES

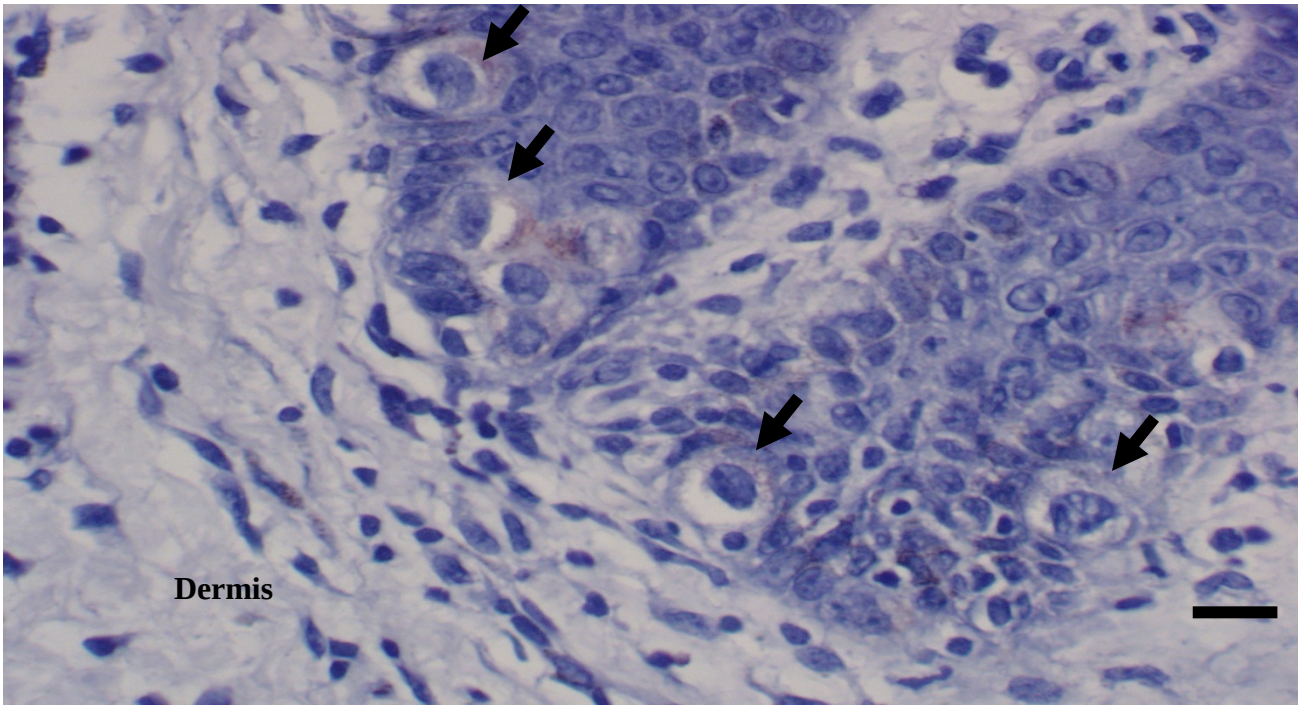

Figure 1. Hematoxylin and Eosin staining of intraepidermal Paget' cells (arrows) with atypical nuclei and pale abundant cytoplasm in the lower strata of the epidermis. Bar = 30 μm

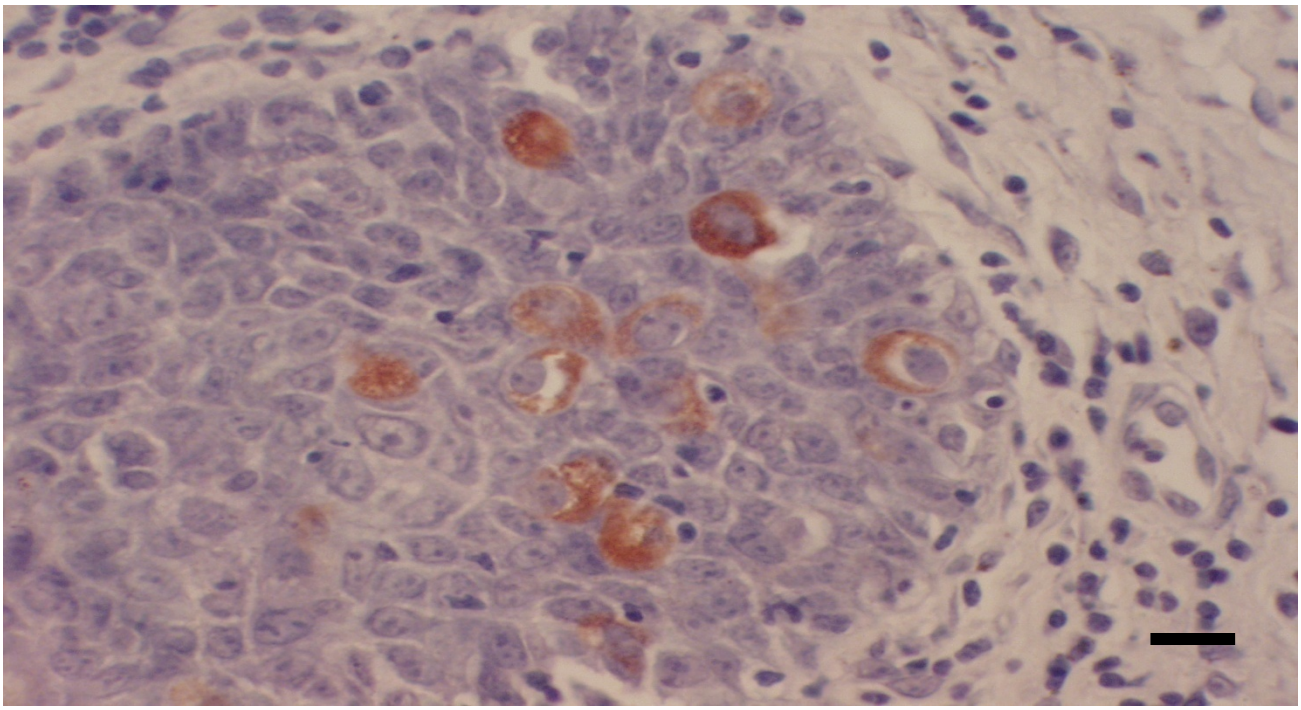

Figure 2. Paget's cells with Nova Red staining. This indicates presence of MUC1 antigen. Bar = 30μm

