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13. ABSTRACT *(Maximum 200 words)*

We have identified MUC1 and KSA as the dominant cell surface proteins or glycoproteins on most breast and ovarian cancers, and conjugation of antigens to keyhole limpet hemocyanin and mixture with the immunological adjuvant QS21 as the optimal approach to immunization. Conjugate KSA vaccines have not yet been tested in humans. We have recently prepared KSA vaccines with KSA of baculovirus origin conjugated to KLH and unconjugated. The protocol for the use of these KSA vaccines in ovarian cancer patients has been IRB, FDA and tentatively DAMD approved. Patient accrual will begin over the next month. MUC1 32 (amino acid) conjugate vaccines have induced high titer antibodies against the immunizing peptide in breast cancer patients, but only low titer antibodies against MUC1 positive tumor cells and no T-cell immunity against these cells. For induction of more relevant immune responses, longer MUC1 peptides may be required as immunogens and these peptides may need to be glycosylated. We have constructed a 106 amino acid MUC1 peptide which is currently being glycosylated for vaccine preparation. A protocol for the use of 106 aa MUC1 (glycosylated or not) conjugated to KLH for use in breast cancer patients is being written.

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Philip Livingston MD

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ANNUAL REPORT

Clinical Trials with a Polyvalent Breast Cancer Vaccine

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Introduction

Glycoprotein and protein antigens expressed at the cell surface of breast cancers and ovarian cancers are tempting targets for immunotherapy because they can be the focus for immunotherapy aimed at induced both antibodies and cytotoxic T-cells. Over the past year we have used DAMD funding to screen breast cancer and ovarian cancer biopsy specimens for expression of a series of 12 such antigens with monoclonal antibodies using immunohistology (1). The antigens expressed most abundantly were MUC1 and KSA. We have no previous experience with KSA which is a 40,000 molecular weight glycoprotein and has not been readily available for vaccine production, but we have had significant experience immunizing breast cancer patients in the past with MUC1. The result was high titer antibodies induced against the synthetic MUC1 peptide but these antibodies reacted only weakly with breast cancer cells or ovarian cancer cells expressing MUC1. Our conclusion was that the fault lay in the 32 amino acid MUC1 peptide used as immunogen in these studies (2,3). A longer peptide might permit a more appropriate secondary structure and the formation of a more relevant MUC1 epitope. It is also possible that glycosylation of the serines and threonines in MUC1 (as is normally encountered on cell bound MUC1) might further enhance this goal. Consequently we have prepared a 106 amino acid MUC1 peptide *(not a simple task)* and are in the process of glycosylating it with GalNAc epitopes (Tn). Also, through a collaboration with Jenner Biotherapies Inc. (San Ramon California) KSA has become available to us and we have initiated vaccine production. Therefore having identified the glycosylated 106 amino acid MUC1 and the 40,000 MUC weight KSA molecular as the most appealing target antigens on cancers of the breast and ovary through DAMD supported studies over the last and now having adequate sources of these antigens, we propose to vaccinate small groups of patients with these antigens over the next year and study their antibody and T-cell responses to these immunizations.

Body

Initially we screened a series of cancers including seven breast cancer biopsy specimens and five ovarian cancer biopsy specimens for the expression of a series of peptide or glycopeptide antigens thought to be expressed at the cell surface. These included MUC1, MUC2, MUC3, MUC4, MUC5ac, MUC5b, MUC7, KSA, PSMA, CEA, βhCG and HER-2/neu. We found that on both breast cancer and ovarian cancer biopsy specimens MUC1 and KSA were the most widely expressed and intensely expressed antigens. This was the basis for refocusing our attention over the last year on these two antigens as targets for immunotherapy through vaccines. The galley proof for this study is attached in the appendix.

FDA approval for the KSA vaccine has been obtained and a revised protocol which is now IRB approved and which has incorporated the modifications suggested by the DAMD as well as the FDA is attached in the appendix. We plan to begin immunizing patients with ovarian cancer according to this protocol over the next month. A protocol for MUC1 peptide (un-glycosylated) has previously been approved by IRB and FDA and I am told by the FDA that it will require only an amendment to include the glycosylated version. It will however require a new IRB protocol and this is currently being drafted. We will send this protocol to the DAMD over the next month, before it is submitted to the IRB.

We had previously immunized groups of breast cancer patients with two 32 amino acid MUC1 peptides representing one and one half repeats of the 20 amino acid MUC1 tandem repeat. High titer antibodies against MUC1 peptide were induced but these reacted only weekly with tumor cells expressing MUC1 (3). Our conclusion from these studies was that the high titer antibodies induced by our vaccine were predominantly against an epitope on the non-glycosylated 32 aa peptide which is not expressed on the much longer and glycosylated MUC1 molecules that coat the cancer cell surface. Consequently we have prepared a 106 amino acid MUC1 peptide containing 5 plus repeats and are in the process of glycosylating it. The methods are described below.

