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13. ABSTRACT (Maximum 200 Words)

The objective is to identify gangliosides associated with prostate cancer (CaP) and their ability to induce immune responses. Resorcinol-HCl and specific monoclonal antibodies were used on 2D-chromatograms, and to visualize on the cell surface with confocal-fluorescence microscopy. IgM against eight gangliosides from sera of patients with BPH, organ-confined and unconfined CaP, and age-matched healthy men were analyzed by ELISA double-blinded and compared using ANOVA and Fisher's least significant difference methods. Endogenous IgM to gangliosides were measured in patients with confined CaP. CaP cells expressed GM1b, GM2, GD2, GD1a, and GM3. GM1a, GD1b and GT1b were undetectable. GM1b and GD1a were more prominent in AR-negative than in AR-positive cells. CaP patients differed from healthy and BPH patients in increased anti-GD2 and antiGD1a IgM and decreased anti-GD3. Other anti-ganglioside IgMs showed no difference among groups. The augmentation of anti-GD1a IgM in patients with organ-confined CaP but not in patients with unconfined CaP or BPH or in healthy controls is striking ($p < 0.025$). The unique ganglioside profiles identify HH870 cell line a potential component of a polyvalent-vaccine for immunotherapy of CaP. Augmentation of anti-GD1a IgM in confined CaP may signify an earliest immune response to eliminate GD1a from tumor microenvironment and circulation.

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INTRODUCTION

Metastatic prostate Cancer (CaP) can be treated by immunotherapy, which has been clinically documented to regress melanomas and renal carcinomas without toxicity, improve the quality of life and prolong the survival of the patients. The feasibility of extending immunotherapy for CaP depends on (1) recognition of heterogeneity of tumor antigens and (2) identification of appropriate target antigens. Our focus is on the cell surface glycoantigens, which were used as targets for immunotherapy human melanoma. Of the various cell surface glycoantigens, gangliosides are unique in that they are overexpressed in tumor cells than on the surface of normal. Furthermore, when they are released from tumor cells into tumor microenvironment and circulation, they block antitumor T-cell functions. However, the specific tumor gangliosides involved these interactions remain to be identified. Although gangliosides are reported in normal and benign prostate tissues, there is meager information on Prostate Cancer (CaP)-associated gangliosides. The purpose of this proposal is to identify the ganglioside signatures of CaP from confined and unconfined (metastatic) tumors and to identify the immunogenic species which can serve as targets for active specific immunotherapy. We hypothesized that *the profile CaP-associated gangliosides and their ability to induce immune response will be beneficial to formulate an immunotherapy protocol for CaP.* If we could identify the CaP-associated gangliosides that are highly immunogenic in patients, it would enable us to develop a cellular vaccine for CaP with balanced expression of immunogenic gangliosides. We will also assess whether the gangliosides are released into circulation during tumor growth. Our results not only revealed the unique gangliosides of androgen-receptor negative and androgen-receptor positive CaP but also the differences between confined and unconfined CaP. In addition, we have observed that the tumor gangliosides as early as in confined state of the disease (stage T1 & T2) act as danger signals and elicit immune response against them. While we could not detect any significant increase in the level of serum gangliosides in patients with localized disease, their profile for IgM antibodies against specific CaP-associated gangliosides are elevated, thus indicating that these endogenous immune response to CaP-associated gangliosides have the potential to serve as early biomarkers for confined prostate cancer.

BODY

Task # 1. To determine the nature of gangliosides expressed in human CaP cell lines and biopsied CaP tissue (months 1-12).

a. Established CaP cell lines (DU 145, LNCaP-FGC & LNCaP-FGC-, NCI-H660, PC-3) obtained from American Type Culture Collection (ATCC), HH870 (Hoag Cancer Center) and six prostate cell lines developed at JWCI will be grown in appropriate media supplemented with hormones, as recommended by ATCC. The cells will be collected, washed and lysed by a freeze-thaw procedure, and processed by cell-lysate ELISA and biochemical analysis of the gangliosides. This study will provide an overall qualitative and quantitative profile of gangliosides in CaP cell lines. (months 1-6).

b. Single-cell suspensions prepared from 11 cryopreserved CaP biopsy specimens obtained from JWCI's tissue bank or from study collaborators. They will be cultured in suitable media for five passages to remove adherent nontumor cells. The recovered cells will be analyzed only by immunoassays for ganglioside expression because the cell density is not sufficient for biochemical assay. (months 1-6).

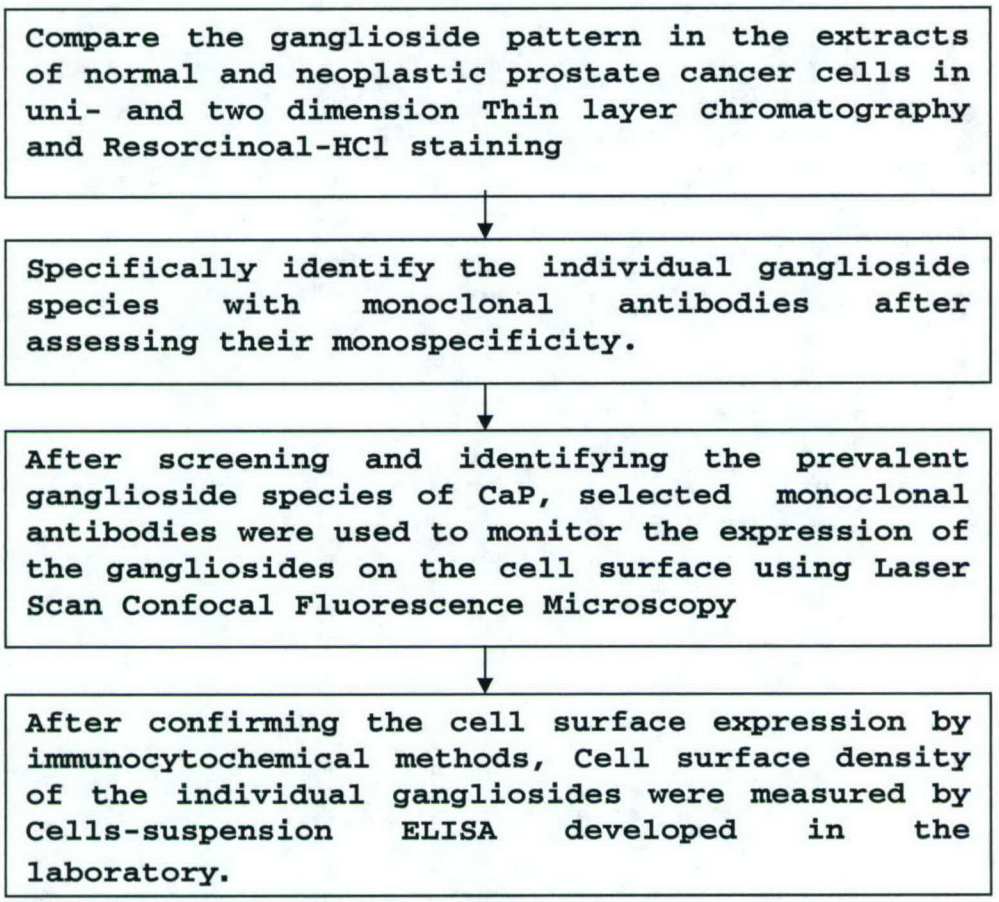
c. Total and specific gangliosides will be analyzed by the following procedures:
   i. Ganglioside extraction, isolation and thin layer chromatography followed by densitometry;
   ii. Cell-surface ELISA using specific affinity-purified monoclonal antibodies for GM2, GD3, GD2, GM2, GD1b and GT1b.
   iii. Cell-lysate ELISA using specific affinity-purified monoclonal antibodies (months 6-10).
   iv. Fresh or frozen sections will also be used as and when available for expression of gangliosides using a battery of monoclonal antibodies

These analyses will confirm the heterogeneity of the ganglioside profile observed among cell lines.

d. Data analyzed, report written, manuscript on heterogeneity of cell-surface expression of gangliosides in CaP will be outlined (months 10-12).

The investigation is completed to the fullest extent. More than 70% of the observations made were presented in the manuscript accepted for publication (Biochemical and Biophysical Research Communications (in Press) [see Appendix #1]. About 30% of observations particularly observations made were not published yet. Some of them relate to observations made on paraffin sections, which require revalidation with immunostaining with monospecific monoclonal antibodies.

Summary of the strategy planned for this investigation:



The outcome of the investigation can be summarized as follows: (Summary of the manuscript accepted for publication in BBRC

Prior development of a unique androgen-receptor (AR)-negative cell line (HH870) from organ-confined (T2b) human prostate cancer (CaP) enabled comparison of the gangliosides associated with normal and neoplastic prostate epithelial cells, organ-confined *versus* metastatic (DU 145, PC-3), and AR-negative *versus* AR-positive CaP cell lines. Resorcinol-HCl and specific monoclonal antibodies were used to characterize gangliosides on 2D-chromatograms, and to visualize them on the cell surface with confocal-fluorescence microscopy. AR-negative cells expressed GM1b, GM2, GD2, GD1a, and GM3. GM1a, GD1b and GT1b were undetectable. GM1b and GD1a were more prominent in AR-negative than in AR-positive cells. PC-3 and HH870 cells were unique in the expression of O-AcGD2 and two □2,3sialidase-resistant, alkali-susceptible GMR17-reactive gangliosides. Expression of GD1a, GM1b, doublets of GD3, GD2 and O-AcGD2, and the presence of an additional alkali-labile-14.G2a-reactive ganglioside, two alkali-susceptible and three alkali-resistant GMR17-reactive

gangliosides makes HH870 a potential component of a polyvalent-vaccine for active-specific immunotherapy of CaP.

Mepur H. Ravindranath, Sakunthala Muthugounder, Naftali Presser, Senthamil R. Selvan, Jacques Portoukalian, Stanley Brosman, and Donald L. Morton Gangliosides of Organ-Confined versus Metastatic Androgen Receptor-Negative Prostate Cancer. **Biochem Biophys Res Commun, in press.** [See Appendix # 1]

Task # 2. To measure the level and profile of serum gangliosides in patients with various stages of CaP (months 13-21),

a. The Division of Serology at JWCI will coordinate the release of sera from the serum bank to the laboratory. Initially a total of 150 serum vials will be released in coded fashion. At least 30 coded serum vials for each stage of the disease will be issued. Based on the results and statistical power estimation to obtain a significance of < 0.05, the sample size required will be immediately calculated and additional sera will be analyzed. Sera will be accrued continuously from collaborators starting from month 1 (months 13-15).

b. Each serum specimen will be analyzed for total gangliosides by biochemical procedures and antiganglioside IgM by ELISA (mon13-15).

c. Specific gangliosides in the sera will be analyzed as follows:
   i. Ganglioside extraction, isolation and thin-layer chromatography followed by densitometry
   ii ELISA using specific affinity-purified monoclonal antibodies for gangliosides (mon 16-21)

d. Data analyzed, PSA values will be obtained from patient charts, serum ganglioside levels and PSA levels will be compared, report written, manuscript on serum gangliosides in different stages of CaP is outlined (mon 18-21).

The strategy Planned for this investigation

Compare the levels of serum total PSA and total gangliosides among patients with different stages of CaP and BPH and with that of normal and healthy volunteers

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If the differences in the level of serum total gangliosides could be detected among different stages, we will screen for individual gangliosides; If not, we will examine whether there are any immune signals from the CaP-associated gangliosides.

The results of the analyses pertaining serum total gangliosides and PSA done under this investigation are presented in Appendices # 2 to 4. The results showed that serum total ganglioside level is independent of PSA. The results of the statistical analyses may be summarized as follows.

1. Correlation between PSA level and Serum Total ganglioside level (N=35, data 1) is not significant as evidenced by the p values obtained with Spearman correlation co-efficient=0.1987, P=0.2525.
2. Kruskal-Wallis test for comparison of the serum total ganglioside among BPH, Cancer, Healthy and Prostatitis group showed significant difference in the level of the serum gangliosides among the above groups (p=0.0065). The serum total ganglioside level in patients with prostatitis is significantly lower than that of healthy and CaP patients.