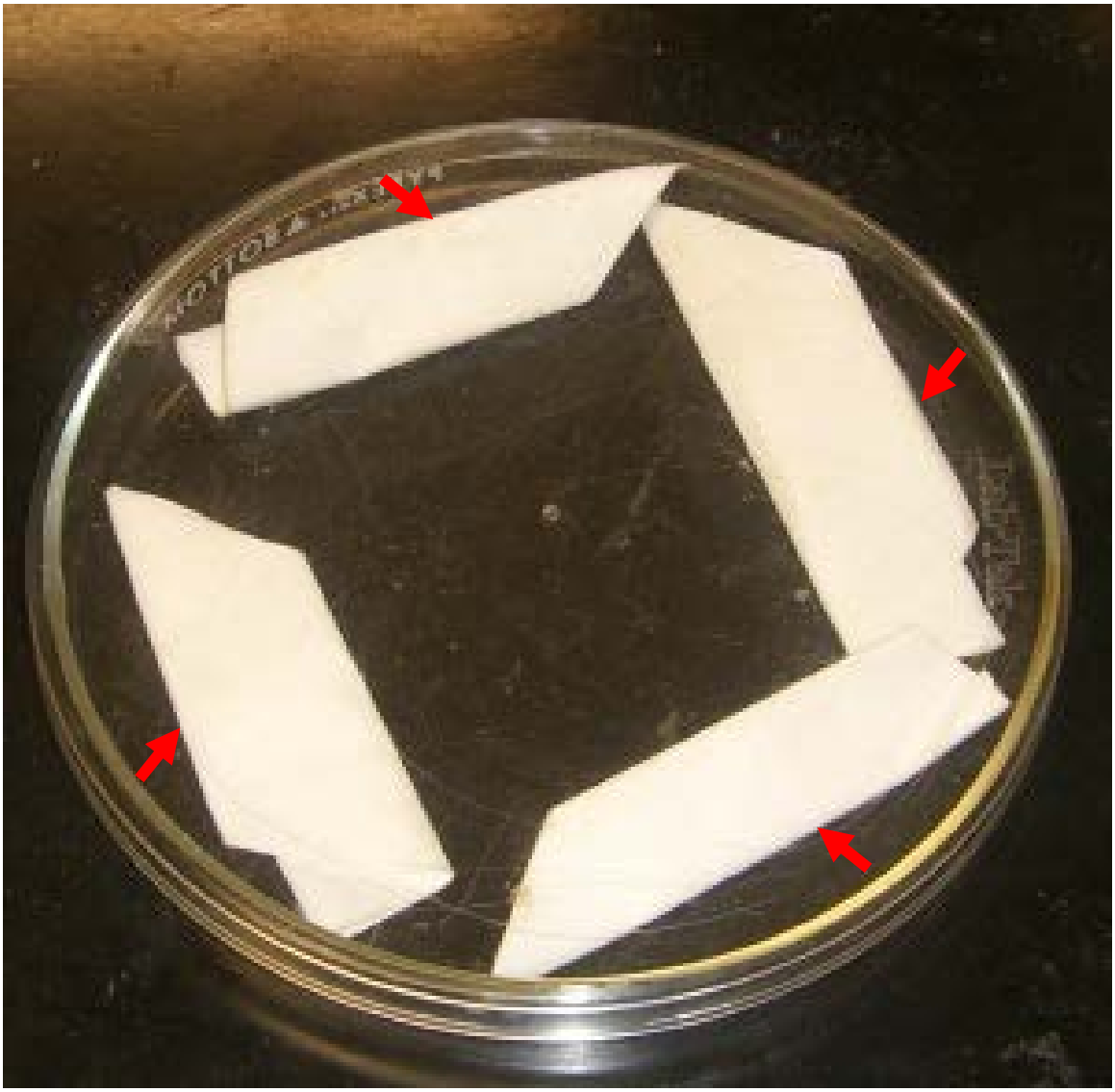

Figure 3. Hydration Chamber consisting of a covered petri dish and white folded filter paper (red arrows).