Peptide Synthesis:

a. The MUC1 peptide is synthesized on an Applied Biosystems Model 431A solid phase peptide synthesizer. L amino acids for peptide synthesis were purchased from Applied Biosystems and used without further purification. Quantities and identities of all solvents and other reagents are listed on the attached page. COAs for the lots of KLH, MBS and DMF used here are attached. Resin samples are taken after every cycle to ensure efficient coupling. After coupling acetic anhydride is delivered to the reaction vessel to terminate the non coupled (n-1) peptide chain.

After synthesis the peptide is cleaved from the solid support using one of a number of different cleavage cocktails depending on the sequence. The crude product is then

injected onto a 4.6 mm i.d. reverse phase high pressure liquid chromatograph (RP-HPLC) system utilizing a diode array detector, monitoring wavelengths of 214, 253, 277 and 297 nanometers. The UV spectra (chromatogram) are inspected for the peptide of interest (peptides that do not contain tyrosine or tryptophan should not have absorbance at any other wavelength other than 214 nm). The fractions are collected via a fraction collector and then mass analyzed by matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI TOF MS). The molecular weight for the major peptide in all lots will be within one and one-half daltons of the projected weight.

The corrected peptide is then preparatively purified using either a 1 cm. or 2 cm. RP-HPLC column. The gradient of either 1%/min or 2%/min (depending on the purity of the crude) is eluted in an A buffer (0.1% TFA) and B buffer (70%MeCN/0.9% TFA). The preparative fractions are screened analytically for homogeneity by both HPLC and CZE (Capillary Zone Electrophoresis). Fractions that are mixed are subjected to a second round of HPLC. Once the peptide is dried the powder is dissolved in UPI grade PBS. The fractions containing pure peptide are pooled in HPLC buffer in a lyophilization tube and lyophilized. This was resuspended in 50% methanol and water and relyophilized twice more. Cold sterilization will then be performed by filtration and the concentration determined by quantitative amino acid analysis. MUC1 peptide or glycosylated peptide will be utilized for conjugate formation only after the proper sequence has been confirmed and it has been purified to homogeneity as defined by migration as a single band by HPLC and CZE with no evidence (less than 5%) of contaminants. The lyophilized peptide/glycopeptide is brought to Dr. Livingston's lab and stored at 4° and used within several days for conjugation. Aside from gradual dimerization, the peptide is quite stable. It is because of the gradual dimerization that we perform the conjugation within several days. The sequence of the 106 amino acid MUC1 peptide used here is GVTS(APDTRPAPGSTAPPAHGVTS)$_5$C. The C-terminal cystine is not part of the MUC1 sequence but is placed there to facilitate covalent attachment to a carrier protein such as KLH.

Glycosylation of MUC1:

Full Glycosylation with Tn epitopes requires a series of three enzymes (4). These have been cloned and expressed by Dr. Henrik Clausen (University of Copenhagen) to whom we have sent this peptide. This will be glycosylated and returned to us for further purification and NMR confirmation of the final structure. It will then be conjugated to KLH for vaccine construction.

Conclusions:

1. The dominant cell surface antigens for both breast and ovarian cancer are KSA and MUC1.

2. KSA and glycosylated MUC1 have recently become available to us for vaccine preparation.

3. KSA vaccines have recently been prepared, and the protocol IRB, FDA and tentatively DAMD approved. Patient accrual will begin over the next month.

4. We have constructed a 106 amino acid MUC1 peptide which is currently being glycosylated for vaccine preparation.

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4. Clausen, H., Bennett, E., Wandall, H. and Hassan, H. Prediction of o-glycosylation Sites of MUC1 Tandem Repeats by Analysis of Substrate Specificities of a Family of GalNAc-Transferases. Program: 5th International Workshop on Carcinoma-Associated Mucins. Robinson College, Cambridge. 18th-23rd July, 1998.

Appendix

KSA protocol: IRB, FDA approved. Final DAMD approval pending.

Zhang et al. Immunohistology paper: galley proof.