P-values from pair-wise comparison

	BPH	Cancer	Healthy men
Cancer	**0.0141**		
Healthy men	**0.0005**	**0.0627**	
Prostatitis	0.1341	0.8457	0.4714

Group	N	Mean	Std	Max	Median	Min
BPH	14	12.8000	2.3846	16.2	13.0	8.7
Cancer	51	15.2471	3.3751	21.6	15.6	6.2
Healthy Men	20	16.8570	3.1528	22.7	16.9	12.1
Prostatitis	4	15.5750	4.3821	20.6	15.7	10.3

1. Kruskal-Wallis test for comparison of the serum total ganglioside among different stages of prostate cancer showed statistically significant difference in the level of serum total gangliosides among different stages of T stage disease ($p < 0.015$)

P-values from pair-wise comparison

	Stage T1 (n=19)	Stage T2 (n=9)	Stage T3 (n=14)
Stage T2 (n=9)	0.2968		
Stage T3 (n=14)	0.3946	0.7724	
Stage T4 (n=9)	**0.0013**	**0.0479**	**0.0147**

Group	N	Mean	Std	Max	Median	Min
Stage T1	19	13.9947	3.2877	21.6	13.00	7.5
Stage T2	9	15.3222	3.9673	18.9	16.50	6.2
Stage T3	14	14.9357	2.8835	18.4	14.75	9.7
Stage T4	9	18.3000	1.8200	21.0	18.10	15.5

Task # 3. To identify the interaction between CaP gangliosides and cytokines associated with CTL stimulation (months 22-36).

a. Experiment # 1. Ganglioside-cytokine binding assay by ELISA. Purified gangliosides characteristic of prostate (or that isolated from prostate cancer) will be coated on microtiter plates for cytokine ELISA and determine the binding to IL-2, TNF-□. IFN-□. IL-12 and IL10, we will also irrelevant gangliosides will be used as negative controls (months 22-26).

b. Experiment # 2. Ganglioside-cytokine binding assay by TLC-autoradiography. Purified gangliosides characteristic of prostate (or that isolated from prostate cancer) will be fractionated or spotted on silica gel coated plastic plates. Labeled (Radiolabeled or Fluorescent dye conjugated) IL-2, TNF-α. IFN~α. IL-12 and IL10 will be used for detection. A panel of irrelevant gangliosides will be used as negative controls (months 27-30).

c. Experiment # 3. [^{125}I] IL-2 receptor binding assay in the presence and absence of prostate specific and irrelevant gangliosides (months 31-34). Human leukemia cell T cell lines will be used.

d. Experiment # 4 Ganglioside-induced IL-10 production by human T cells. Peripheral blood lymphocytes taken from healthy volunteers will be used

e. Data analyzed, report written, manuscript on CaP gangliosides interacting with cytokines is outlined (months 35-36).

Important changes in the strategy of specific aim# 3.

Since serum total ganglioside level is not significantly different among stages T1, T2 and T3, instead of characterizing the ganglioside profiles in the serum, we have screened the sera for antibodies against the most prevalent gangliosides of prostate Cancer, which has yielded a serendipitous discovery of very early endogenous immune response to gangliosides. This has become an important finding to proceed further to analyze the interaction between gangliosides and patients immune status.

The results of the findings were submitted as a manuscript to International Journal of Cancer. The communication is awaited. The manuscript is included as Appendix # 6. The summary of the finding is as follows:

The endogenous IgM antibodies to gangliosides associated with tumor cells are correlated with disease progression in cancer patients. This study determines whether endogenous IgM to gangliosides occur in patients with confined CaP, after defining the ganglioside-profiles of the CaP cell lines. Using ELISA, the

titers of IgM against eight gangliosides from sera of patients with benign prostatic hyperplasia (BPH) (n=10), organ-confined (T1/T2, n=20) and unconfined (T3/T4, n=7) CaP, and age-matched healthy men (n=11) were determined double-blinded. Using ANOVA and Fisher's least significant difference methods, the log-titers among different groups were compared. Immune- and resorcinol staining of the ganglioside extracts of normal (PrEC) and neoplastic androgen-insensitive (PC-3, DU145) and sensitive (LNCaP FGC and LNCaP FGC-10) prostatic epithelial cells revealed the presence of gangliosides, GM3, GM2, GD3, GD2 and GD1a but not GM1a, GD1b or GT1b. CaP patients (stage T) differed from healthy and BPH patients in increased titers against GD2 and GD1a, decreased titers against GD3. Antibodies to other gangliosides showed no difference in the titers between CaP patients and others. The augmentation of anti-GD1a IgM in patients with organ-confined CaP (stage T1/T2) but not in patients with unconfined CaP (stage T3/T4) or BPH or in healthy controls is striking ($p < 0.025$). This finding together with identification of GD1a as a major ganglioside in CaP cell lines and with the reports on the immunosuppressive nature of GD1a suggests that augmentation of anti-GD1a IgM in confined CaP may signify an earliest endogenous immune response to eliminate GD1a from tumor microenvironment and circulation.

Mepur H. Ravindranath, Sakunthala Muthugounder, Naftali Presser, Xing Ye, Stanley Brosman, and Donald L. Morton Endogenous Immune Response to Gangliosides in Patients with Confined Prostate Cancer. **Intl J Cancer *(submitted) (Appendix # 5)***

Based on both the ganglioside profiles of prostate cancer and endogenous IgM response to the CaP-associated gangliosides, we consider GD1a is the most important Prostate associated gangliosides. Following which we consider GM1b is very important. Although GM3 and GM2 are major gangliosides of prostate cancer, they are also the major gangliosides of normal prostatic epithelial cells. Therefore further investigations on the interaction of the gangliosides with cytokines we have focused GD1a and GM1b. GD1a is available commercially, where as GM1b had to be extracted from the prostate cancer cell lines. Since GD1a is available commercially, we have launched an ELISA assay to test whether GD1a binds to IL-2. The results were negative as indicated in the figure. We intend to screen the affinity of GD1a for other cytokines.

Table 1. Recombinant IL-2 recognizes and binds specifically to the Galβ1,3GalNAc β1,4 residues of GD_{1b} but not to Prostate Cancer-associated GD_{1a}.

Glycolipids	Expression in CaP[1]	IL-2+anti-IL-2 MAb [2]	anti-IL-2Mab (neg control)
Ethanol control	-	≤0.1	≤0.1
GD_3	Very low	≤0.1	≤0.1
GM_3	high	≤0.1	≤0.1
GM_2	high	≤0.1	≤0.1
AsialoGM_1	Not tested	0.522	0.820
GM_{1a}	absent	≤0.1	≤0.1
GD_{1a}	high	≤0.1	≤0.1
GD_{1b}	absent	1.280	0.150
GT_{1b}	absent	≤0.1	≤0.1
GD_2	high	≤0.1	≤0.1

[1]Expression in Prostate Cancer cells compared to normal prostatic epithelia; [2]Values are the mean of duplicates obtained by two independent investigators, expressed as absorbance difference between 490 nm and 650 nm after correcting for respective negative controls (Anti-IL-2 MAb without IL-2). The amount of ganglioside is 3 nmol/well and IL-2 used is 0.24 μg/well. The dilution of biotinylated anti-IL-2 antibody is 1/1000 at 100 □L/well. Gangliosides suspended in ethanol were added into the wells and dried under vacuum, therefore wells similarly treated with ethanol were used as background.

Complete List of Work accomplished, outcome and work strategy changed.

Task #1

1. Compared the ganglioside signatures in the extracts of normal (PrEC) and neoplastic androgen-insensitive (PC-3, DU145, HH870) and sensitive (LNCaP FGC and LNCaP FGC-10).
2. Used the monoclonal antibodies to gangliosides to screen paraffin sections of prostate biopsies of varying Gleason grade.

Outcome:

1. Confirmed that the major gangliosides of prostate cancer, not found in normal prostatic epithelial cells are: O-acetyl GD2, GD2, GD1a and GM1b.
2. Confirmed the absence of GD1b, GT1b. GM1a could not be detected. GD3 is poorly expressed. GM3 and GM2 though overexpressed in prostate cancer, the gangliosides are also the major gangliosides of normal prostatic epithelial cells.
3. HH870 is selected as a potential component of a polyvalent cellular vaccine for active specific immunotherapy of prostate cancer fro the following reasons: (i) Expression of GD1a, GM1b, doublets of GD2 and O-AcGD2, (ii)the presence of an additional alkali-labile-14.G2a-reactive ganglioside, and (iii) two alkali-susceptible and three alkali-resistant gangliosides reactive to monoclonal antibody GMR17 (specific for Gm1b, GD1a and GT1b).
4. Biosynthetic pathway of the major gangliosides of prostate cancer is presented in Biochem. Biophys. Res. Commun. (in press).

Task # 2

1. Levels of serum total PSA and total gangliosides were measured in healthy men (n=20), patients with Cancer (n=51), benign prostatic hyperplasia (n=14) and prostatitis (n=4). For stages of prostate cancer, we have obtained sera from stage T1 (n=19), stage T2 (n=9), stage T3 (n=14), stage T4 (n=9). We have obtained 150 sera from patients and volunteers, but we could complete only 95 sera (51 cancer patients, 20 BPH patients, 4 prostatitis patients and 20 healthy volunteers with budget allocated for analyses).
2. Levels of IgM titers for antiganglioside antibodies against the following gangliosides were measured: GM3, GM2, GM1a, GD3, GD2, GD1a, GD1b, GT1b. In addition, we have carried out Immunoblotting for IgM against GM1b.

Outcome

1. There is not correlation between PSA and serum total ganglioside level as assessed by Spearman correlation coefficient ($p<0.25$).
2. Sera of patient with BPH had significantly lower level of serum total gangliosides compared to healthy men and cancer

patients. This is a seminal finding relevant to distinguish BPH from CaP.

3. Serum level of gangliosides in cancer patients did not differ from that of the health men.
4. Of the various antiganglioside antibodies screened CaP patients (stage T) differed from healthy and BPH patients in increased titers against GD2 and GD1a, decreased titers against GD3.
5. Antibodies to other gangliosides showed no difference in the titers between CaP patients and others.

Task # 3 and modification of strategy

1. Prostate ganglioside cytokine binding assay was carried out for IL-2.
2. Further binding assays were not done and the course of investigation is redirected to create a seminal finding on early detection of confined CaP.

The main reason for changing the strategy from studying cell mediated to humoral response is the finding that the level of serum total gangliosides are too low to evaluate the presence of individual gangliosides. Since we could not screen the individual gangliosides in the sera, we have resorted to screen IgM antibodies to different gangliosides. Although the antiganglioside IgM profile in patients confirmed the findings made in Task # 1, that the major and the most important gangliosides of prostate cancer are GD1a and GD2, the very presence of IgM antibodies in the sera suggest that the antibodies neutralize any of the prostate cancer gangliosides released into circulation. This explains why we could not observe any significant difference in the level of serum gangliosides. Although stage T4 showed significantly higher ganglioside level than other stages, all patients taken into consideration did not show difference from healthy and age-matched men volunteers. Therefore we have modified our approach to determine that humoral responses in relation to stages of the disease.

Outcome:

1. The augmentation of anti-GD1a IgM in patients with organ-confined CaP (stage T1/T2) but not in patients with unconfined CaP (stage T3/T4) or BPH or in healthy controls is strikingly significant ($p < 0.025$).
2. This finding together with identification of GD1a as a major ganglioside in CaP cell lines and with the reports on the immunosuppressive nature of GD1a suggests that augmentation of anti-GD1a IgM in confined CaP may signify an earliest endogenous immune response to eliminate GD1a from tumor microenvironment and circulation.

KEY RESEARCH ACCOMPLISHMENTS (Bulleted list)

- O The ganglioside signatures of human prostatic normal and neoplastic epithelial cells were characterized.
- O Development of a unique androgen-receptor (AR)-negative cell line (HH870) from organ-confined (T2b) human prostate cancer (CaP) enabled comparison of organ-confined *versus* metastatic (DU 145, PC-3) CaP cell lines.
- O GM1b and GD1a were more prominent in Androgen Receptor-negative than in AR-positive prostate cancer cells.
- O GM1a, GD1b and GT1b were undetectable in both androgen-receptor positive and negative cells.
- O PC-3 and HH870 cells were unique in the expression of O-AcGD2 and two □2,3sialidase-resistant, alkali-susceptible gangliosides reactive to the monoclonal antibody to GMR17 (reacts with GM1b, GD1a & GT1b).
- O Expression of GD1a, GM1b, doublets of GD2 and O-AcGD2, and the presence of an additional alkali-labile-14.G2a-reactive ganglioside, two alkali-susceptible and three alkali-resistant GMR17-reactive gangliosides makes HH870 a potential component of a polyvalent-vaccine for active-specific immunotherapy of CaP.
- O After defining the ganglioside-profiles of the CaP cells, this study determines whether endogenous IgM to gangliosides occur in patients with confined CaP.
- O Using ANOVA and Fisher's least significant difference methods, the log- IgM titers against eight gangliosides were compared among sera of patients with benign prostatic hyperplasia (BPH) (n=10), organ-confined (T1/T2, n=20) and unconfined (T3/T4, n=7) CaP, and age-matched healthy men (n=11) double-blinded.
- O CaP patients (stage T) differed from healthy and BPH patients in increased titers against GD2 and GD1a, decreased titers against GD3.
- O Antibodies to other gangliosides showed no difference in the titers between CaP patients and others.
- O The augmentation of anti-GD1a IgM in patients with organ-confined CaP (stage T1/T2) but not in patients with unconfined CaP (stage T3/T4) or BPH or in healthy controls is striking ($p < 0.025$).
- O This finding together with identification of GD1a as a major ganglioside in CaP cell lines and with the reports on the immunosuppressive nature of GD1a suggests that augmentation of anti-GD1a IgM in confined CaP may signify an earliest endogenous immune response to eliminate GD1a from tumor microenvironment and circulation.