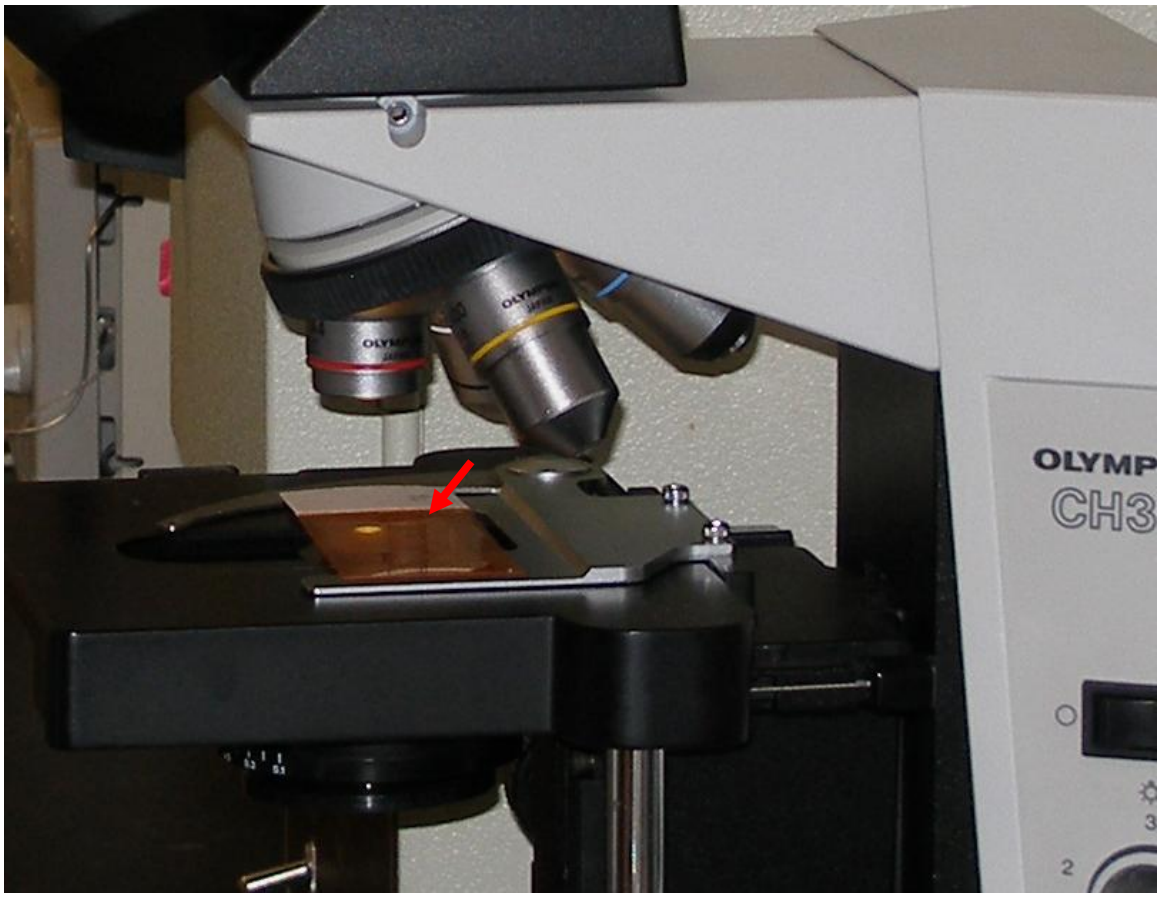

Figure 4. Photograph of arrangement of two sided orange plastic (arrow) over slide for examination under the microscope.

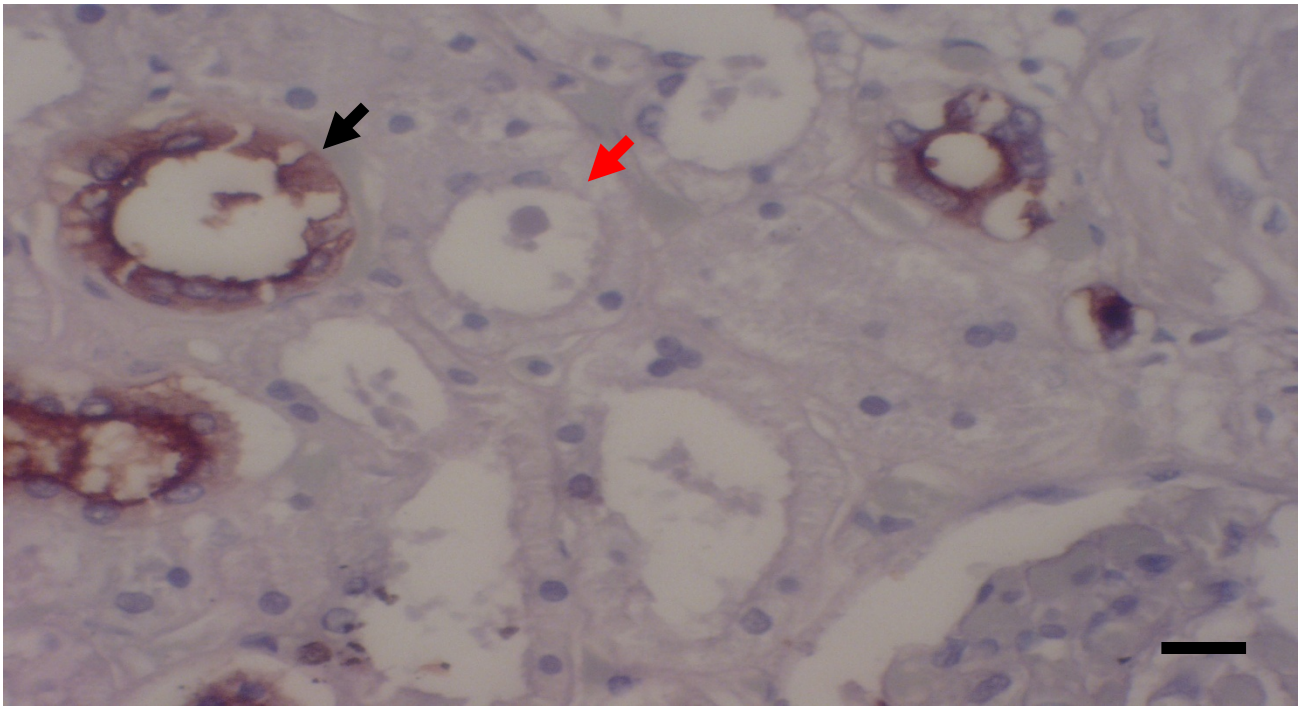

Figure 5. Control for MabW ZCE113 at 1:100 dilution. Only the distal tubules of the kidney (black arrow) are stained indicating the presence of the MUC1 antigen. The proximal tubules are not stained (red arrow). Bar = 30μm