PAGE 1-4, 20, 21 Best Available Copy

Selection of Tumor Antigens As Targets For Immune Attack Using Immunohistochemistry: III. Protein Antigens[1]

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ABSTRACT

The relative expression of mucin antigens MUC1, MUC2, MUC3, MUC4, $MUC5_{AC}$, $MUC5_{B}$, and MUC7 and glycoprotein antigens KSA, carcinoembryonic antigen, PSMA, HER-2/*neu*, and human chorionic gonadotropin-β on different cancers and normal tissues is difficult to determine from available reports. We have compared the distribution of these antigens by immunohistology on a broad range of malignant and normal tissues. MUC1 expression was most intense in cancers of breast, lung, ovarian, and endometrial origin; MUC2 was most intense in cancers of colon and prostate origin; and $MUC5_{AC}$ was most intense in cancers of breast and gastric origin. MUC4 was intensely expressed in 50% of cancers of colon and pancreas origin, and MUC3, $MUC5_{B}$, and MUC7 were expressed in a variety of epithelial cancers, but not so intensely. KSA was intensely and uniformly expressed on all epithelial cancers; carcinoembryonic antigen was expressed in most cancers of breast, lung, colon, pancreas, and gastric origin; and PSMA was expressed only in cancers of prostate origin. Human chorionic gonadotropin-β was expressed on the majority of sarcomas and cancers of breast, lung, and pancreas origin, although intense staining was not seen. Staining on normal tissues was restricted to one or many normal epithelial tissues ranging from MUC3, MUC4, and PSMA, which were expressed only on epithelia of pancreas, stomach, and prostate origin, respectively, to MUC1 and KSA, which were expressed on most normal epithelia. Expression was restricted to the secretory borders of these epithelia with stroma and other normal tissues were completely negative. These results plus the results of the two previous papers (S. Zhang *et al.*, Int. J. Cancer, *73:* 42–49, 1997; S. Zhang *et al.*, Int. J. Cancer, *73:* 50–56, 1997) in this series provide the basis for selection of multiple cell surface antigens as targets for antibody-mediated attack against these cancers.

INTRODUCTION

This is our third and final immunohistochemistry study comparing the expression of a series of cell surface antigens (selected as potential targets for immunotherapy) on a range of normal and malignant tissues. The previous two studies (1, 2) focused on carbohydrate epitopes expressed in glycolipids, mucins, and other glycoproteins. Here, we focus on the peptide epitopes of seven mucins and five glycoproteins, each of which is available for vaccine construction as a consequence of simple peptide synthesis (MUC1–MUC7) or expression in *Escherichia coli* or baculovirus (3–7). Each of these antigens is either known to be expressed at the cell surface as a consequence of a demonstrated transmembrane domain (MUC1, KSA, CEA,[3] PSMA, and HER-2/*neu*; Refs. 8–12) or is thought to be shed by tumor cells and be either adherent to or abundant in the vicinity of tumor cells (MUC2, MUC3, MUC4, $MUC5_{AC}$, $MUC5_{B}$, and MUC7 and βhCG; Refs. 13–19). Although the expression of each of these antigens on human tumors and normal tissues has been described, previous studies were limited in terms of number and types of tissues studied, involved mAbs against only one to three antigens without direct comparison to expression of other antigens, and used different immunostaining procedures (indirect immunofluorescence, indirect immunoperoxidase, or ABC immunoperoxidase; Refs. 13 and 19–29). Consequently, the comparative distribution of these antigens on cancers and normal tissues is difficult to determine from available reports, although this is precisely the information required for selecting target antigens for immunotherapy. This is especially important with the recent development of conjugate vaccines capable of inducing antibodies in most patients against a variety of well-defined tumor antigens (30) and with the recent evidence that the induction of these antibodies correlates with a more favorable prognosis (30–32).

MATERIALS AND METHODS

Tissue Samples. Frozen specimens embedded in Tissue-Tek O.C.T. compound (Diagnostic Division, Elkhart, IN) were provided with pathological reports by the Tissue Procurement Service of Memorial Sloan-Kettering Cancer Center (New York, NY), with the exception of four frozen specimens of metastatic prostate cancer, which were kindly provided by Dr. G. Steven Bova (PELICAN Laboratory, Johns Hopkins University, Baltimore, MD). Cryostat sections were cut at 5 μm, dried in air, and fixed with neutral buffered 10% formalin solution

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[3] The abbreviations used are: CEA, carcinoembryonic antigen; βhCG, human chorionic gonadotropin-β; mAb, monoclonal antibody; ABC, avidin-biotin complex; GI, gastrointestinal; [illegible]

Table 1 Mouse mAbs used for immunohistology

mAb	Ig class	Antigen	Antigen structure	Ref.
HMFG-2	IgG1	MUC1	VTSAPDTRPAPGSTAPPAHG repeating	41, 42
LDQ10	IgM	MUC2	PTTTPISTTTTVTPTPTPTGTQT repeating	37
M3.2	IgG2a	MUC3	HSTPSFTSSITTTETTS repeating	20
MUC4.275	IgG1	MUC4	TSSASTGHATPLPVTD repeating	43
CLH2	IgG1	$MUC5_{AC}$	TTSTTSAP repeating (interrupted)	27
PANH2	IgG1	$MUC5_{B}$	No peptide repeats	44, 45
PANH3	IgG1	MUC7	TTAAPPTPSATTPAPPSSSAPPE repeating	44, 45
NCL-CEA	IgG1	CEA	Glycoprotein (*M* 180,000)	Vector Laboratories
Cyt351	IgG	PSMA	Glycoprotein (*M* 100,000)	28, 46
GA733-2	IgG2a	KSA	Glycoprotein (*M* 40,000)	47
FB12	IgG1	βhCG	145-amino acid glycoprotein	48
NCL-CBE1	IgG2a	HERv2/*neu*	Protein (*M* 185,000)	8