REPORTABLE OUTCOMES

Abstracts:

1. Ravindranath MH, Muthugounder S, Verma M, Portoukalian J, Morton DL, (2002) Evidence for IgM response to GD1a and GT1b in patients with early stage Prostate carcinoma and Melanoma. Abstract of the talk presented at the **International Workshop on Immunotherapy,** Center for Molecular Immunology, Havana 11600, Cuba.
2. Ravindranath MH, Muthugounder S, Verma, M, Selvan RR, Portoukalian J, Brosman S, Morton DL, (2003) Neoplastic transformation changes the ganglioside profile of prostatic epithelial cells. **Proc. Amer. Assoc. Cancer Res.** 44:2407.
3. Ravindranath MH, Muthugounder S, Presser N, Brosman S, Morton DL, (2004) Endogenous IgM Response to Tumor-associated Ganglioside GD1a in Patients with Prostate Cancer or Melanoma. Abstract of the talk to be presented at the 4th **International Congress on Autoimmunity,** Budapest, Hungary.

Full Length Papers:

4. Ravindranath MH, Muthugounder S, Presser N, Selvan SR, Portoukalian J, Brosman S, Morton DL, (2004) Gangliosides of Organ-Confined versus Metastatic Androgen Receptor-Negative Prostate Cancer. **Biochem. Biophys. Res. Commun.** (in Press).
5. Ravindranath MH, Muthugounder S, Presser N, Ye X, Brosman S, Morton DL, (2004) Endogenous Immune Response to Gangliosides in Patients with Confined Prostate Cancer. **Intl J. Cancer** (submitted).

CONCLUSIONS

Development of Immunotherapy of prostate Cancer (CaP) depends on (1) recognition of heterogeneity of CaP-antigens and (2) identification of appropriate target antigens. Of the various cell surface glycoantigens, gangliosides are unique in that they are overexpressed in tumor cells than on the surface of normal. Although gangliosides are reported in normal and benign prostate tissues, there is meager information on Prostate Cancer (CaP)-associated gangliosides. The objective of this proposal is to identify the profile CaP-associated gangliosides and their ability to induce immune response, which will be beneficial to formulate an immunotherapy protocol for CaP. If we could identify the CaP-associated gangliosides that are highly immunogenic in patients, it would enable us to develop a cellular vaccine for CaP with balanced expression of immunogenic gangliosides. Development of a unique androgen-receptor (AR)-negative cell line (HH870) from organ-confined (T2b) human prostate cancer (CaP) enabled comparison of the gangliosides associated with normal and neoplastic prostate epithelial cells, organ-confined *versus* metastatic (DU 145, PC-3), and AR-negative *versus* AR-positive CaP cell lines. Resorcinol-HCl and specific monoclonal antibodies

were used to characterize gangliosides on 2D-chromatograms, and to visualize them on the cell surface with confocal-fluorescence microscopy. AR-negative cells expressed GM1b, GM2, GD2, GD1a, and GM3. GM1a, GD1b and GT1b were undetectable. GM1b and GD1a were more prominent in AR-negative than in AR-positive cells. PC-3 and HH870 cells were unique in the expression of O-AcGD2 and two □2,3sialidase-resistant, alkali-susceptible gangliosides reactive to monoclonal antibody GMR17. Expression of GD1a, GM1b, doublets of GD3, GD2 and O-AcGD2, and the presence of an additional alkali-labile-14.G2a-reactive ganglioside, two alkali-susceptible and three alkali-resistant GMR17-reactive gangliosides makes HH870 a potential component of a polyvalent-vaccine for active-specific immunotherapy of CaP. After defining the ganglioside-profiles of the CaP cell lines, this study examines whether endogenous IgM to gangliosides occur in patients with confined CaP, Using ELISA, the titers of IgM against eight gangliosides from sera of patients with benign prostatic hyperplasia (BPH) (n=10), organ-confined (T1/T2, n=20) and unconfined (T3/T4, n=7) CaP, and age-matched healthy men (n=11) were determined double-blinded. Using ANOVA and Fisher's least significant difference methods, the log-titers among different groups were compared. CaP patients (stage T) differed from healthy and BPH patients in increased titers against GD2 and GD1a, decreased titers against GD3. Antibodies to other gangliosides showed no difference in the titers between CaP patients and others. The augmentation of anti-GD1a IgM in patients with organ-confined CaP (stage T1/T2) but not in patients with unconfined CaP (stage T3/T4) or BPH or in healthy controls is striking ($p < 0.025$). This finding together with identification of GD1a as a major ganglioside in CaP cell lines and with the reports on the immunosuppressive nature of GD1a suggests that augmentation of anti-GD1a IgM in confined CaP may signify an earliest endogenous immune response to eliminate GD1a from tumor microenvironment and circulation.

REFERENCES

This report summarizes the achievement of the tasks and the outcome. It also explains the need to change the strategy during the course of the experiments. We refer the details of our findings to two of our publications.

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All the relevant references are cited in both the manuscripts.

APPENDICES

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APPENDIX # 1

August 31, 2004

Ms. No.: BBRC-04-2319

Title: Gangliosides of Organ-Confined versus Metastatic Androgen Receptor-Negative Prostate Cancer
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Dear Dr. Ravindranath,

We are pleased to inform you that your manuscript referenced above has been accepted for publication in Biochemical and Biophysical Research Communications.

Many thanks for submitting your fine paper to Biochemical and Biophysical Research Communications.

With kind regards,

Naoyuki Taniguchi
Editor
Biochemical and Biophysical Research Communications

Gangliosides of Organ-Confined *versus* Metastatic Androgen Receptor-Negative Prostate Cancer

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Prior development of a unique androgen-receptor (AR)-negative cell line (HH870) from organ-confined (T2b) human prostate cancer (CaP) enabled comparison of the gangliosides associated with normal and neoplastic prostate epithelial cells, organ-confined *versus* metastatic (DU 145, PC-3), and AR-negative *versus* AR-positive CaP cell lines. Resorcinol-HCl and specific monoclonal antibodies were used to characterize gangliosides on 2D-chromatograms, and to visualize them on the cell surface with confocal-fluorescence microscopy. AR-negative cells expressed GM1b, GM2, GD2, GD1a, and GM3. GM1a, GD1b and GT1b were undetectable. GM1b and GD1a were more prominent in AR-negative than in AR-positive cells. PC-3 and HH870 cells were unique in the expression of O-AcGD2 and two α2,3sialidase-resistant, alkali-susceptible GMR17-reactive gangliosides. Expression of GD1a, GM1b, doublets of GD3, GD2 and O-AcGD2, and the presence of an additional alkali-labile-14.G2a-reactive ganglioside, two alkali-susceptible and three alkali-resistant GMR17-reactive gangliosides makes HH870 a potential component of a polyvalent-vaccine for active-specific immunotherapy of CaP.

Key Words: Gangliosides, Prostate Cancer, Androgen-Receptor, Organ-confined, metastasis, O-acetylGD2, 2D-chromatogram, HPLC, Confocal-Fluorescence-Microscopy

Gangliosides are a unique class of glycoantigens implicated in tumor progression, proliferation, invasion, angiogenesis and immunosuppression [1-9]. These amphophilic molecules have a hydrophilic head group (sialic acids, lactose) and a hydrophobic tail group (sphingosine and a long-chain fatty acid) [10]. Gangliosides are overexpressed on the tumor cell surface; these T-cell independent antigens [11] may serve as targets for passive [12] and active specific [13, 14] immunotherapy. The nature and distribution of gangliosides differ between normal and neoplastic cells and among specific cancers [15].

Although GM3, GM2, GD3 and GD2 have been identified in human prostate cancer (CaP) tissue [16-18], the ganglioside profile of established human CaP cell lines is not known. Of the four American Type Culture Collection (ATCC) cell lines, two (LNCaP FGC and LNCaP FGC-10) are androgen receptor (AR) positive (+) cells from lymph node metastases, and two (DU 145 and PC-3) are AR-negative (-) cells from brain and bone metastases. Recently, Selvan and others [19] have developed an AR (-) cell line (HH870) from an organ-confined CaP (stage T2b). This cell line provides an opportunity to compare the ganglioside signatures of CaP cell lines derived from organ-confined *versus* metastasized AR-negative CaP.

This investigation used monoclonal antibodies (MAbs), resorcinol staining and mobility assessment to characterize the ganglioside signatures

of human CaP cell lines. Our objective was to identify a cell line with optimal expression of major CaP gangliosides for active specific immunotherapy of CaP.

MATERIAL AND METHODS

Prostate cancer cell lines. Three AR (-) CaP cell lines were used in this study: PC-3 (ATCC, CRL-1435) was initiated from a bone metastasis, DU 145 (ATCC, HTB-81) was isolated from a brain lesion, and HH870 (Hoag Cancer Center, Newport Beach, CA) was developed from an organ-confined primary tumor that had been resected from the right mid-gland and apex of the prostate in a 56-year-old, previously untreated white male. This tumor was Gleason grade 3/4, with no evidence of vascular or perineural invasion or extracapsular extension (stage T2b). At the time of surgery, the patient had a serum prostate-specific antigen level of 5.65 ng/ml. Bone scans and chest x-rays revealed no metastatic disease. Androgen receptor was not detectable in the cell line by immunocytochemistry or by Western blot analysis (19).

Two CaP cell lines that expressed receptors for 5-α-dihydrotestosterone were used as AR (+) lines: LNCaP clones FGC (ATCC, CRL-1740) and FGC-10 (ATCC, CRL-10995). Normal prostate epithelial cells (PrEC) were obtained from Cambrex (BioScience Walkersville, Inc, MD) and cultured in their respective growth media (PrEGM, CC-4177, Cambrex). Murine YAC-1 lymphoma cell line (ATCC No: TIB-160) was used to isolate GM1b.

Cells were grown in RPMI medium 1640 with glutamine (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum, HEPES buffer, gentamycin (5 mg %) and fungizone (0.5 mg %), at 37°C in a humidified atmosphere (95% air/5% CO_2). The cells were detached with sterile EDTA-dextrose (137 mM sodium chloride, 5.4 mM potassium chloride, 5.6 mM dextrose, 0.54 mM ethylene diamine tetra acetate (EDTA), 7.1 mM sodium bicarbonate) at 37°C for 5 minutes, recovered with cold RPMI 1640-human serum albumin (4%; HSA, Gropo Grifols de America, Inc. Miami, FL), and resuspended in the same medium. Harvested cells were cryopreserved in RPMI-7.5% HSA containing 10% dimethylsulfoxide (DMSO), and stored under liquid nitrogen. When required, the cryopreserved cells were half-thawed at 37°C in a water bath for 15-30 sec and further thawed at room temperature. The cell suspension was washed with RPMI-4% HSA, centrifuged and resuspended in the same medium. Cell count was measured using a hemocytometer and viability by trypan blue (0.2%) dye exclusion method.

Glycolipid extraction from cell pellets. Glycolipids were extracted following a protocol described earlier [20]. Freshly harvested or cyropreserved cells resuspended in RPMI-4% HSA, were counted and rewashed in PBS. Methanol (M) was added to the pellets (v/v, 1/10),

vortexed, mixed with equal volume of chloroform and allowed to stand at room temperature for 5 hrs. After centrifugation, the supernatant was separated and evaporated to dryness under nitrogen. The dried moiety was resuspended in 2 ml of chloroform/methanol (v/v, 2/1). After the suspension was centrifuged, the supernatant collected, dried over nitrogen and redissolved in 4 ml chloroform/methanol (v/v, 1/1). The final ratio of the suspension was adjusted to chloroform/methanol/ PBS (v/v/v, 1/1/0.7). The extract was centrifuged; the upper phase containing gangliosides was recovered and the lower phase was re-extracted with methanol/PBS. The upper phase was recovered thrice and pooled.

Isolation of Gangliosides. Gangliosides were isolated on columns (ENVI-Chrom P, Supelco, Bellefonte, PA) containing a resin made of small, non-ionic, highly cross-linked styrene-divinylbenzene beads [21]. The columns were fixed in Visiprep solid phase extraction vacuum manifold (Supelco). Initially the column was conditioned by adding methanol followed by PBS. The upper phase containing gangliosides was layered on the column, the flow rate adjusted in drops, and the solution was collected. The column was washed with distilled water twice in order to remove non-lipid contaminants such as salts, sugars, and amino acids. Water was removed completely. Gangliosides were eluted from the column by adding 3 ml of methanol and then 3 ml of chloroform:methanol (C:M) (v/v, 2/1). Eluent was dried over nitrogen and the gangliosides were dissolved in chloroform/methanol (v/v, 2/1) and stored at -20°C.