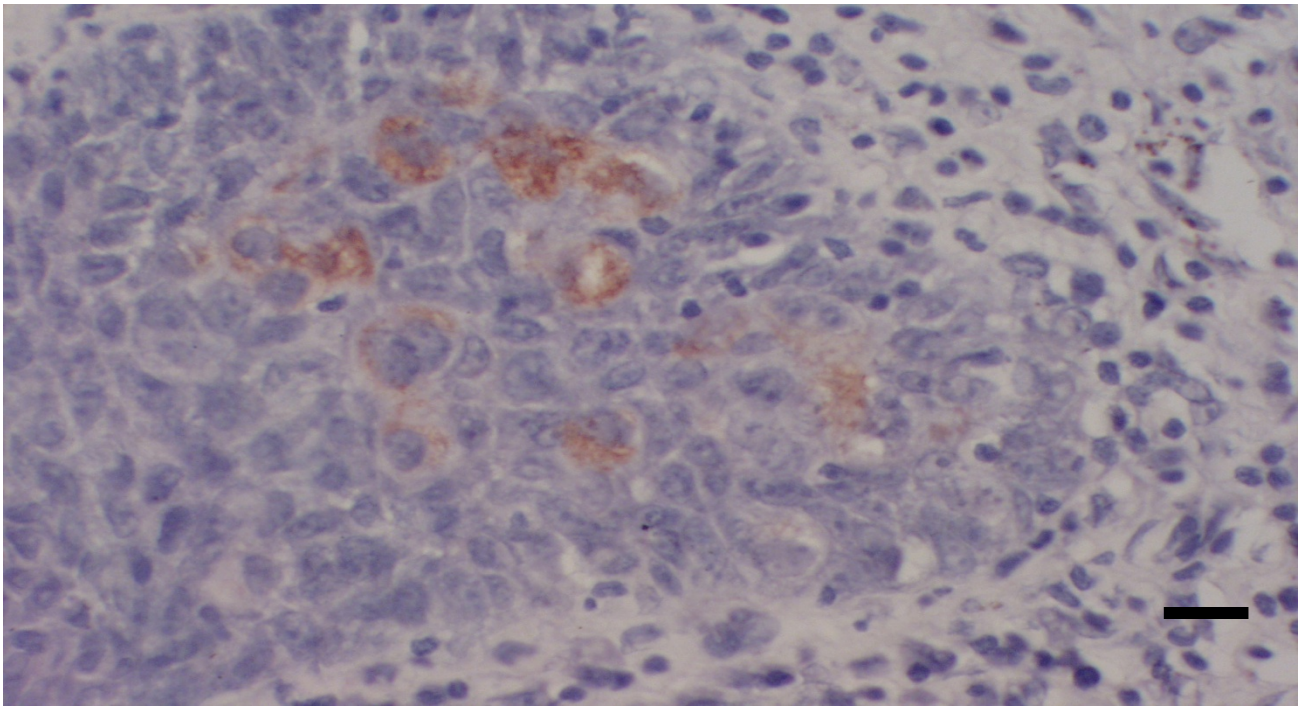

Figure 6. Limiting Dilution staining for MabW ZCE113 at 1:5000. Paget's cells are very lightly stained. Bar = 30μm

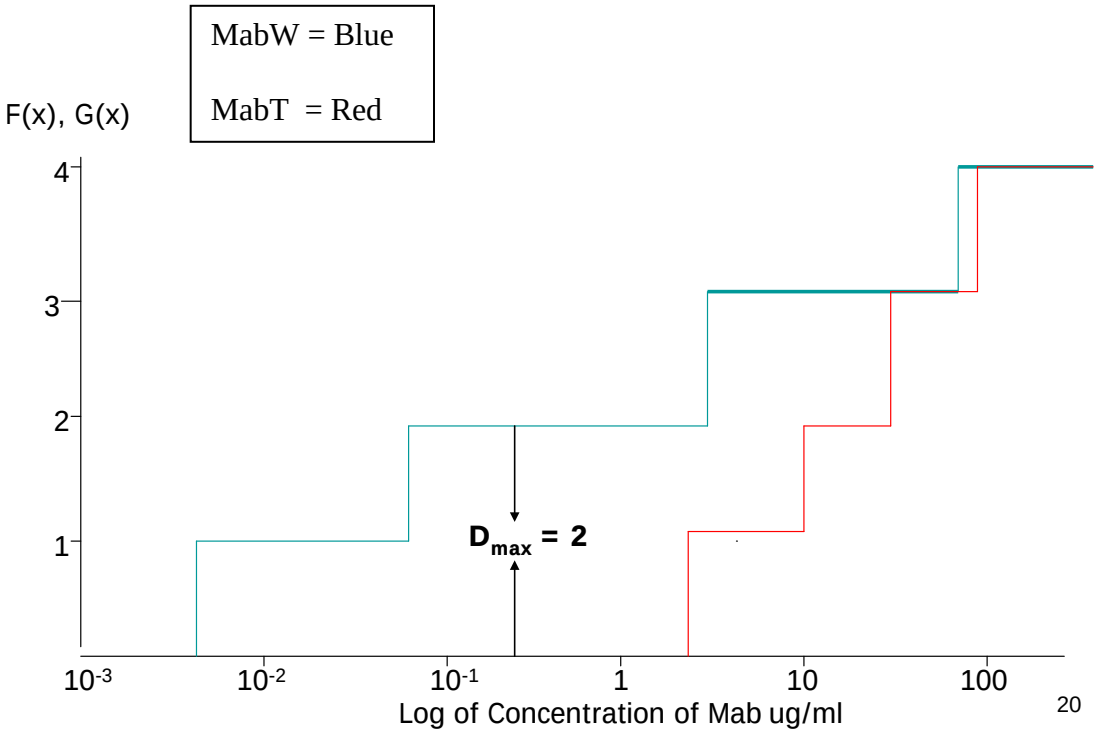

Figure 7. Graph of cumulative empirical function distributions for the two groups.