Table 2 Proportion of cancer specimens with ≥50% positive cancer cells (≥2+ staining intensity) by immunohistology[a]

Cancer	Antigen (mAb)											
	MUC1 (HMFG-2)	MUC2 (LDQ10)	MUC3 (M3.2)	MUC4 (M4.275)	$MUC5_{AC}$ (CLH2)	$MUC5_{B}$ (PANH2)	MUC7 (PANH3)	KSA (GA733-2)	PSMA (Cyt351)	CEA (NCL-CEA)	βhCG (FB12)	HER-2/*neu* (NCL-CBE1)
Melanoma	0/5	1/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5
Sarcoma	0/5	0	0	0	0	1/5	0	0	0	0	3/5	0
Neuroblastoma	0	1/5	0	0	0	0	0	0	0	0	1/5	0
B-cell lymphoma	0/5	0	0	0	0	0	0	0	0	0	0	0
Small cell lung	1/5	0	0	0	0	0	0	5/5[b]	0	3/5	1/5	0
Breast	5/7[b]	3/7	1/7	1/7	5/6[b]	4/6	1/6	5/7[b]	0/7	4/7[b]	4/7	1/7
Metastatic prostate	3/5	4/5[b]	0	0	1/5	0	0	5/5[b]	3/5[b]	0	1/5	1/5
Lung	4/5[b]	0	1/5	4/5	0	0	0	5/5[b]	0	4/5[b]	3/5	1/5
Colon	3/8	6/8[b]	4/8	4/8[b]	1/8	3/8	4/8	8/8[b]	0	6/8[b]	2/8	0/8
Pancreas	2/5	2/5	0	3/5[b]	2/5	1/5	0	5/5[b]	0	3/5[b]	4/5	0
Gastric	1/5	0	3/5	2/5	4/5[b]	1/5	1/5	5/5[b]	0	5/5[b]	0	
Ovarian	5/5[b]	2/5	4/5	3/5	0	0	0	5/5[b]	0	2/5	2/5	0
Endometrial	3/5[b]	0	2/5	0	1/5	2/5	0	5/5[b]	0	1/5	2/5	0

[a] All tumor tissues were stained by ABC immunoperoxidase methods.
[b] Median staining intensity was 4+ for ≥80% of cells.

(Sigma Co., St. Louis, MO) for 10 min before H&E or immune staining.

mAb and Immunohistochemistry. The murine mAbs and the antigens they recognize are summarized in Table 1. mAb HMFG-2 was provided by J. Taylor-Papadimitriou (Imperial Cancer Research Fund, London, United Kingdom); LDQ10 was provided by F. X. Real (Institut Municipal d'Investigacio Medica, IMIM, Barcelona, Spain); M3.2 and MUC4.275 were provided by V. Apostolopoulos (Austin Research Institute, Victoria, Australia); CLH2, PANH2, and PANH3 were provided by H. Clausen (University of Copenhagen, Copenhagen, Denmark); Cyt351 was provided by W. Heston (Memorial Sloan-Kettering Cancer Center); FB12 was provided by D. Bellet (Institut Gustave-Roussy, Villejuif, France); and GA733-2 was provided by D. Herlyn (The Wistar Institute, Philadelphia, PA). mAbs NCL-CEA and NCL-CBE1 were purchased from Vector Laboratories, Inc. (Burlingame, CA).

The ABC immunoperoxidase method was performed as described previously (33). Briefly, the sections were quenched with 0.1% H_2O_2 in PBS for 15 min, blocked with avidin and biotin reagents (Vector Laboratories) for 10 min each, incubated in 10% serum of horse or goat from which the second antibody was raised, and incubated with various mAbs for 1 h at optimal concentration. The optimal mAb concentration was selected based on the strongest reactivity against the known positive target cells with little or no background against stroma. The concentrations of mAbs used were: FB12, 0.5 μg/ml; Cyt351 and GA733-2, 2 μg/ml; HMFG-2, M3.2, MUC4.275, CLH2, PANH2, and PANH3 (supernatants), between 1:3 and 1:6; LDQ10 and NCL-CBE1 (ascites), 1:15; and NCL-CEA, 1:50. The sections were subsequently incubated with 1:600 biotinylated horse antimouse IgG or 1:300 goat antimouse IgM antibodies (Vector Laboratories) for 40 min and then incubated in 1:50 ABC reagent (Vector Laboratories) for 30 min. Reactions were developed with 0.02% H_2O_2 and 0.1% diaminobenzidine tetrahydrochloride (Sigma) for 2–5 min. Slides were then counterstained with Harris modified hematoxylin (Fisher Scientific, Fair Lawn, NJ) for 1–3 min. The immunoreactivities were graded based on the percentage of positive cells and staining intensity above that seen on the negative control: 1+ (weak), 2+ (moderate), 3+ (strong), and 4+ (very strong or intense). Staining intensities of 2+ or stronger were considered positive (Table 2 and Fig. 1). Known positive and negative control slides