High-performance Thin-layer Chromatography (HPTLC). Ganglioside signatures of CaP cells were analyzed by HPTLC as described earlier [20]. HPTLC plates (10x10 cm) precoated with Silica Gel 60 (glass or aluminium backing) (E. Merck, Darmstadt, Germany) were used. Two dimensional HPTLC was performed using two different solvent systems. The sample was separated in the first dimension in chloroform/methanol/ 0.2%$CaCl_2$ (v/v/v, 55/45/10,). Solvent systems were equilibrated several hours before use in TLC chamber. The plate was dried in a vacuum desiccator overnight and run in the second dimension in chloroform/methanol/2.5M NH_4OH in 0.25% KCl (v/v/v, 50/40/10).

The following gangliosides were screened for purity and homogeneity: GM3 (Sigma: G 5642), GM2 (Sigma: G 8397), N-glycol-GM3 (Gift from Dr. Adriana Carr, Center for Molecular Immunology, Habana, Cuba), GM1a (Sigma: G 7641), GD3 (Calbiochem: 345752), GD1a (Sigma: G 2392), GD2 (Advanced Immunochem, Long Beach, IG6), GD1b (Sigma: G 8146) and GT1b (Sigma: G 3767). These gangliosides were used as reference standards for TLC and as antigens for ELISA. The gangliosides were spotted onto the plates using Linomat (CAMAG Scientific Inc., Wilmington, NC). The plates were pre-run in chloroform to eliminate neutral lipid and other contaminants that may interfere with the mobility of gangliosides. Gangliosides were visualized by heating at 100°C after spraying with

resorcinol-HCl reagent (10 ml of 2% resorcinol in water, 40 ml concentrated HCl, 0.125 ml 0.1M copper sulphate). Each chromatogram represented ganglioside extract from 25 x 10^6 cells.

Specificity of monoclonal antibodies and immunostaining. Gangliosides were separated on aluminium-backed silica gel plates, and the plates were dipped in 0.2% solution of polyisobutyl-methacrylate in hexane for 1 minute. After drying, plates were blocked with PBS-1% HSA for 30 minutes, washed with PBS and dried. Immunostaining was performed with antiganglioside murine monoclonal antibodies (MAbs) [22, 23] (**Figure 1A)** whose specificity had been tested with an ELISA protocol described elsewhere (24). Each plate was overlaid with a MAb diluted in PBS-1% HSA for 2 h at 37°C. The dilution used for immunostaing and the protein concentration of the primary antibodies used for ELISA are as follows: GMB16 (anti-GM1a; 1/400 for immunostaining; 200 ng/well for ELISA); KM696 (anti-GM2; I/1000, 500 ng/well); 14.G2a (anti-GD2; 1/250, 250 ng/well); MB3.6 (anti-GD3; 1/250, 250 ng/well); GGR12 (anti-GD1b; 1/200, 1 μg/well); 14F7 (1/1000, 40 ng/well); GD1a-1 (1/500, 250 ng/well); GMR17 (anti-GD1a/GM1b; 1/800, 200 ng/well); GMR5 (anti-GT1b; 1/500, 200 ng/well) and GMR11 (anti-GT1a; 1/400). The plates were then washed with PBS (for 3 min) thrice, dried and incubated with biotinylated rabbit anti-mouse IgG (Jackson Immunoresearch, West Grove, PA; 315-065-008) or rabbit anti-mouse IgM (315-065-049) diluted at 1/500 for 1 h at 37°C. The plates were washed thrice in PBS, dried and incubated for 45 minutes in strepdavidin-peroxidase (Jackson Immunoresearch; 016-030-084) diluted in PBS at 1/2500. The plates were developed with 4-chloro-1-naphthol solution (10 ml) and H_2O_2 (5 μl).

Immunostaining with GMR17 after sialidase treatment. Gangliosides isolated from PC-3 cell lines were treated with 50 mU of α2, 3 neuraminidase from *Macrobdella decora* (CalBiochem, Cat #480706 (lot #B52582) suspended in sodium acetate buffer (pH 5.5) for 24 hours at 37°C. The control extracts were treated only with sodium acetate buffer (pH 5.5) for 24 hours at 37°C. The gangliosides were desalted in columns (ENVI-Chrom P, Supelco, Bellefonte, PA). The repurified gangliosides were separated by 2D chromatography as described above. Both experimental and control chromatograms were stained with GMR17, GMB16 and GD1a-1.

Base Treatment of Gangliosides. O-acetyl groups of gangliosides were removed by base treatment following an earlier procedure (25). Ganglioside extracts from 50 million cells (HH870 or PC3, two analyses/preparation) were suspended in 100 μl of chloroform:methanol (v/v, 1/1) and equal amount of concentrated NH_4OH (14.8 N) mixed well and incubated for 1 hr at room temperature (25°C). Ammonia was evaporated over N_2.

HPLC purification of gangliosides. Gangliosides purified from PC-3 were applied to a Shimadzu HPLC (Shimadzu, Kyoto, Japan) C-18 column (4.6 mm x 25 cm) and fractionated. Individual gangliosides were then eluted with a programmed gradient of propanol:hexane (55:45) and propanol: water (55:45). The flow rate was adjusted to 0.25 ml/min and 0.5 ml each of the effluent was successively collected. The mono- and disialoganglioside fractions (tubes 25 to 49) were analyzed by TLC immunostaining.

Immunochemical Localization of gangliosides with Laser Scan Confocal Microscopy. Live cells were immunostained to examine the distribution of the major gangliosides. For this purpose, CaP and PrEC cells were cultured in sterile 8 well Nunc Lab-Tek II Chamber Slide System (Cat No 154534; Nalge Nunc International, Naperville, IL 60563). For immunochemical observations, 10,000 PrEC or CaP cells were suspended in RPMI 1640 (0.5 ml) with glutamine supplemented with 10% fetal calf serum, HEPES buffer, and antibiotics; the cells were incubated at 37°C in a humidified (95% air/5% CO_2) chamber. After 24 hrs, the medium was removed and the cells attached to the bottom of the chamber were washed in cold D-MEM/F-12 medium (Invitrogen; Cat No 11039-021) once and incubated in the same cold medium for 30 min. Further experiments were carried on an ice chamber without exposing the cells directly to ice. The cells were blocked with cold PBS (pH 7.2)-human serum albumin (HSA, 2%) for 2 hr and rinsed with PBS-HSA (1%). Primary antibody was diluted in cold PBS-HSA (1%) and the concentration of the antibody was adjusted to 2 ng/ml. The cells were incubated on ice with primary antibody for 90 min, and then washed in PBS-HSA (1%) for 5 min, thrice. The cells were then incubated for 30 min in rhodamine red conjugated goat anti-mouse IgM/ fluorescein-isothiocyanate FITC conjugated goat anti-mouse IgG (diluted in cold PBS-1% HSA 1:200)(Jackson Immunoresearch; Cat No : 115-295-075; 115-095-071). After washing the cells in the same cold buffer thrice, the slide was removed from the chamber and mounted with 95% glycerol with 5% phosphate buffer. In some preparations, after final wash, the cells were fixed for one minute in 4% paraformaldehyde (cold). The cells were washed with PBS (without HSA) buffer. The glycerol-mounted slides were refrigerated for a day or two and examined with a laser scan confocal fluorescence microscope (LSCFM, LSM 510 Carl Zeiss, Oberkochen, Germany) equipped with a 514λ argon and a 543λ helium-neon laser. The emitted light reflected from the sample and fluorescence was collected by an oil immersion lens and imaged onto a photomultiplier tube after passing through a confocal aperture at an optical filter.

RESULTS

Specificity of Monoclonal antibodies in ELISA and TLC

Figure 1A shows the specificity of eight MAbs. Seven of them were monospecific for their respective gangliosides. GMR17 recognized both GD1a and GT1b in ELISA (**Figure 1A**) and in the thin-layer chromatogram (**Figure 1B**). GMR17 failed to stain GM1a, GM3 and GD3 (**Figure 1C**); however it stained standard GD1a and GM1b isolated from YAC-1 (murine lymphoma cells) (**Figure 1A**). GM1b or GD1a was not recognized by GMB16, a MAb specific for GM1a (**Figure 1C,** upper, leftl). MB3.6 recognized GD3 but not GM3 in the same chromatograms at any concentration (**Figure 1C,** Lower, middle).

Immunostaining of Cell surface Ganglioside with monoclonal antibodies: Assessment with LSFM

The monoclonal antibodies to GM1a (GMB16) GD1b (GGR12) GT1b (GMR5) and GD3 (MB3.6) did not stain PrEC or CaP cells. The results obtained with other MAbs are presented in **Figure 2.** GMR17 stained the cell surface and the cytoplasm of PrEC cells (**Figure 2A**) and predominantly the surface of PC-3 cells cells (**Figure 2B**). In addition to uniform staining, distinct fluorescent spherules on the cell surface indicated clustering of the gangliosides during immunostaining. Cell surface distribution of GMR17-positive gangliosides during telophase of HH870 cells (**Figure 2C**), DU 145 cells (**Figures 2D & 2E**) showed uniform distribution of GD1a on the cell surface during telophase. MAb 14.G2a, specific for GD2 stained intensely the cell surface of CaP cells (**Figure 2F**). In contrast, no such cell surface distribution of GD2 (14.G2a immunoreactivity) is observed with normal PrEC cells (**Figure 2G**). The cytoplasm of normal cells stained weakly. The surface and cytoplasm of PrEC and CaP cells were stained by anti-GM2 monoclonal antibody KM696. There is not much difference in the distribution of GM2 between normal and CaP cells, although the gangliosides appeared as distinct clusters on the cell surface (**Figure 2I, 2J & 2K**). Both GMR17 and KM696 are IgM antibodies. IgM and IgG isotype controls did not stain the cells (**Figure 2H).**

Identification of Gangliosides in normal and neoplastic prostate epithelial cells by Resorcinol-HCl

We used ganglioside extracts from 25 million cells per lane in one-dimensional (1D) or two-dimensional (2D) chromatograms. Commercial bovine brain gangliosides (3 nmol/lane) were used for comparison. The staining intensity of the gangliosides was low in normal cells (PrEC) compared to CaP cells (**Figure 3**).

The resorcinol staining intensity and the mobility of standard gangliosides in 1D-chromatograms tentatively identified the gangliosides in the following order, in PrEC cells **(Figures 3):** GM3 > GD1a = GT1b > GM2 = GD3 = GD1b > GM1. Gangliosides in PC-3, DU 145 and HH870 cells were:

GD1a > GM3 > GM2 > GM1 >GD2 > GD3 > GT1b (GD1b not identified). Resorcinol staining intensity and mobility of the gangliosides from normal and CaP cells in 2D-chromatograms were compared with that of bovine gangliosides. GD1a was the most prevalent ganglioside in all CaP cell lines. Further, these cell lines expressed abundant quantities of GM1, GM2 and GM3 and the presence of GD3 and GD2.

Several gangliosides appeared as doublets, which signify the differences in length, number of double-bonds and hydroxylation of fatty acid chains and alterations in the sialic acids (10). Human tissues usually contain N-acetyl neuraminic acid (NeuAc) but may contain N-glycolyl neuraminic acid (NeuGc), O-acetyl neuraminic acid (O-Ac NeuAc) or neuraminyl lactone [10], and the last two are susceptible to base treatment [25]. **Figure 4A** illustrates three distinct resorcinol-stained spots for GM3 in HH870 cells, whereas in standard (bovine brain gangliosides) only one spot was observed (**Figure 4C**). The different spots of GM3 in HH870 could be due to differences in the fatty acids or sialic acids.

Identification of Gangliosides by Immunostaining.

Since none of the murine MAbs for GM3 were specific for GM3, no immunostaining was done to verify the presence of GM3. Moreover GM3 is common in all extraneural tissues and in both normal and malignant prostate cells.

Murine MAb KM696, specific for GM2, reacted with two distinct spots in the 2-D chromatograms of the ganglioside extracts of PC-3, DU 145 and HH870 (**Figure 4B**). The doublets of GM2 reflect differences in the nature of fatty acids (length, presence of double bonds, or hydroxyl groups).