TABLES

Table 1

Limiting dilution results for the MabW clones.

Monoclonal Antibody	***Limiting Dilution***	***Antibody Concentration (µg/ml)***	***Dilutions Tested***
ZCE113	1:5000	0.0024	1/100 1/500 1/1000 1/3000 1/8000 1/16000
GP1.4	1:90	3.3000	1/100
3G145	1:90	55.5000	1/100 1/80 1/70
0.N.273	1:3800	0.0260	1/100 1/200

			1/300 1/400 1/2000 1/3000 1/3500 1/3900

Table 2

Limiting dilution results for the MabT clones

Monoclonal Antibody	***Limiting Dilution***	***Antibody Concentration (µg/ml)***	***Dilutions Done***
VU4H5	1:400	13.75	1/100 1/200 1/300 1/500
SM3	1:100	10.00	1/100 1/200
3G196	1:50	68.00	1/100

			1/40 1/60
0.N.272	1:100	2.00	1/50 1/80 1/90 1/200

Appendix A: Solutions

A. 1 Phosphate Buffered Saline [PBS]

400ml distilled water

0.11M sodium chloride (Fisher Scientific, Fair Lawn, NJ, USA)

0.002M monobasic sodium phosphate (Fisher Scientific, Fair Lawn, NJ, USA)

0.006 M dibasic sodium phosphate heptahydrate (Fisher Scientific, Fair Lawn, NJ, USA)

Mix until in solution

Adjust pH to 7.4

A. 2 0.3% Hydrogen Peroxide in Methanol

31.5 ml absolute methanol (Sigma-Aldrich, Milwaukee, WI, USA)

3.5 ml drugstore (3%) hydrogen peroxide (Walgreen Co., Deerfield, IL, USA)

Mix into solution

Use immediately

A. 3 Antibody Diluting Solution

10 ml PBS

150 µl (3 drops) normal (unimmunized) horse serum (Vector Laboratories, Burlingame, CA, USA)

Mix until in solution

References

Allen, S., McLaren, K., & Aldridge, R. D. (1998). Paget's disease of the scrotum: a case exhibiting positive prostate specific antigen staining and associated prostatic adenocarcinoma. *British Journal of Dermatology , 138* (4), 689-691.

Bacchi, C. E., Goldfogel, G. A., Greer, B. E., & Gown, A. M. (1992). Paget's disease and melanoma of the vulva. Use of monoclonal antibodies to identify cell type and to microscopically define adequacy of surgical margins. *Gynecologic Oncology , 46* (2), 216-221.

Baldus, S. E., Goergen, D., Hanisch, F. G., & Dienes, H. P. (2001). Epitope-dependent differential immunoreactivities at anti-muc1 monoclonal antibodies in human carcinomas. *International Journal of Oncology , 18* (3), 507-513.

Becker-Wegerich, P. M., Fritsch, C., Schulte, K. W., Megahad, M., Neuse, W., Goerz, G., et al. (1998). Carbon dioxide laser treatment of extramammary paget's disease guided by photodynamic diagnosis. *British Journal of Dermatology , 138* (1), 169-172.

Bodnar, M., Miller, O. F., & Tyler, W. (1999). Paget's disease of the male breast associated with intraductal carcinoma. *Journal of the American Academy of Dermatology , 40* (5), 829 - 831.

Brugger, W., Buhring, H., Grunebach, F., Vogel, W., Kaul, S., Muller, R., et al. (1999). Expression of muc-1 epitopes on normal bone marrow: Implications for detection of micrometastatic tumor cells. *Journal of Clinical Oncology , 17* (5), 1535-1544.

Burchell, J., Gendler, S., Taylor-Papadimitriou, J., Girling, A., Lewis, A., Millis, R., et al. (1987). Development and characterization of breast cancer reactive monoclonal antibodies directed to core protein of human milk mucin. *Cancer Research , 47*, 5476-5482.

Burrows, N. P., Hudson, P. M., & Pye, R. J. (1995). Treatment of extramammary paget's disease. *British Journal of Dermatology , 132* (6), 970-972.

Cohen, C., Guarner, J., & DeRose, P. B. (1993). Mammary paget's disease and associated carcinoma-an immunohistochemical study. *Archives of Pathology and Laboratory Medicine , 117* (3), 291-294.

Coldiron, B. M., Goldsmith, B. A., & Robinson, J. K. (1991). Surgical treatment of extramammary paget's disease. A report of six cases and a reexamination of Mohs micrographic surgery compared with conventional surgical excision. *Cancer , 67* (4), 933-938.

Conover, W. J. (1980). *Practical Nonparametric Statistics, 3rd ed.* New York: John Wiley & Sons.

Costello, T. J., Wang, H. H., Schnitt, S. J., Ritter, R., & Antonioli, D. A. (1988). Paget's disease with extensive invovlement of the female genital tract initially detected by cytosmear. *Archives of Pathology and Labortory Medicine , 112* (9), 941-944.