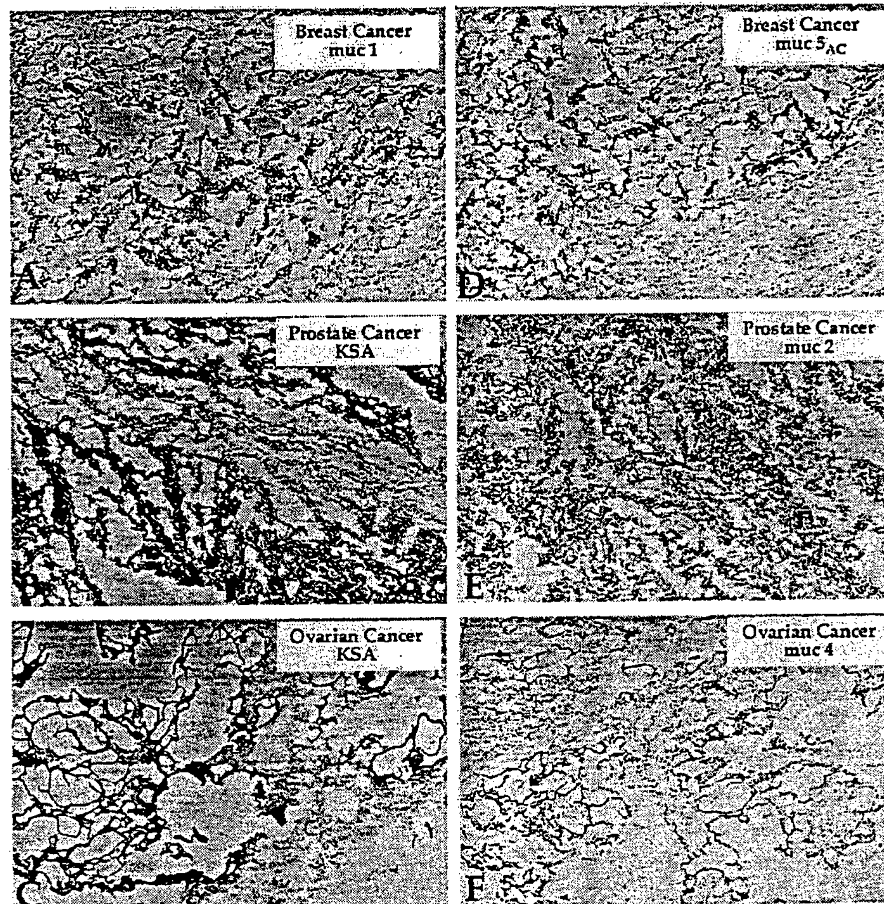

Fig. 1 Expression of protein antigens on breast cancer (*A* and *D*), prostate cancer (*B* and *E*), and ovarian cancer (*C* and *F*). The pattern of staining of cell membrane bound antigens MUC1 (*A*) and KSA (*B* and *C*) is indistinguishable from secreted antigens $MUC5_{AC}$ (*D*), MUC2 (*E*), and MUC4 (*F*). Staining intensity in these sections is graded as follows: *A*, 2–3+, 80% of tumor cells positive; *B*, 4+, 100%; *C*, 4+, 100%; *D*, 3+, 80%; *E*, 3+, 80%; *F*, 3+, 80%. Magnification, ×70.

were used in each experiment. Results with the several IgM, IgG3, and IgG2 mAbs included in the panel of antibodies tested ruled out nonspecific adherence of particular subclasses of antibodies.

An indirect immunoperoxidase assay was performed as described previously (34) on normal liver, kidney, and stomach samples because these tissues reacted strongly with ABC reagent directly, producing high background. Briefly, the sections were quenched with 0.1% H_2O_2 in PBS for 15 min, blocked with 10% serum, and incubated with mAbs for 1 h at the optimal concentration. The sections were incubated with 1:100 rabbit antimouse immunoglobulin labeled with peroxidase (DAKO Corp., Carpinteria, CA) for 1 h and developed as described for the ABC method.