Murine IgG MAb 14.G2a reported to be specific for GD2 [26] stained GD2 distinctly in 2-D chromatograms of all the cell lines (**Figure 4D**). While 14.G2a stained only one spot in DU 145 (which is GD2), it reacted with two spots in PC-3 (A1 & GD2 in **Figure 4D**) and three spots in HH870 (A1/A2, GD2 & B in **Figure 4D**). The mobility of spot A is faster than the main GD2 spot. Such faster mobility could be due to O-acetylation of gangliosides as reported earlier (25). Spot B was not stained by anti-GT1a IgM (GMR11). It is possible that this spot could be GT2 that reacts often with antibodies to GD2 as in neuroblastoma tumors (Portoukalian, personal communication). Alkali-treatment of the gangliosides from HH870 abolished spots A1, A2 and B (**Figure 4E**), indicating that A1 and A2 could be doublets of O-AcGD2 and B could be O-acetyl GT1a and not reactive to GMR11. There was a distinct spot (doublet) that was resistant to alkali-treatment. But it showed a shift in mobility to the position of standard GD1a or GD3 (**Figure 4E**). The spot was immunostained with MAb 14.G2a but do not react to monoclonal antibodies to GD1a (GD1a-1) or GD3 (MB 3.6) and therefore it is GD2.

Murine MAb to GD3 (MB3.6) was applied to 1-dimensional chromatograms of all CaP lines. MB3.6 identified GD3 in the extracts of HH870 (as a doublet in **Figure 4F**) and DU 145 but not in PC-3 cells.

Murine MAb from Seikagaku clone GD1a-1 reacts with GD1a and GM1b but not with any other gangliosides. This MAb was used to stain the 2D chromatograms of gangliosides purified from HH870. Based on the immunostaining and relative mobility with standard, GD1a (doublet) is the major ganglioside of HH870 (**Figure 4G**). A spot higher than GD1a and comparable to the position of GM1, was observed and required further analysis (*vide infra*).

Status of GM1a, GD1b and GT1b in CaP cells

MAb GMR17 is known to react with GD1a and GT1B (**Figure 1C & 5A**). However, GMR17 failed to stain any of the gangliosides in PrEC cells suggesting the absence of the ganglioside GD1a and GT1b in PrEC cells (**Figure 5B**). Similarly, MAb 14.G2a failed to react with any of the gangliosides in PrEC cells (data not shown) while it stained a single spot of GD2 in AR-positive LNCaP FGC cells (**Figure 5C**). It should be noted that GM1a could not be detected in CaP cells by immunostaining (GMB16) (data not shown). Similarly, Murine MAbs to GD1b (GGR12) and GT1b (GMR5) revealed that these gangliosides were not present in detectable quantities in the extracts from all cell lines (**Figure 5D, E**).

Evidence for the presence of GM1b in CaP cells.

Immunostaining of the 2D chromatograms confirmed that GM2, GD2 and GD1a were the most prevalent gangliosides in the three CaP lines. Although a ganglioside comparable in mobility to GM1 was present in all the cell lines, GMB16 MAb failed to stain GM1a in any of the cell lines. Therefore the ganglioside migrating to the position corresponding to GM1 might not be GM1a; instead it might be GM1b. Since GM1b is a major ganglioside of murine YAC-1 cells, gangliosides were extracted and isolated from this cell line (**Figure 5A**). GMR17 MAb, which binds to both GD1a and GT1b, also recognized GM1b in YAC-1 cells as well as in all CaP cell lines (**Figure 5A**). The relative mobility of GM1b was slightly lower than GM1a (**Figure 6F**).

To further investigate GM1b in CaP cells, the 2D-chromatograms of the gangliosides isolated from all three cell lines were stained in resorcinol-HCl (**Figures 6A & 6D**) and immunostained with GMR17 (**Figures 6B & 6D**). The immunoprofile revealed GMR17-reactive gangliosides in five different positions (five spots). The major spot #1 stained very intensely in HH870 corresponded to standard GD1a (**Figure 6B**), whereas in PC-3 and in DU 145, the GMR17 staining was moderate (**Figure 6D**). In HH870, the relative mobility of the spot # 1 is comparable to that of the standard GD1a (**Figure 6B**). Spots #2 occurs as a doublet in HH870 and as a single spot in PC-3 and DU 145. In PC-3, both spots # 2 and #3 were more intense than spot # 1. In DU 145 all spots appeared somewhat uniform and faint (**Figure 6B**). Spot # 5 is seen only in HH870 and PC-3 (**Figure 6B and 6D**).

These different spots may share a common or a general epitope involving sialic acid. In order to understand the nature of gangliosides, we have treated the gangliosides purified from PC-3 and HH870 with α2, 3

sialidase as well as subjected to alkali-treatment and resolved in 2D chromatograms. The results obtained with PC-3 and HH870 were identical. Presence of O-acetylated gangliosides was assessed by the base-treatment of the sample. The extracts of HH870 were subjected to NH_4OH, chromatographed and immunostained with GMR17. The immunoprofile after base-treatment revealed (**Figure 6C**) that the spots #3 and #4, but not spots # 1, #2 and #5, were susceptible to base-treatment indicative of the presence of O-acetyl groups, which explains as to why these gangliosides were resistant to sialidase treatment. **Figure 6E** represents the profile of GMR17-positive spots in PC-3 after α2, 3 sialidase treatment. There is a decrease in the number of spots stained by GMR17 after sialidase treatment; Spots #1, # 2 and #5 have disappeared. Spots #3 and #4 were resistant to sialidase treatment. This finding further supports the contention that these spots could be due to O-acetylated gangliosides because the O-acetyl groups prevent binding of the sialidase.

Spot #2 corresponds to a position lower to GM1a and could be GM1b (**Figure 5A)**. GM1b purified from YAC-1 cells, was also immunostained with clone GD1a-1 MAb indicating that it is GM1b. To further confirm the presence of GM1b in ganglioside extracts of these cell lines, we used Shimadzu-HPLC to isolate the GM1b fractions (Vials 33-43) (**Figure 6F**). The HPTLC profiles were compared with that obtained from YAC-1 cells (data not shown) to confirm the identity of GM1b. Immunostaining with GMR17 further confirmed that the fractions were indeed GM1b.

The immunostaining with different MAbs, together with the observations made with resorcinol staining intensity and the relative mobility with bovine brain gangliosides, provide evidence for the presence of the prostate gangliosides in the following order: **Normal PrEC:** GM3 > GM2 >> GD3 >>>GD1a; **PC-3, DU 145 and HH870 cells:** GM3 > GD2 > GD1a > GM2 > GM1b >> GD3. Our observations reveal that GD2, O-AcGD2, GD1a and GM1b are the most prevalent gangliosides of AR (-) CaP cells. GD1a and GM1b were present in low concentrations in extracts from AR (+) LNCaP FGC and LNCaP FGC-10 cells (**Figure 5A**). However, these cells expressed high levels of GD2 (**Figure 5C**) but no additional 14.G2a-reactive spots, including O-AcGD2, were observed.

DISCUSSION

Current treatments for AR (-) CaP have not significantly improved survival [27]. Hormonal ablation, the basis of systemic therapy, usually fails to stop eventual progression of metastatic disease [28]. Novel treatment strategies that target specific signaling pathways, apoptosis, differentiation, or membrane components represent promising alternatives [28]. Tumor-associated gangliosides are membrane antigens that have been successfully used for active immunotherapy of melanoma and colon cancer (29). Their successful application for active immunotherapy of CaP requires a better

understanding of the ganglioside profile of AR (-) CaP cells, particularly the profiles of organ-confined *versus* metastatic CaP.

Although several AR (-) cell lines from metastatic tumors or viral-transformed prostate epithelial cells (RWPE-1, RWPE-2, 22Rv-1) are commercially available, development of a cell line from AR (-), organ-confined CaP is difficult because little tissue is available after histopathology. The HH870 CaP cell line is remarkable because it is AR (-) and was developed from a stage T2b organ-confined tumor (19).

Our findings revealed a unique pattern for the ganglioside profile of AR (-) CaP cells. The presence of GM3 is not surprising because it is the most common ganglioside of extraneural tissues [13]. Previous investigators have observed GM3 in normal and neoplastic prostate tissues [16, 17]. Our results confirm the reports of Livingston's team (18) on the presence of GM2 in normal and neoplastic prostatic epithelial cells. Results of LSCFM as well as TLC immunostaining with KM696 confirm the presence of GM2 both in normal and neoplastic prostate epithelial cells. Zhang and co-investigators (18) have immunostained frozen sections of normal prostatic epithelial cells and CaP cells with KM696 and reported that both the type of tissue stained in equal intensity (4+). Our LSCFM observations confirm their findings on PrEC and CaP cell lines. Since GM2 is expressed in equal density in both normal and neoplastic prostatic epithelial cells, we may infer that GM2 may not be a useful target for antibody-mediated passive or active specific immunotherapy of CaP. However, TLC-immunostaining with KM696 documents that the intensity of GM2 in PrEC cells is too low compared to that found in CaP cell lines. It is not clear whether tissue culture conditions induce overexpression of GM2 specifically in CaP cells under tissue culture conditions, as reported earlier in melanoma cells grown *in vitro* (35). 2D-chromatograms revealed doublets of GM2 in CaP cells (1, 2 in **Figure 4B**), which is due to differences in the nature of the fatty acids [10, 30]. Such a difference is not noticed in normal PrEC cells. While the length and nature of fatty acids in a ganglioside are critical for expression of epitope on the cell surface (10), their length may also alter their exposure to targeting antibodies and the rate of shedding in tumor microenvironment (30). GD3 was less common in HH870, PC-3 and DU 145 cells. It also occurred as a doublet, which could represent differences in the nature of its fatty acids [30]. GD3 might be the precursor of GD2, the most prominent ganglioside in all three CaP lines.

The 14.G2a MAb is specific for the GD2 epitope [25] and has been employed in passive immunotherapy [31, 32]. In our study, 14.G2a was a valuable tool to distinguish differentiation among CaP cell lines. The most striking difference between organ-confined cells (HH870) and bone (PC-3) or brain (DU 145) metastatic CaP was in the number and nature of 14.G2a-reactive fractions. In DU 145 cells, 14.G2a stained a single spot of GD2; in PC-3 and HH870 cells, 14.G2a stained a fraction above GD2. In PC-3, the upper fraction is a single spot (A1), whereas in HH870 cells, the upper

fraction is a distinct doublet (A1 and A2). The doublet of upper 14.G2a positive fraction distinguishes HH870 from PC-3. The singlet (A1) in PC-3 and the doublet (A1 & A2) were susceptible to base-treatment therefore could be O-AcGD2. We documented previously that the base-treatment de-O-acetylates and reverts O-acetylated gangliosides into the non-O-acetyl gangliosides (25). HH870 also differed from PC-3 in the presence of a slow-moving, intensely staining 14.G2a-reacting fraction below GD2 and closer to the standard GT1b; this fraction did not immunostain with anti-GT1a MAb. Sometimes, extraction procedure may lactonize gangliosides. Lactonized gangliosides differ in their mobility identical to O-acetyl gangliosides and are susceptible to base-treatment. If such an artifact were to occur, it will be observed in all cell lines since all of them were treated alike. But, we found the number of spots varied among cell lines. Spot A occurs as a single band in DU 145 and as a doublet in other cell lines. Therefore, we infer that the alkali-susceptibility is not be due to lactone formation but is due to O-acetylated GD2. Similarly, the susceptibility of spot B to base-treatment suggested that it could be an O-acetylated and not a lactonized ganglioside. Diffused and feeble distribution of GD2 in the cytoplasm of PrEC cells and prominent staining and restricted distribution of 14.G2a reactivity on the cell surface of CaP cells suggest that GD2 and O-AcGD2 are tumor cell surface-associated gangliosides and could be a potential target for both antibody-mediated passive and active specific immunotherapy. Because overexpression of GD2 is attributed to cancers with high metastatic potential [33, 34], strong expression of GD2 and expression of O-AcGD2 and another 14.G2a-positive fraction (Spot B) suggest that organ-confined HH870 cells could be a well differentiated tumor type with potential invasive characteristic.

The most interesting aspect of the ganglioside signature of AR (-) CaP cell lines was their reactivity to GMR17. MAb GMR17 stained both GD1a and GT1b of the mixture of bovine brain gangliosides (**Figures 1A & 5A**), GM1b in YAC-1 cells (**Figure 5A**), and five different spots (**Figures 6B & 6D**) in HH870 and PC-3 cell lines, but nothing in PrEC cells (**Figure 5B**). In 2D-chromatograms, we observed that one of the GMR17 positive spots (#1) was GD1a. Spot #2 corresponded with GM1b of YAC-1 cells. These observations point out that the gangliosides GD1a and GM1b are indeed CaP-associated antigens. Their distribution on the cell surface was confirmed by LSCFM observations using GMR17 (**Figures 2C, 2D & 2E**). Therefore, both GD1a and GM1b could be potential targets for immune recognition. None of the CaP cell lines reacted to GMR5 (anti-GT1b) (**Figure 5E**) confirming that GMR17-positivity is not due to GT1b.