Croce, M., Rabassa, M. E., Pereyra, A., & Segal-Eiras, A. (2005). Influence of sialic acid removal on muc1 antigenic reactivity in head and neck carcinoma. *Pathology & Oncology Research , 11* (2), 74-81.

Croce, M., Rabassa, M. E., Price, M. R., & Segal-Eiras, A. (2001). Muc1 mucin and carbohydrate associated antigens as tumor markers in head and neck squamous cell carcinoma. *Pathology & Oncology Research , 7* (4), 284-291.

Crocker, H. R. (1889). Paget's disease affecting the scrotum and penis. *Transactions of the Pathological Society of London , 40*, 187-191.

Curtin, J. P., Rubin, S. C., Jones, W. B., Hoskins, W. J., & Lewis, J. L. (1990). Paget's disease of the vulva. *Gynecologic Oncology , 39* (3), 374-377.

Demitsu, T., Gonda, K., Tanita, M., Takahira, K., Inoue, T., Okada, O., et al. (1999). Extramammary paget's disease in two siblings. *British Journal of Dermatology , 141* (5), 951-953.

Finn, O. J., Jerome, K. R., Henderson, R. A., Pecher, G., Domenech, N., Magarian-Blander, J., et al. (1995). Muc1 epithelial tumor mucin-based immunity and cancer vaccines. *Immunological Reviews , 145*, 61-89.

Geisler, J. P., Gates, R. W., Shirrell, W., Parker, S. M., Maloney, C. D., Wiemann, M. C., et al. (1997). Extramammary paget's disease with diffuse invovlement of the lower female genitourinary system. *International Journal of Gynaecologic Cancer , 7* (1), 84-87.

Gendler, S. J., Lancaster, C. A., Taylor-Papadimitriou, J., Duhig, T., Peat, N., Burchell, J., et al. (1990). Molecular cloning and expression of human tumor-associated polymorphic epithelial mucin PEM. *Journal of Biological Chemistry , 265* (25), 15286-15293.

Glass, G. V., Peckham, P. D., & Sanders, J. R. (1972). Consequences of failure to meet assumptions underlying the analysis of variance and covariance. *Review of Educational Research , 42* (3), 237-288.

Goldblum, J. R., & Hart, W. R. (1998). Perianal paget's disease- a histologic and immunohistochemical study of 11 cases with and without associated rectal adenocarcinoma. *American Journal of Surgical Pathology , 2*, 170-179.

Goldblum, J. R., & Hart, W. R. (1997). Vulvar paget's disease: a clinicopathologic and immunohistochemical study of 19 cases. *American Journal of Surigcal Pathology , 21*, 1178-1187.

Guarner, J., Cohen, C., & DeRose, P. B. (1989). Histogenesis of extramammary and mammary paget's cells. An immunohistochemical study. *American Journal of Dermatopathology , 11* (4), 313-318.

Guarner, J., Cohen, C., & DeRose, P. B. (1989). Histogenesis of extramammary and mammary paget's cells. An immunohistochemical study. *American Journal of Dermatopathology , 11* (4), 313-318.

Guldhammer, B., & Norgaard, T. (1986). The differential diagnosis of intraepidermal malignant lesions using immunohistochemistry. *American Journal of Dermatopathology , 8* (4), 295-301.

Hanisch, F. G., & Muller, S. (2000). Muc-1: the polymorphic appearance of a human mucin. *Glycobiology , 10* (5), 439-449.

Hanisch, F. G., Egge, H., Peter-Katalinic, J., & Uhlenbruck, G. (1986). Primary structure of a major sialyl-saccharide alditol from human amniotic mucins expressing mucins the tumor-associated sialyl-Lewis antigenic determinant. *FEBS Letters , 200*, 42-46.

Hanisch, F. G., Egge, H., Peter-Katalinic, J., Uhlenbruck, G., Dienst, C., & Fangmann, R. (1985). Primary structures and Lewis blood-group-dependent expression of major sialylated saccharides from mucus glycoproteins of human seminal plasma. *European Journal of Biochemistry , 152*, 343-351.

Helm, K. F., Goellner, J. R., & Peters, M. S. (1992). Immunohistochemical stains in extramammary paget's disease. *American Journal of Dermapathology , 14* (5), 402-407.

Hilkens, J., Buijs, F., Hilgers, J., Hageman, P., Calafat, J., Sonnenberg, A., et al. (1984). Monoclonal antibodies against human milk-fat globule membranes detecting differentiation antigens of the mammary gland and its tumors. *International Journal of Cancer , 34* (2), 197-206.

Hilkens, J., Ligtenberg, M. J., Vos, H. L., & Litvinov, S. V. (1992). Cell membrane associated mucins and their adhesion modulating property. *Trends in Biochemical Sciences , 17* (9), 359-363.

Hitchcock, A., Topham, S., Bell, J., Gullick, W., Elston, C. W., & Ellis, I. O. (1992). Routine diagnosis of mammary paget's disease. A modern approach. *American Journal of Surgical Pathology , 16* (1), 58-61.

Hounsell, E. F., Lawson, A. M., Feeney, J., Gooi, H. C., Pickering, N. J., Stoll, M. S., et al. (1985). Structural analysis of the O-glycosidically linked core-region oligosaccharides of human meconium glycoproteins which express oncofetal antigens. *European Journal of Biochemistry , 148*, 367-377.

Jones, R. E. (1985). Mammary Paget's disease without underlying carcinoma. *American Journal of Dermatopathology , 7*, 361-365.

Jones, R. E., Austin, C., & Ackerman, A. B. (1979). Extramammary paget's disease. A critical reexamination. *American Journal of Dermatopathology , 1*, 101-132.

Jones, R. R., Spaull, J., & Gusterson, B. (1989). The histogenesis of mammary and extramammary paget's disease. *Histopathology , 14* (4), 409-416.