RESULTS

Reactivity of mAbs with Tumor Tissues. Table 2 summarizes the staining on tumor tissue samples observed with the panel of mAbs. Eighty-two neoplastic tissue specimens representing 13 tumor types were analyzed with each of the 12 antibodies. None of these mAbs reacted consistently with melanoma, neuroblastoma, or B-cell lymphoma specimens, and only FB12 against βhCG reacted weakly (2+) with some sarcomas. KSA was very strongly expressed (median 4+) on small cell lung cancer and all or most specimens of all of the epithelial cancers. At the other extreme was PSMA, expressed only on primary and metastatic prostate cancer (median, 3+–4+). βhCG was expressed moderately (median, 2+) on some samples of most tumor types, but strong expression (3+) on occasional specimens, such as three of five lung cancer specimens, was also seen. CEA, MUC1, MUC2, and MUC4 were strongly expressed on the majority of some epithelial cancers (median, 3+–4+) but not expressed at all on others. MUC3, $MUC5_B$, and MUC7 were moderately expressed on the majority of several cancers (median, 2+). $MUC5_{AC}$ was strongly expressed on only breast and gastric cancers. Confidence in all of these results was bolstered by the very strong expression (4+) seen on some specimens with each of these mAbs and complete lack of staining on other specimens. Strong (3+) HER-2/*neu* expression was only seen on one prostate cancer specimen, and the other two positive specimens were 2+, despite using the available ascites at a 1:15 dilution. Consequently, in the absence of a clear positive control, it is not clear whether the lack of staining of more specimens with NCL-CBE1 against HER-2/*neu* was a consequence of low levels of antigen expression, inactive antibody, or problems with the assay. Representative examples of these reactions and our grading of percentage positive tumor cells and staining intensity are shown in Fig. 1. Staining of stroma with all 12 of these mAbs was uniformly negative.

Table 3 Antigen expression on normal tissues defined by immunohistology[a]

Normal tissue (no.)[b]	Antigen (mAb)											
	MUC1 (HMFG-2)	MUC2 (LDQ10)	MUC3 (M3.2)	MUC4 (M4.275)	$MUC5_{AC}$ (CLH2)	$MUC5_{B}$ (PANH2)	MUC7 (PANH3)	KSA (GA733-2)	PSMA (Cyt351)	CEA (NCL-CEA)	βhCG (FB12)	HER-2/*neu* (NCL-CBE1)
Spleen (2)												
White pulp	–	–	–	–	–	–	–	–	–	–	–	–
Red pulp	–	–	–	–	–	–	–	–	–	2+[c]	–	–
Striated muscle (2)	–	–	–	–	–	–	–	–	–	–	1+	–
Epithelia												
Lung (2)	2+	–	–	–	–	–	–	3+	–	1+	1+	1+
Breast (2)	1+	–	–	–	–	–	–	3+	–	2+	–	–
Prostate (6)	±	2+	–	±	–	–	–	4+	3+	3+	3+	2+
Color (2)	2+	3+	–	3+	–	3+	1+	4+	–	4+	1+	–
Stomach (2)	1+	–	–	–	4+	–	–	–	–		2+	–
Pancreas (2)	2+	2+	2+	–	–	–	1+	4+	–		2+	–
Uterus (2)	1+	–	–	–	–	–	–	3+	–	1+	–	–
Ovary (2)	1+	–	–	–	–	–	–	3+	–		–	–
Liver (2)	–	–	–	–	–	–	–	–	–		–	–
Kidney (2)	2+	–	–	–	–	–	–	1+	–		–	–
Testis (2)	–	–	–	–	–	1+[d]	–	2+	–		2+	–
Tissues negative for all 12 antigens												
Brain (3); gray matter, whitematter												
Lymph nodes (2)												
Smooth muscle (2)												
Connective tissue (2 each): lung, breast, prostate, colon, stomach, pancreas, uterus, ovary, liver, and kidney												

[a] All tissues were stained by avidin-biotin complex immunoperoxidase method, except stomach, liver, and kidney, which were stained by the indirect immunoperoxidase method.
[b] The numbers in parenthesis indicate the number of different specimens tested.
[c] Histiocytes in the red pulp were predominantly stained.
[d] Seminiferous tubules were stained.