Expression of some of the gangliosides might be an *in vitro* artifact of tissue culture conditions. In an earlier study on melanoma-associated gangliosides, expression of GM2 and GD2 could have been due to overexpression of GM2 synthase in tissue culture [35]. High GM2 content in cultured cells may not reflect the native composition of the CaP

gangliosides. The same logic cannot be applied to GD2 or GD1a because expression of these gangliosides differed markedly between organ-confined and metastatic cell lines and between AR (+) and AR (-) cell lines, although these cell lines were cultured under identical conditions.

We conclude that CaP cells express GM3, GD3, GM2, GD2, GD1a and GM1b. The gangliosides GM1a, GD1b and GT1b were undetectable. Unlike AR (+) CaP cells, AR (-) CaP cells strongly express GD1a and GM1b and may express O-acetylated versions of these gangliosides. LSCFM observations on immunostaining of gangliosides confirmed the distribution of the major CaP-associated gangliosides on the cell surface. Therefore GD2, O-AcGD2, GD1a and GM1b could be ideal targets for passive and active specific immunotherapy of CaP. Since GM2 expression is high and identical in both PrEC and CaP cells grown *in vitro* and *in* vivo (Ref. 18 reports 4+ intensity on normal PrEC), it may not be an ideal target for immunotherapy of prostate cancer, unless variants of GM2 (O-acetylation, N-glycolyl groups, differences in the length of the fatty acid) are identified in CaP cells. It is in this context, absence of GD2 and GD1a in detectable quantities in normal prostatic epithelial cells and high and varied (O-acetylated) expression of these two gangliosides make them potential targets for passive and active specific immunotherapy of CaP.

HH870 overexpressed GD1a, expressed doublets of several gangliosides including GD3, and has three different gangliosides reactive to 14.G2a (GD2, O-AcGD2 and unindentified alkali-labile ganglioside) and two alkali-susceptible and three alkali-resistant gangliosides reactive to GMR17 MAb. Therefore, this cell line may be an ideal component of a polyvalent vaccine for active specific immunotherapy of CaP, as M10, M24 and M101 cell lines for melanoma (14, 29).

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LEGENDS

Figure 1. Specificity of antiganglioside murine monoclonal antibodies. A. All monoclonal antibodies except 14F7 are commercially available. The clones used include GMB16 (anti-GM1), KM696 (anti-GM2), 14.G2a (anti-GD2), MB3.6 (anti-GD3), GGR12 (anti-GD1b), 14F7 (anti-GM3-NeuGc), GD1a-1 (anti-GD1a), GMR17 (anti-GD1a) and GMR5 (anti-GT1b). All monoclonal antibodies except GMR17 showed remarkable specificity for their respective target antigens. Eight gangliosides (purified to >98%) were coated onto microtiter plates. Antibody titers were measured in ELISA microtiter plate reader at 490 nm, absorption Maxima (λ) of quinone formed in the presence of OPDE/H_2O_2 and 6N sulfuric acid, and at 650 nm, for background correction. **B.** Immunostaining standard gangliosides with GMR17. Resorcinol profile is given for comparison. Although all the gangliosides were present, GMR17 immunostained only GD1a and GT1b. The intensity of GD1a to GT1b is 5/1. This observation confirms that GMR17 is not specific for GD1a but it recognizes the GD1a epitope on GT1b. **C.** Immunostaining of one-dimensional thin-layer chromatograms with GMB16 (anti-GM1), GMR17 (anti-GD1a) and MB3.6 (anti-GD3). Bovine brain standard gangliosides were applied 1, 3, 5, 7 &/or 9 nmol/lane. Upper left: staining of GM1 by GMB16. GM1b isolated from YAC-1 did not stain with GMB16. Upper right: staining of GD1a by GMR17. GM1b isolated from YAC-1 also stained with GMR17. Lower panel: staining of GM3 and GD3 with resorcinol-HCl (left), and staining of GD3 but not GM3 with MB3.6. GMR17 failed to stain GM3 and GD3 and stained standard GD1a.

Figure 2. Confocal fluorescence microscopic assessment of gangliosides in normal prostate epithelial cells (PrEC) and AR (-) prostate Cancer cell lines (DU 145, PC-3 and HH870). GMR17 is a monoclonal antibody that reacts to GD1a and GT1b in bovine brain gangliosides and to GD1a, GM1b and three other unidentified gangliosides in prostate cancer cell lines. A. GMR17 immunostaining of the cell surface and the cytoplasm of PrEC cells. Ganglioside GD1a but not GM1b is detectable in chromatograms of ganglioside extracts of PrEC. B. GMR17 stains predominantly the surface of PC-3 cells. Cell-surface clustering of the gangliosides is shown by the bright fluorescent spherules. C-E. Cell surface distribution of GMR17 positive gangliosides during telophase of HH870 (C), DU 145 (D, E). F, G: FITC-14.G2a immunostaining. 14.G2a is known to be specific for GD2 but it also stains one or two additional unknown gangliosides in CaP cells. F. Cell surface distribution of immunoreactivity of 14.G2a suggests the distribution of GD2 on the cell surface of CaP cells. G. In contrast, no such cell surface distribution of GD2 (14.G2a immunoreactivity) is observed with normal PrEC. The cytoplasm of normal cells stains weakly. H. Isotype control for IgM antibodies. Both GMR17 and

KM696 are IgM antibodies. Therefore, a non-specific murine IgM was used as isotype control. The isotype control is distinctly negative. I-K: KM696-Rhodamine Red staining. KM696 is monospecific for GM2. Cell surface staining for GM2 is observed in PC-3 (I), normal PrEC (J) and DU 145 (K). Vertical line refers to 15 μM (for A, B, F, H and J) and 10 μM (for C, D, E, G, I and K). H-K: These preparations were treated with 4% paraformaldehyde rinse prior to glycerol mounting. The vertical bars refer to 20 microns.

Figure 3. Ganglioside profiles in normal Prostate epithelial cells (PrEC) and AR (-) CaP cell lines stained by resorcinol-HCl in one-dimensional chromatograms. Each lane contains a ganglioside extract from 25 million cells. The concentration of each ganglioside in the bovine brain standard is 3 nm. Left to Right: PrEC, PC-3, HH870, DU 145, purified bovine brain GM2 and GD1a, purified bovine brain gangliosides (mixture). The bottom line is the point of application. Solvent system: Chloroform/methanol/0.2% $CaCl_2$ (v/v/v: 55/45/10).

Figure 4. Ganglioside signatures of AR-negative CaP cell lines in 2-D chromatograms. Each chromatogram contains ganglioside extract obtained from 25 million cells. In the standards (bovine brain gangliosides), the concentration of each ganglioside is 3 nm. **A.** Resorcinol-HCl staining of gangliosides from HH870 in the 2D chromatogram. Vertical line represents direction of flow of the first solvent system, chloroform/methanol/0.2% $CaCl_2$ (55/45/10, v/v/v). Horizontal line represents the direction of flow of the second solvent system, chloroform/methanol/2.5M ammonia in 0. 25% KCl 50/40/10,v/v/v). Note the presence and position of GM3, GM2, GM1, GD1a and GD2. GM3, GM2 and GD1a appear to be the most prevalent gangliosides. **B.** Immunochemical identification of GM2 with KM696 MAb. The solvent systems used for first and second runs were the same as in Figure 2. Interestingly there were two variants of GM2. Right larger spot (1) and Left smaller spot (2). **C.** Resorcinol-HCl staining of gangliosides from bovine brain. The conditions were the same as in A. **D.** DU 145, PC-3 and HH870 were stained with 14.G2a MAb, which is known to be specific for GD2; however the 2-D chromatogram of PC-3 and HH870 showed two and three distinct spots, respectively. A1 and A2 are unidentified spots that stain with 14.G2a. In HH870 the spot B may represent long chain gangliosides which may include O-AcGT1a, since it did not stain with GMR11 (anti-GT1a IgM antibody), or GQ1b since the mobility lower than GT1b or GT2. **E.** 14.G2a-reactivity after NH_4OH-treatment of ganglioside extract from HH870. Spots A1 and A2 have disappeared indicating that it could be O-AcGD2. Spot B also disappeared suggested that it could be O-acetylated GT1a. After base-treatment, GD2-doublets show faster mobility. **F.** Immunostaining of HH870 with MB3.6 MAb against GD3. Clearly HH870 expresses GD3. GD3 appears as a doublet due to differences in the length of fatty acids of the ganglioside. **G.** Immunostaining of HH870 with anti-

GD1a MAb. Two spots were identified. The more intense spot corresponded to GD1a. The upper weak spot correspond to the position of GM1b.

FIGURE 5. Pattern of immunostaining of Gangliosides derived from AR (+) CaP cells with GMR17 and 14.G2a. A. One-dimensional chromatograms of gangliosides from AR (-) (PC-3, DU 145, HH870), AR (+) (LNCaP FGC, LNCaP FGC-10) and murine YAC-1 cells immunostained with GMR17 MAb. GM1b, characteristic of YAC-1 cells stained with GMR17. GMR17 stained feebly the AR (+) cells, however GD1a is visible. **B.** One-dimensional chromatogram of gangliosides from normal prostatic epithelium (PrEC). GD1a is not detectable with GMR17. **C.** 2D chromatogram of gangliosides from AR (+) LNCaP FGC cells shows one distinct spot and a weak upper spot. **D.** Immunostaining of one-dimensional chromatograms of AR (-) cells with GGR12 (anti-GD1b). GD1b was not detectable in any of the cell lines tested. **E.** GMR5 (anti-GT1b) showing the absence of GD1b and GT1b at detectable level in CaP cells but their strong presence in standards.

Figure 6. Immunochemical identification of GD1a, its precursor and their variants in the ganglioside extracts of CaP cell lines. The solvent systems used for first and second runs are reported in the legend for Figure 2. Immunostaining of 2-D chromatograms of CaP cell lines was done with GMR17 MAb, which reacts with GD1a and GT1b. GT1b was not detected in any cell line. **A** 2-D chromatogram of standard bovine gangliosides (each ganglioside 3 nmol) and DU 145 stained in resorcinol-HCl. **B.** 2-D chromatogram of standard bovine GD1a (3 nmol), DU 145 and HH870 immunostained with GMR17. Four (DU 145) and five (HH870) spots are immunostained with GMR17. Spot #1 is intense in HH870 and corresponds to standard GD1a. Spot #2 was confirmed to be GM1b using gangliosides extracted from murine YAC-1 cell line. **C.** GMR17-reactivity after NH_4OH-treatment of ganglioside extract from HH870. Spots #1, #2 and #5 remain indicating that they are base-resistant. Disappearance of spots #3 and #4 could be due to loss of O-acetyl groups or loss of lactone derivatives of the ganglioside GD1a or GM1b. After base-treatment, base-resistant gangliosides showed faster mobility. **D.** 2-D chromatogram of PC-3 stained with resorcinol-HCl and with GMR17. Note staining of five spots with GMR17. **E.** Sialidase treatment of 2-D chromatogram of PC-3. The sialidase treated chromatogram was immunostained with GMR17. Spot #1 corresponds to GD1a; Spot # 2 corresponds to GM1b; spots #1, #2 and #5 have disappeared, whereas spots #3 and #4 were resistant. The spots that are resistant to the enzyme are O-acetylated gangliosides (**C**) in which the O-acetyl group prevents binding by the enzyme. **F.** PC-3 gangliosides were fractionated by HPLC. GM1b and GD1a fractions were stained with GMR 17 to confirm the presence of the gangliosides.

Figure 7. Two series of ganglioside biosynthetic pathways. GM2, GM1, GD1a and GT1a belong to gangliosides series called "a-series"; the "b-series" comprises GD3, GD2, GD1b, and GT1b, and GQ1b. The parent of all gangliosides appears to be lactosylceramide (LacCer). In addition to the two series, LacCer gives rise to two neutral lipids, gangliosides 3 and 4 (Gg3 and Gg4). Sialylation of the terminal gal of Gg4 results in GM1b or GM1α. GM1b may give rise to GD1c and GD1α because there is no GM1a. In prostate tissues, though GM1a is not detectable, GD1a might have been derived from GM1a or by simultaneous bisialylation of Gg4. GMR17 is a unique MAb that identifies a class of gangliosides as indicated. GD1a, stained by GD1a-1 is also reactive with GMR17. Among b-series GD1b and GT1b are not detectable in CaP Cells. Three double-lined boxes encompass gangliosides characteristic of CaP cells.