Karsten, U., & Cao, Y. (2001). Binding patterns of 51 monoclonal antibodies to peptide and carbohydrate epitopes of the epithelial mucin (muc1) on tissue sections of adenolymphomas of the parotid (Warthin's tumours: role of epitope masking by gylcans. *Histochemistry and Cell Biology , 115* (4), 349-356.

Karsten, U., Serttas, N., Paulsen, H., Danielczyk, A., & Goletz, S. (2004). Binding patterns of DTR-specific antibodies reveal glycosylation-conditioned tumor-specific epitope of the epithelial mucin (muc1). *Glycobiology , 14* (8), 681-692.

Kodama, S., Kaneko, T., Saito, M., Yoshiya, N., Honma, S., & Tanaka, k. (1995). A clinicopathologic study of 30 patients with paget's disease of the vulva. *Gynecologic Oncology , 56* (1), 63-70.

Koga, F., Gotoh, S., & Suzuki, S. (1997). A case of invasive bladder cancer with pagetoid skin lesion of the vulva and anogenital paget's disease. *Nippon Hinyokika Gakkai Zasshi , 88* (4), 503-506.

Kondo, Y., Kashima, K., Daa, T., Nakayama, I., & Yokohama, S. (2002). The ectopic expression of gastric mucin in extramammary and mammary paget's disease. *American Journal of Surgical Pathology , 26* (5), 617-623.

Lamblin, G., Boersna, A., Lhermitte, M., Roussel, P., Mutsaers, J. H., van Halbeek, H., et al. (1984). Further characterization, by combined high-performance liquid chromatography/H-NMR approach, of the heterogeneity displayed by neutral carbohydrate chains of human bronchial mucins. *European Journal of Biochemistry , 143*, 227-236.

Lan, M. S., Batra, S. K., Qui, W. N., Metzgar, R. S., & Hollingsworth, M. A. (1990). Cloning and sequencing of a human pancreatic tumor mucin cDNA. *Journal of Biological Chemistry , 265* (25), 15294-15299.

Liegl, B., Leibl, M., Gogg-Kamerer, M., Tessaro, B., Horn, L. C., & Moinfar, F. (2007). Mammary and extramammary paget's disease: an immunohistochemical study of 83 cases. *Histopathology , 50*, 439-447.

Ligtenberg, M. J., Vos, H. L., Gennissen, A. M., & Hilkens, J. (1990). Episialin, a carcinoma associated mucin, is generated by a polymorphic gene encoding splice variants with alternative amino termini. *Journal of Biological Chemistry , 265* (25), 5573-5578.

Lloyd, J., & Flanagan, A. M. (2000). Mammary and extramammary paget's disease. *Journal of Clinical Pathology , 53* (10), 742-749.

Lloyd, J., Evans, D. J., & Flanagan, A. (1999). Extension of extramammary paget's disease of the vulva to the cervix. *Journal of Clinical Pathology , 52*, 538-540.

Magnani, J. L., Steplewski, Z., Koprowski, H., & Ginsburg, V. (1983). Identification of the gastrointestinal and pancreatic cancer-associated antigen detected by monoclonal antibody 19-9 in the sera of patients as a mucin. *Cancer Research , 43*, 5489-5492.

Misery, L. (2007). Treatment of genital paget's skin disease with topical imiquimod: severe adverse events. *Journal of Dermatological Treatment , 18* (3), 191-192.

Mohs, F. E., & Blanchard, L. (1979). Microscopically controlled surgery for extramammary paget's disease. *Archives of Dermatology , 115* (6), 706-708.

Mutsaers, J. H., van Halbeek, H., Vliegenthardt, J. F., Wu, A. M., & Kabat, E. A. (1986). Typing of core and backbone domains of mucin-type oligosaccharides from human ovarian-cyst glycoproteins by 500 MHz H-NMR spectroscopy. *European Journal of Biochemistry , 157*, 139-146.

Nakamura, G., Shikata, N., Shoji, T., Hatano, T., Hioki, K., & Tsubura, A. (1995). Immunohistochemical study of mammary and extramammary paget's disease. *Anticancer Research , 15* (2), 467-470.

Ordonez, N. G., Awalt, H., & Mackay, B. (1987). Mammary and extramammary paget's disease. An immunocytochemical study and ultrastructural study. *Cancer , 59* (6), 1173-1183.

Paget, J. (1874). On disease of the mammary areola preceding cancer of the mammary gland. *St. Bartholemew Hospital Research London , 10*, 87-89.

Pandey, P., Kharbanda, S., & Kufe, D. (1995). Association of the DF3/MUC1 breast cancer antigen with Grb2 and the Sos/Ras exchange protein. *Cancer Research , 55*, 4000-4003.

Parker, L. P., Parker, J. R., Bodurka-Bevers, D., Deavers, M., Bevers, M. W., Shen-Gunther, J., et al. (2000). Paget's disease of the vulva: pathology,pattern of invovlement, and prognosis. *Gynecologic Oncology , 77* (1), 183-189.

Powell, F. C., Bjornsson, J., Doyle, J. A., & Cooper, A. J. (1985). Genital Paget's disease and urinary tract malignancy. *Journal of the American Academy of Dermatology , 13* (1), 84-90.

Price, M. R., Rye, P. D., Petrakou, E., Murray, A., Brady, K., Imai, S., et al. (1998). Summary report on the isobm td-4 workshop: analysis of 56 monoclonal antibodies against the muc1 mucin. *Tumor Biology , 19* (1), 1-20.

Quinn, A. M., Sienko, A., Basrawala, Z., & Campbell, S. C. (2004). Extramammary paget's disease of the scrotum with features of bowen's disease. *Archives of Pathology and Laboratory Medicine , 128* (1), 84-86.