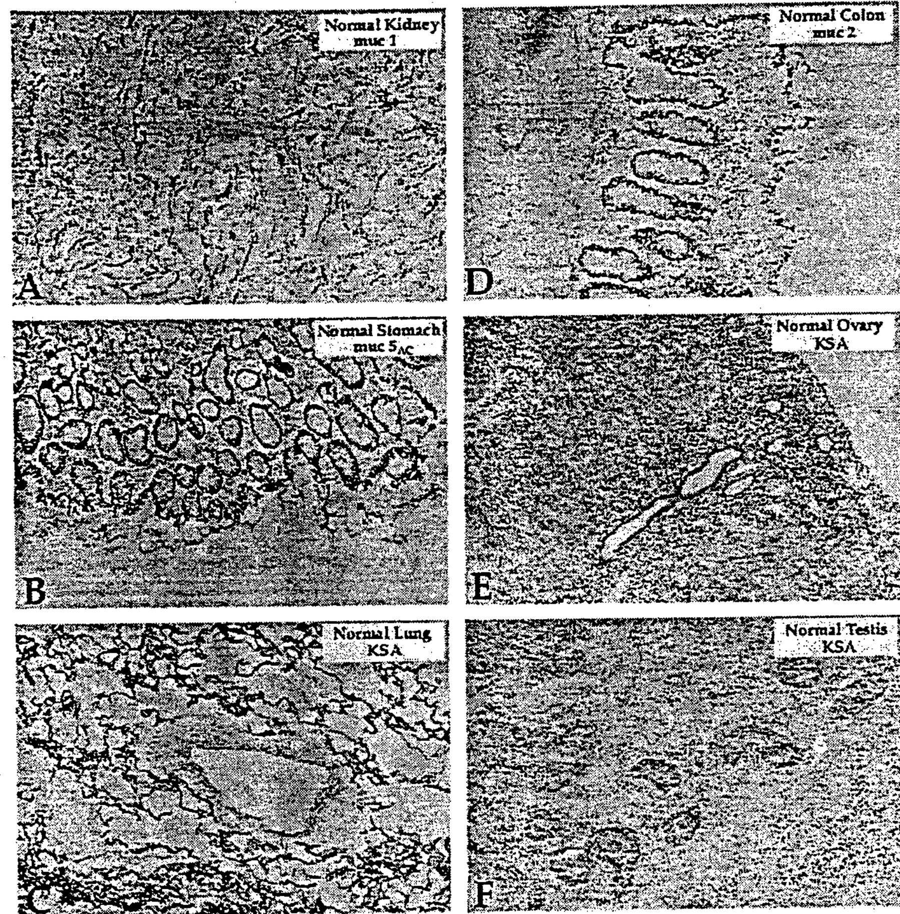

Fig. 2 Expression of protein antigens on normal tissues. Epithelial cells at secretory borders were stained in kidney (*A*) with mAb HMFG2 against MUC1 (2+); in stomach (*B*) with mAb CLH2 against $MUC5_{AC}$ (4+); in lung (*C*), ovary (*E*), and testis (*F*) with mAb GA733-2 against KSA (4+, 2+, and 3+, respectively) and in colon (*D*) with mAb LDQ10 against MUC2 (3+). Magnification, ×70.

Reactivity of mAbs with Normal Tissues. Table 3 summarizes the immunoreactivity on normal tissue samples observed with the panel of mAbs. MUC1 was weakly distributed on the epithelia of all of the tested organs, except liver. MUC2 was observed on the epithelia of prostate, colon, and pancreas. MUC3 was only detected on epithelia of pancreas. MUC4 was expressed on epithelia of colon and prostate (weakly). $MUC5_{AC}$ was very strongly expressed in stomach epithelium. MUC7 and HER-2/*neu* were not expressed on any normal tissues, and $MUC5_B$ was only detected on normal colon epithelium and weakly in the testis. βhCG was detected in epithelia of prostate, stomach, and pancreas and weakly in colon and lung, and it was detected in the testis. PSMA was only detected on prostate epithelia. KSA was strongly expressed on the epithelia of all of the tested organs except stomach and liver and moderately expressed on seminiferous tubules of testis. CEA was strongly expressed in the epithelia of prostate and colon and weakly in lung, uterus and breast. The pattern of expression of each of these antigens on normal epithelia was mainly luminal, with evident polarity. Luminal cells stained most intensively at luminal borders. In addition, CEA was detected in histiocytes in the red pulp of the spleen, an expected consequence not of CEA expression but of the mAb used, NCL-CEA, which cross-reacts with nonspecific cross-reacting antigen on histiocytes (35). Examples of the staining patterns on normal tissues with these mAbs are shown in Fig. 2. Once again, staining of stroma was uniformly negative.

DISCUSSION

One of the striking features of our two previous reports was the clear separation between the carbohydrate antigens expressed by tumors of neuroectodermal origin and the carbohydrate antigens expressed by tumors of epithelial origin (1, 2). This is also the case for the protein antigens studied here. None of the seven mucins were expressed on more than one of the specimens of the five nonepithelial origin cancers, but these mucins were widely and densely expressed on a variety of epithelial cancers. The same applies for the other glycoproteins, except that all small cell lung cancers expressed KSA very strongly and some expressed CEA, and some sarcomas expressed moderate amounts of βhCG. On this basis, melanomas, sarcomas, neuroblastomas, and B-cell lymphomas are quite distinct from the eight epithelial cancers tested. Small cell lung cancer, not surprisingly, is intermediary, with some characteristics of each group.