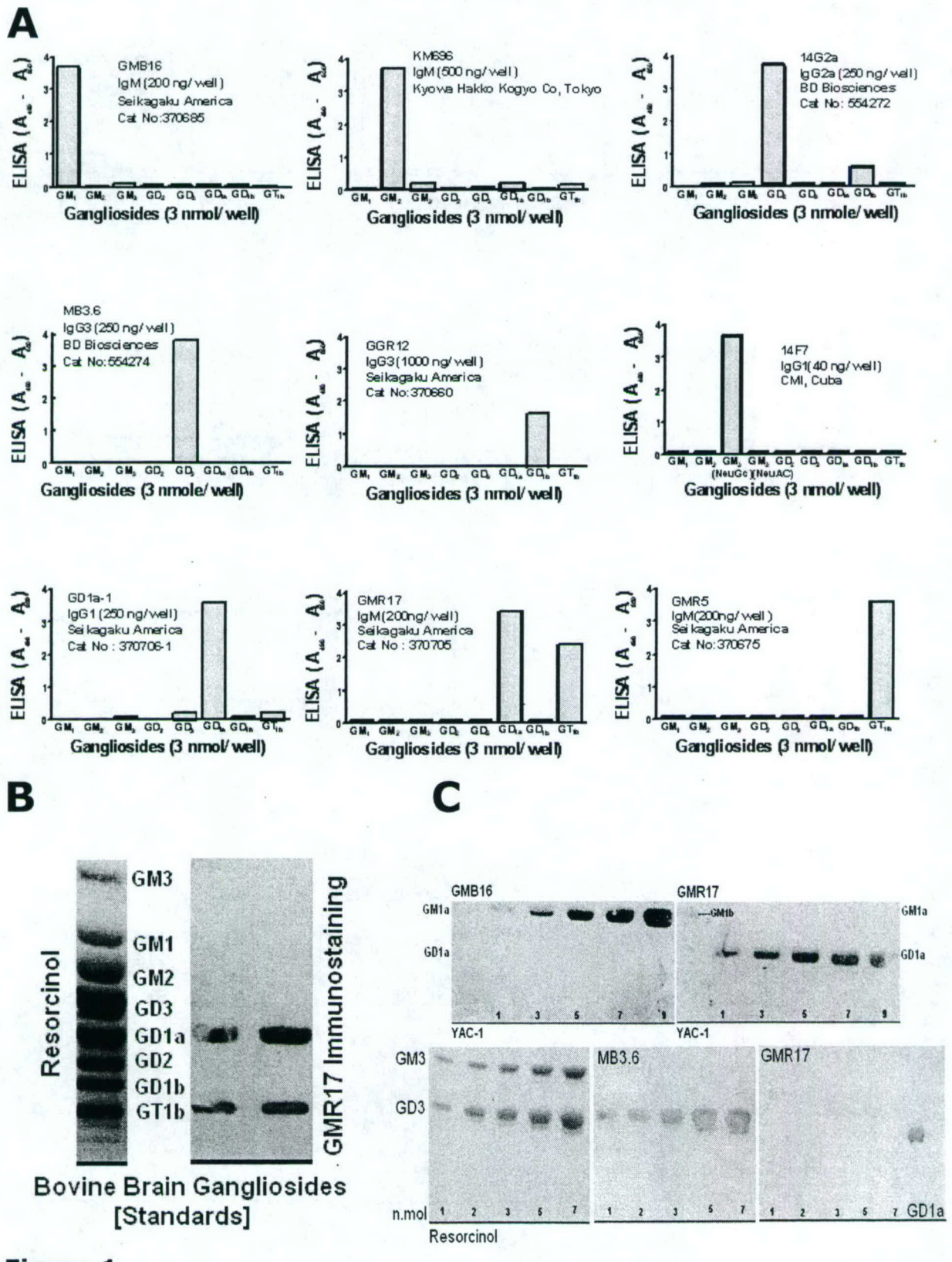
A
GMB16
IgM (200 ng/ well)
Seikagaku America
Cat No:370685
KM696
IgM (500 ng/ well)
Kyowa Hakko Kogyo Co, Tokyo
14G2a
IgG2a (250 ng/ well)
BD Biosciences
Cat No: 554272
MB3.6
IgG3 (250 ng/ well)
BD Biosciences
Cat No:554274
GGR12
IgG3 (1000 ng/ well)
Seikagaku America
Cat No:370660
14F7
IgG1 (40 ng/ well)
CMI, Cuba
GD1a-1
IgG1 (250 ng/well)
Seikagaku America
Cat No : 370706-1
GMR17
IgM (200ng/ well)
Seikagaku America
Cat No : 370705
GMR5
IgM (200ng/ well)
Seikagaku America
Cat No:370675
ELISA (A405 - A490)
Gangliosides (3 nmol/ well)
Gangliosides (3 nmole/ well)
B
Resorcinol
GM3
GM1
GM2
GD3
GD1a
GD2
GD1b
GT1b
GMR17 Immunostaining
Bovine Brain Gangliosides
[Standards]
C
GMB16
GMR17
GM1a
GD1a
YAC-1
GM3
GD3
MB3.6
n.mol
Resorcinol

Figure 1.

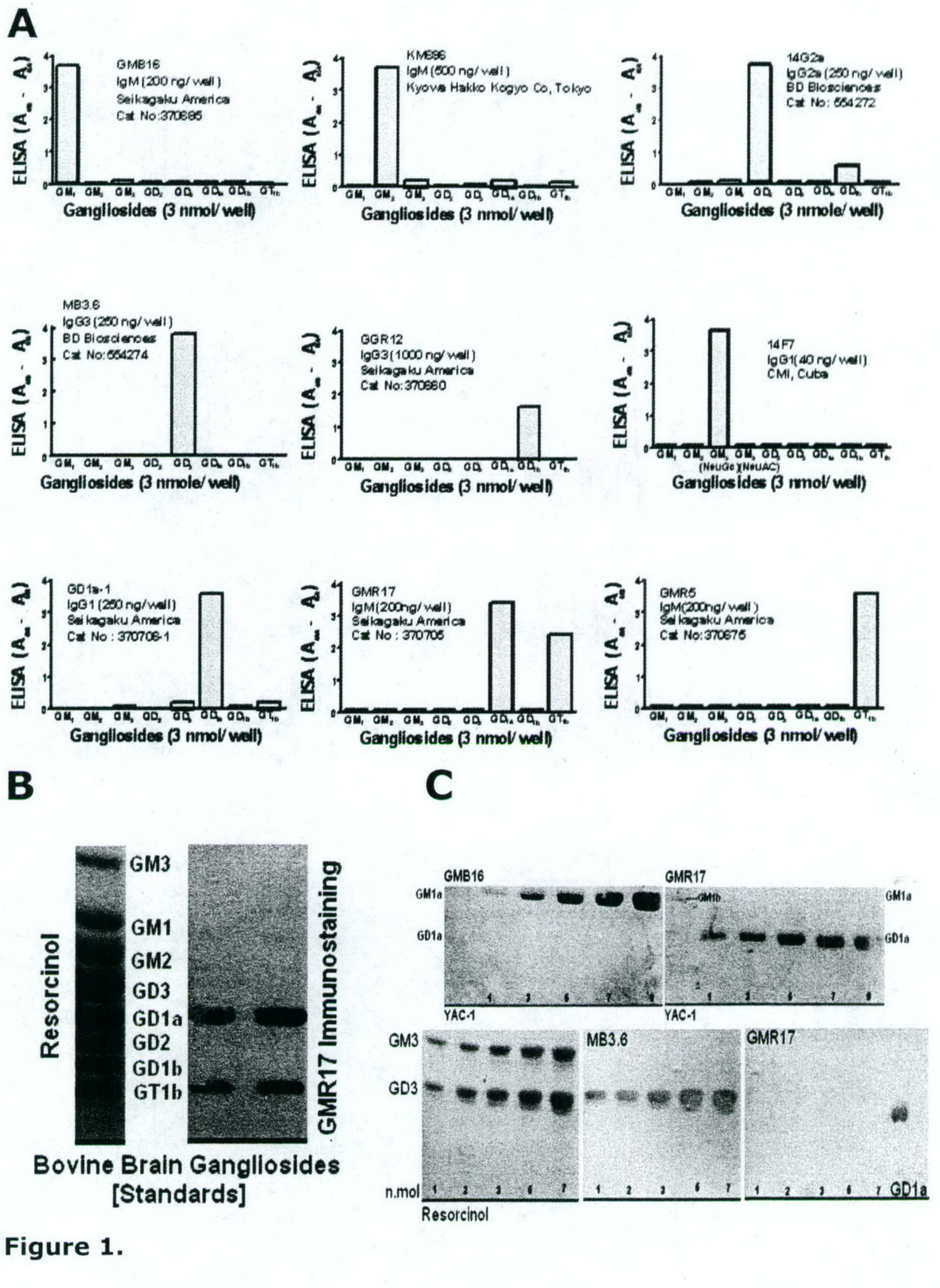
A
GMB16
IgM (200 ng/ well)
Seikagaku America
Cat No:370685
KM696
IgM (500 ng/ well)
Kyowa Hakko Kogyo Co, Tokyo
14G2a
IgG2a (250 ng/ well)
BD Biosciences
Cat No: 554272
MB3.6
IgG3 (250 ng/ well)
BD Biosciences
Cat No:554274
GGR12
IgG3 (1000 ng/ well)
Seikagaku America
Cat No:370680
14F7
IgG1 (40 ng/ well)
CMI, Cuba
GD1a-1
IgG1 (250 ng/ well)
Seikagaku America
Cat No : 370708-1
GMR17
IgM (200ng/ well)
Seikagaku America
Cat No : 370705
GMR5
IgM (200ng/ well)
Seikagaku America
Cat No:370675
ELISA (A405 - A490)
Gangliosides (3 nmol/ well)
Gangliosides (3 nmole/ well)
B
Resorcinol
GM3
GM1
GM2
GD3
GD1a
GD2
GD1b
GT1b
GMR17 Immunostaining
Bovine Brain Gangliosides
[Standards]
C
GMB16
GMR17
GM1a
GD1a
GM1b
YAC-1
GM3
GD3
MB3.6
n.mol
Resorcinol

Figure 1.

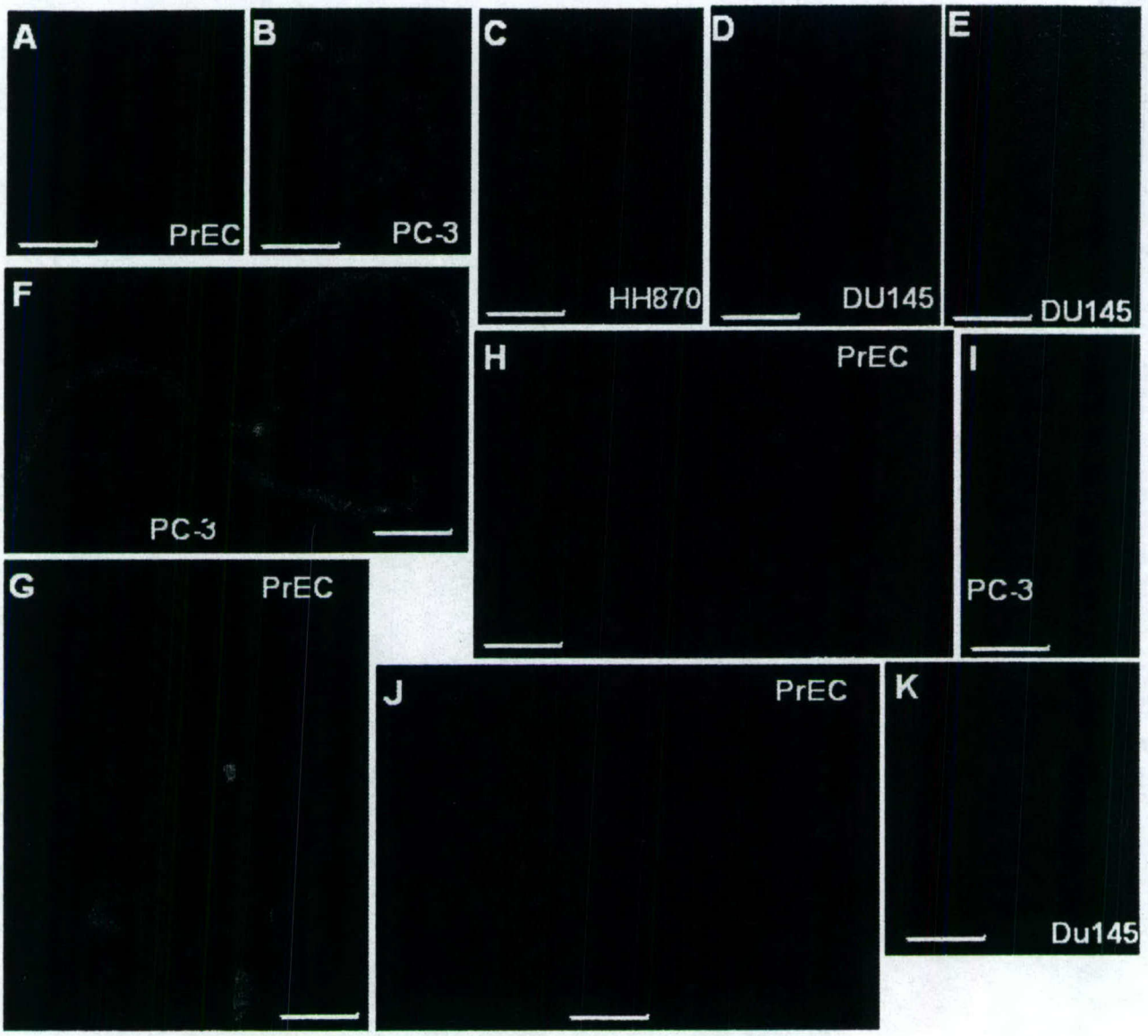
A
PrEC
B
PC-3
C
HH870
D
DU145
E
DU145
F
PC-3
H
PrEC
I
PC-3
G
PrEC
J
PrEC
K
Du145

FIGURE 2.