Reed, W., Oppedal, B. R., & Eeg Larsen, T. (1990). Immunohistology is valuable in distinguishing between paget's disease, Bowen's disease and superficial spreading melanoma. *Histopathology , 16*, 583-588.

Reis, C. A., David, L., Seixas, M., Burchell, J., & Sobrinho-Simoes, M. (1998). Expression of fully and under-glycosylated forms of MUC1 mucin in gastric carcinoma. *International Journal of Cancer, 74* (4), 402-410.

Roth, L. M., Lee, S. C., & Ehrlich, C. E. (1977). Paget's disease of the vulva. A histogenetic study of five cases including ultrastructural observations and review of the literature. *American Journal of Surgical Pathology , 1* (3), 193-206.

Shepherd, V., Davidson, E. J., & Davies-Humphreys, J. (2005). Extramammary paget's disease. *BJOG: An International Journal of Obstetrics and Gynaecology , 112*, 273-279.

Sherblom, A. P., & Moody, C. E. (1986). Cell surface sialomucin and resistance to natural cell-mediated ctyotoxicity of rat mammary tumor ascites cells. *Cancer Research , 46*, 4543-4546.

Shieh, S., Dee, A. S., Cheney, R. T., Frawley, N. P., Zeitouni, N. C., & Oseroff, A. R. (2002). Photodynamic therapy for the treatment of extramammary paget's disease. *British Journal of Dermatology , 146* (6), 1000-1005.

Shimizu, S., Kobayashi, H., Suchi, T., Torii, Y., Narita, K., & Aoki, S. (1991). Extramammary paget's disease arising in mature cystic teratoma of the ovary. *American Journal of Surgical Pathology , 15* (10), 1002-1006.

Sitakalin, C., & Ackerman, A. B. (1985). Mammary and extramammary paget's disease. *American Journal of Dermatopathology , 7* (4), 335-340.

Stephenson, T. J., & Cotton, D. W. (1987). Paget's disease in an epidermal cyst. *Dermatologica , 174* (4), 186-190.

Tebes, S., Cardosi, R., & Hoffman, M. (2002). Paget's disease of the vulva. *American Journal of Obstetrics and Gynecology , 187* (2), 281-283.

Thirlby, R. C., Hammer, C. J., Galagan, K. A., Travaglini, J. J., & Picozzi, V. J. (1990). Perianal paget's disease: successful treatment with a combined chemoradiotherapy. Report of a case. *Diseases of the Colon and Rectum , 33* (2), 150-152.

Thompson, M. S., Green, S. B., Chen, Y., Stockford, S., & Lo, W. (2005). Power of the t-test for normal and mixed normal distributions. *Journal of Modern Applied Statistical Methods , 4* (2), 591-597.

Vanstapel, M. J., Gatter, K. C., De Wolf- Peeters, C. D., Millard, P. R., Desmet, V. J., & Mason, D. Y. (1984). Immunohistochemical study of mammary and extramammary paget's disease. *Histopathology , 8* (6), 1013-1023.

Watring, W. G., Roberts, J. A., Lagasse, L. D., Berman, M. L., Ballon, S. C., Moore, J. G., et al. (1978). Treament of recurrent paget's disease of the vulva with topical bleomycin. *Cancer , 41* (1), 10-11.

Wesseling, J., van der Valk, S. W., & Hilkens, J. (1996). A mechanism for inhibition of E-cadherin-mediated cell-cell adhesion by membrane-associated mucin episialin/MUC1. *Molecular Biology of the Cell , 7* (4), 565-577.

Wesseling, J., van der Valk, S., Sonnenberg, A., & Hilkens, J. (1995). Episialin(MUC1) overexpression inhibits integrin-mediated cell adhesion to extracellular matrix components. *Journal of Cell Biology , 129*, 255-265.

Willman, J. H., Golitz, L. E., & Fitzpatrick, J. E. (2005). Vulvar clear cells toker: precursors of extrammary paget's disease. *American Journal of Dermatopathology , 27* (3), 185-188.

Wreschner, D. H., Hareuveni, M., Tsarfaty, I., Smorodinsky, N., Horev, J., Zaretzky, J., et al. (1990). Human epithelial tumor antigen cDNA sequences. Differential splicing may generate multiple protein forms. *European Journal of Biochemistry , 189*, 463-473.

Yamamoto, M., Bharti, A., Li, Y., & Kufe, D. (1997). Interaction of the DF3/MUC1 breast carcinoma-associated antigen and B-catenin in cell adhesion. *Journal of Biological Chemistry , 272* (19), 12492-12494.

Ye, J. N., Rhew, D. C., Yip, F., & Edelstein, L. (2006). Extramammary paget's disease resistant to surgery and imiquimod monotherapy but responsive to imiquimod combination topical chemotherapy with 5-fluorouracil and retinotic acid: a case report. *Cutis , 77* (4), 245-250.

Yoshii, N., Kitjima, S., Yonezawa, S., Matsukita, S., Setoyama, M., & Kanzaki, T. (2002). Expression of mucin core protein in extramammary paget's disease. *Pathology International , 52* (5-6), 390-399.

Zampogna, J. C., Flowers, F. P., Roth, W. I., & Hassenein, A. M. (2002). Treatment of primary limited cutaneous extramammary paget's disease with topical imiquimod monotherapy: two case reports. *Journal of the American Academy of Dermatology , 47* (4 Suppl), S229-S235.

Zollo, J. D., & Zeitouni, N. C. (2000). The roswell park cancer institute experience with extramammary paegt's disease. *British Journal of Dermatology , 142* (1), 59-65.