This study differs from previous reports on the distribution of these antigens in several ways. Our focus was entirely on potential targets for immunotherapy and, especially, on antibody-mediated immunotherapy. We have compared the expres-

sion of 12 antigens rather than 1 to 3 and explored a wide variety of malignant and normal tissues rather than a few. On the other hand, we tested only five to eight specimens in most cases, and because this was part of a larger study looking at expression of glycolipid antigens as well, specimens were fixed with 10% formalin, which may not be ideal for some protein antigens. However, to the extent that others have studied the expression of these antigens on these cancers, our study is largely in agreement. MUC1 has long been known to be expressed by many normal epithelial tissues and by many or most cancers of breast, ovary, pancreas, prostate, and colon origin (21–23, 26). We concur and add to this list endometrial and non-small cell lung cancer. MUC2 has been previously identified in most colon cancers as well as cancers of the stomach, pancreas, breast, and, recently, prostate (22, 24, 36). We concur, except that we found no evidence of MUC2 in the five gastric cancer specimens we tested. The previously described more restricted expression of MUC2 than MUC1 on normal tissues (22, 24, 36, 37), with MUC2 detected in the GI tract and, recently, the prostate (36) but not most other sites, was also our finding. MUC3 was previously detected on the majority of colon, ovarian, and gastric cancers (20, 24), in agreement with our findings, but also in the GI tract but not the pancreas, which is the reverse of our findings. $MUC5_{AC}$ has previously been detected in the majority of gastric cancers and in normal stomach, as we found, and we add to this strong expression in most breast cancers (22, 27). $MUC5_B$ has been described to be strongly expressed on some colorectal carcinomas and normal colon (22), as we found. We add to this moderate expression of $MUC5_B$ on the majority of breast cancers. Our study breaks little new ground on the distribution of KSA (38) and PSMA (4, 28, 36), except that we were not prepared for the intensity and uniformity of KSA expression on all epithelial cancers tested (and normal epithelial tissues), and we have extended the number of different normal tissues and nonprostate cancers that are negative for PSMA by immunohistology. Likewise, we confirm the strong expression of CEA on most breast, lung, and GI malignancies and the corresponding normal tissues as described previously (35, 39, 40). βhCG mRNA has been described to be strongly expressed in 61% of bladder cancers (which we did not test) and to be moderately expressed in 46% of breast cancers and 20% of prostate cancers (19), which agrees with our findings. We add to this moderate expression in a small proportion of several other cancers and the majority of sarcomas and cancers of the lung and pancreas, as well as a variety of normal tissues.

A benefit of testing many different types of cancers with a broad range of mAbs is that it permits selection of the several antigens most suitable as targets for immune attack against each cancer. Expression on normal tissues is, of course, a consideration in this selection, but expression at the secretory border of epithelial tissues does not appear to be a problem (as discussed at greater length in part I of this series; Ref. 1). Antigens expressed at epithelial secretory borders induce neither immunological tolerance nor detectable autoimmunity once antibodies are administered or induced against them. Consequently, if strong expression [handwritten: on ≥ 80% of tumor cells] of 60% or more of the cancer specimens tested but not on immune accessible tissues are used as selection criteria, the antigens selected as targets for each cancer are shown in Table 4. The results summarized in Table 4 for protein

Table 4 Protein targets for antibody-mediated immunotherapy[a]

Cancer	Antigens[b]
Melanoma	None
Sarcoma	(βhCG)
Neuroblastoma	None
B-cell lymphoma	None
Small cell lung cancer	KSA
Breast	MUC1, $MUC5_{AC}$, (KSA), (CEA)
Prostate	MUC2, KSA, (PSMA)
Lung	MUC1, CEA, KSA, (MUC4), (βhCG)
Colon	MUC2, CEA, KSA, (MUC4)
Pancreas	KSA, (MUC4), (CEA), (βhCG)
Gastric	$MUC5_{AC}$, CEA, KSA, (MUC3)
Ovarian	MUC1, KSA, (MUC3)
Endometrial	KSA, (MUC1)

[a] Targets selected from the 12 antigens tested in this study.
[b] Antigens expressed intensely (4+) on ≥80% of tumor cells in ≥70% of specimens. Antigens in parentheses were expressed [handwritten: on ≥ 80% of tumor cel.] strongly (3+) on at least 50% or moderately (2+) on at least 60% of specimens.

antigens plus the corresponding tables for ganglioside and carbohydrate antigens in parts I and II of this series (1, 2) provide the basis for selection of multiple antigens as targets for antibody-mediated immune attack against these cancers.

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