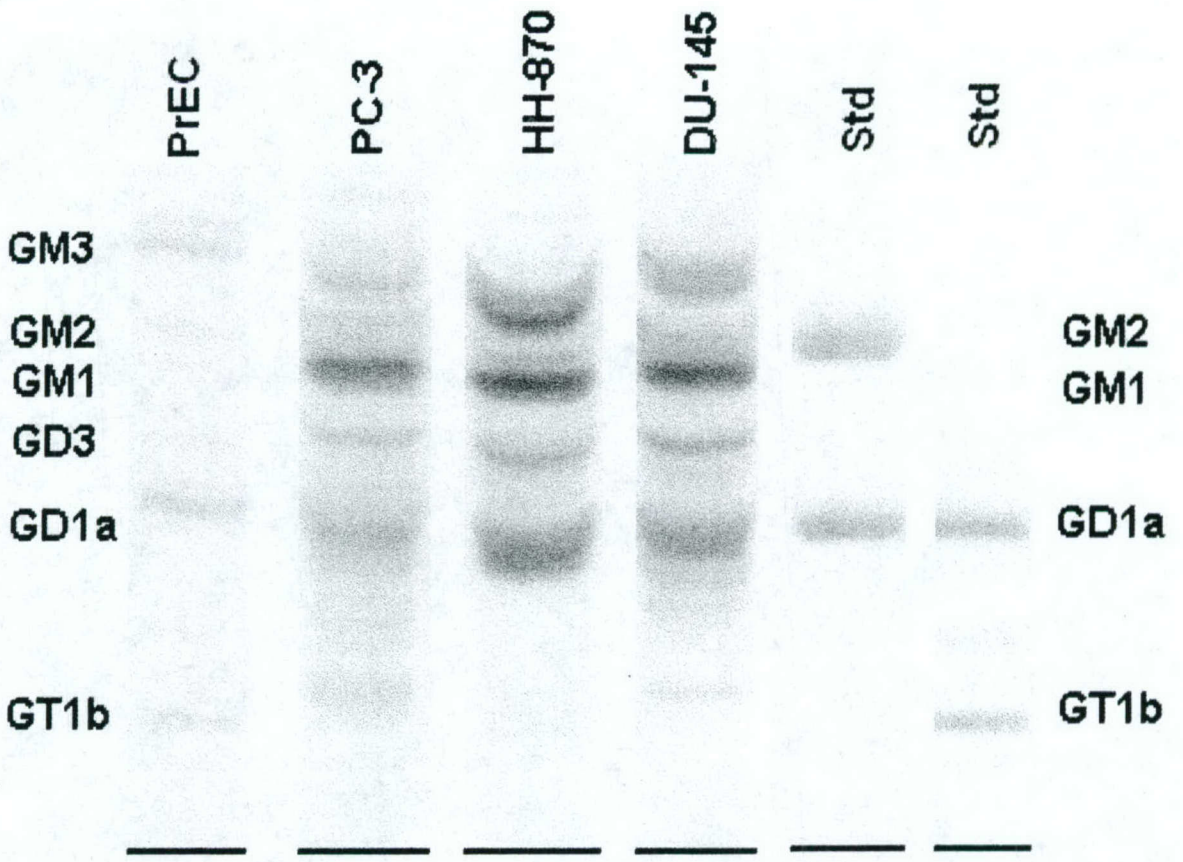
PrEC
PC-3
HH-870
DU-145
Std
Std
GM3
GM2
GM1
GD3
GD1a
GT1b
GM2
GM1
GD1a
GT1b

Figure 3

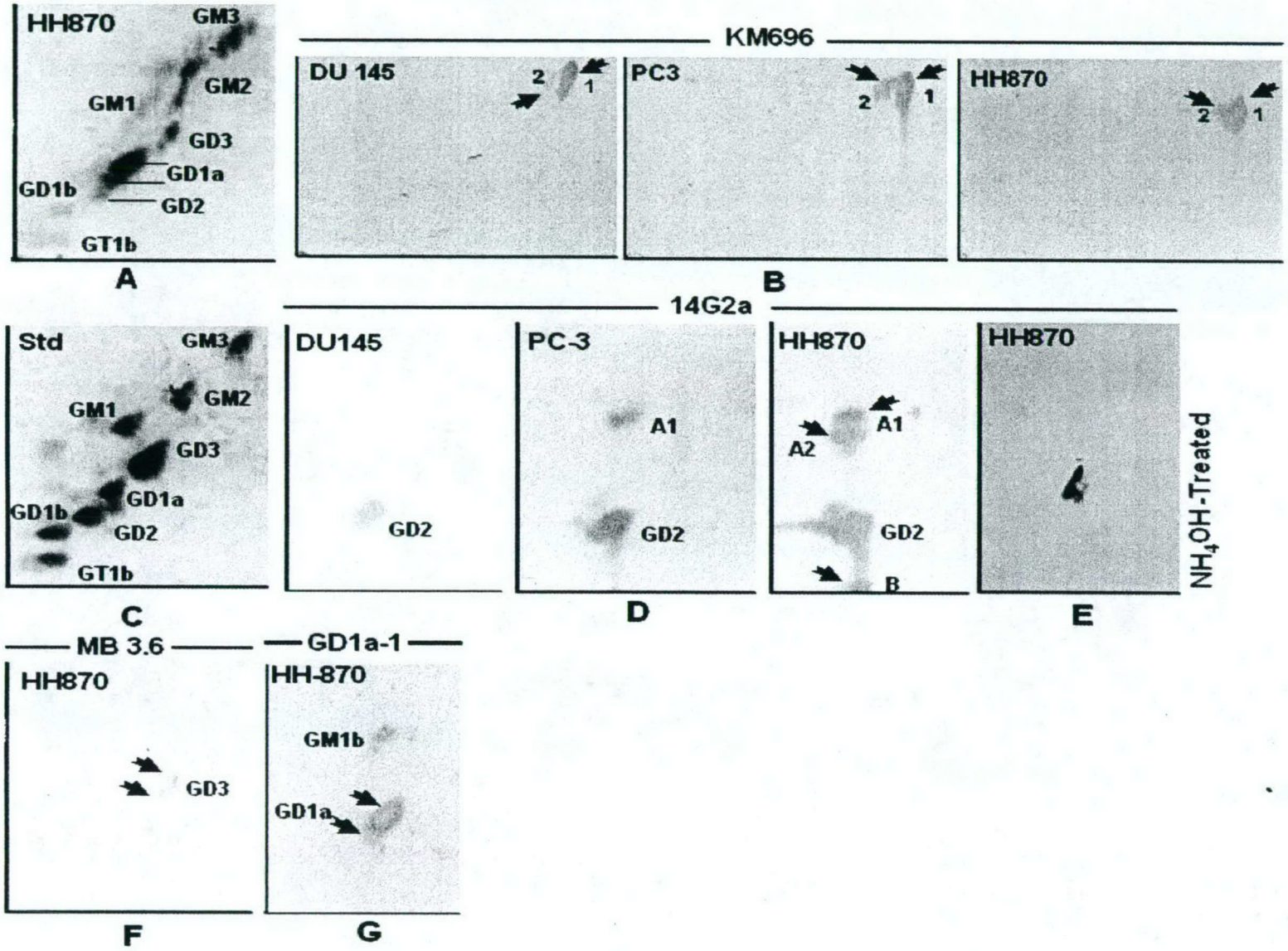
HH870
GM3
GM2
GM1
GD3
GD1a
GD1b
GD2
GT1b
A
KM696
DU 145
2
1
PC3
2
1
HH870
2
1
B
14G2a
Std
GM3
GM2
GM1
GD3
GD1a
GD1b
GD2
GT1b
C
DU145
GD2
PC-3
A1
GD2
D
HH870
A1
A2
GD2
B
HH870
E
NH4OH-Treated
MB 3.6
HH870
GD3
F
GD1a-1
HH-870
GM1b
GD1a
G

Figure 4.

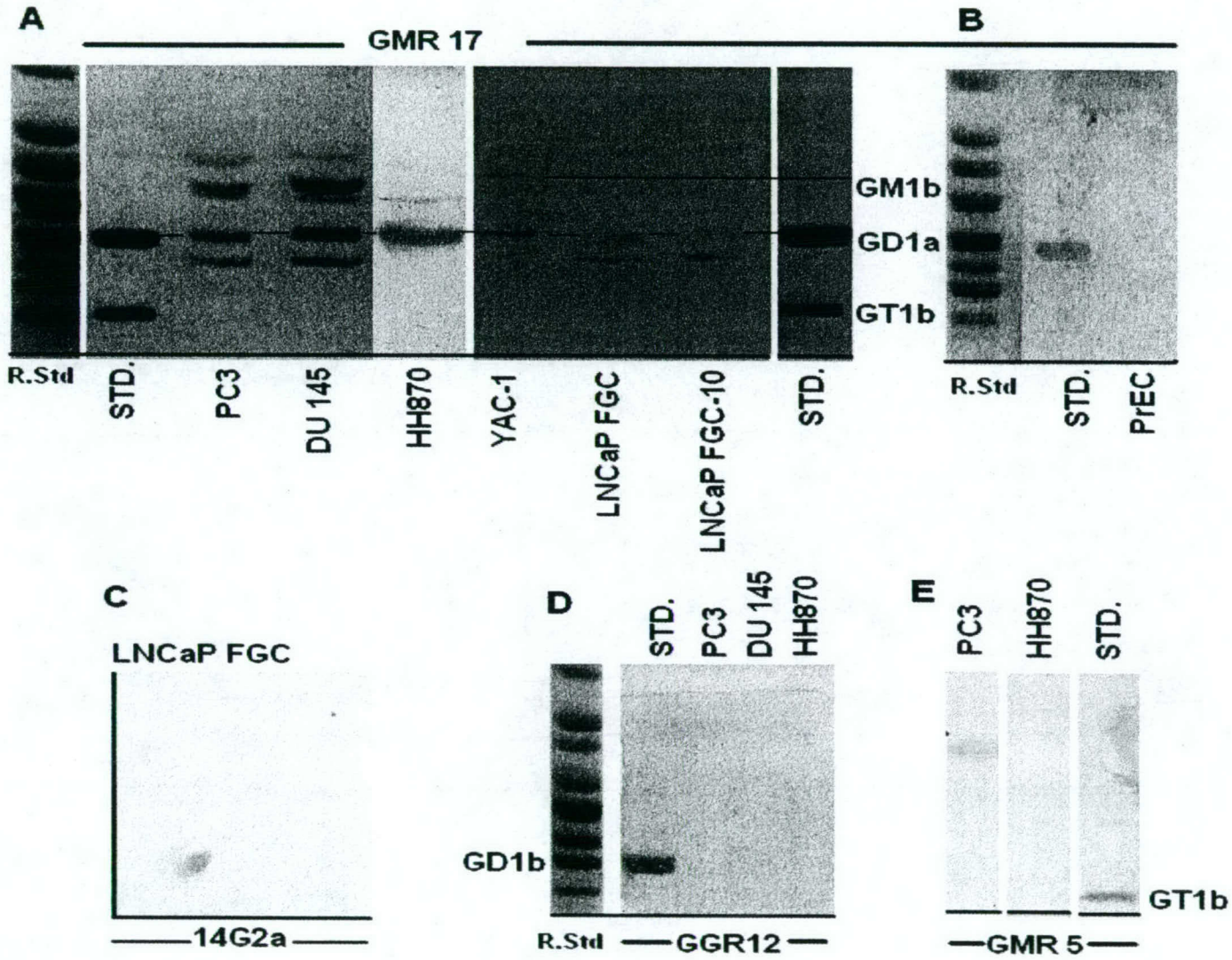
A
GMR 17
B
GM1b
GD1a
GT1b
R.Std
STD.
PC3
DU 145
HH870
YAC-1
LNCaP FGC
LNCaP FGC-10
STD.
R.Std
STD.
PrEC
C
LNCaP FGC
14G2a
D
STD.
PC3
DU 145
HH870
GD1b
R.Std
GGR12
E
PC3
HH870
STD.
GT1b
GMR 5

FIGURE 5

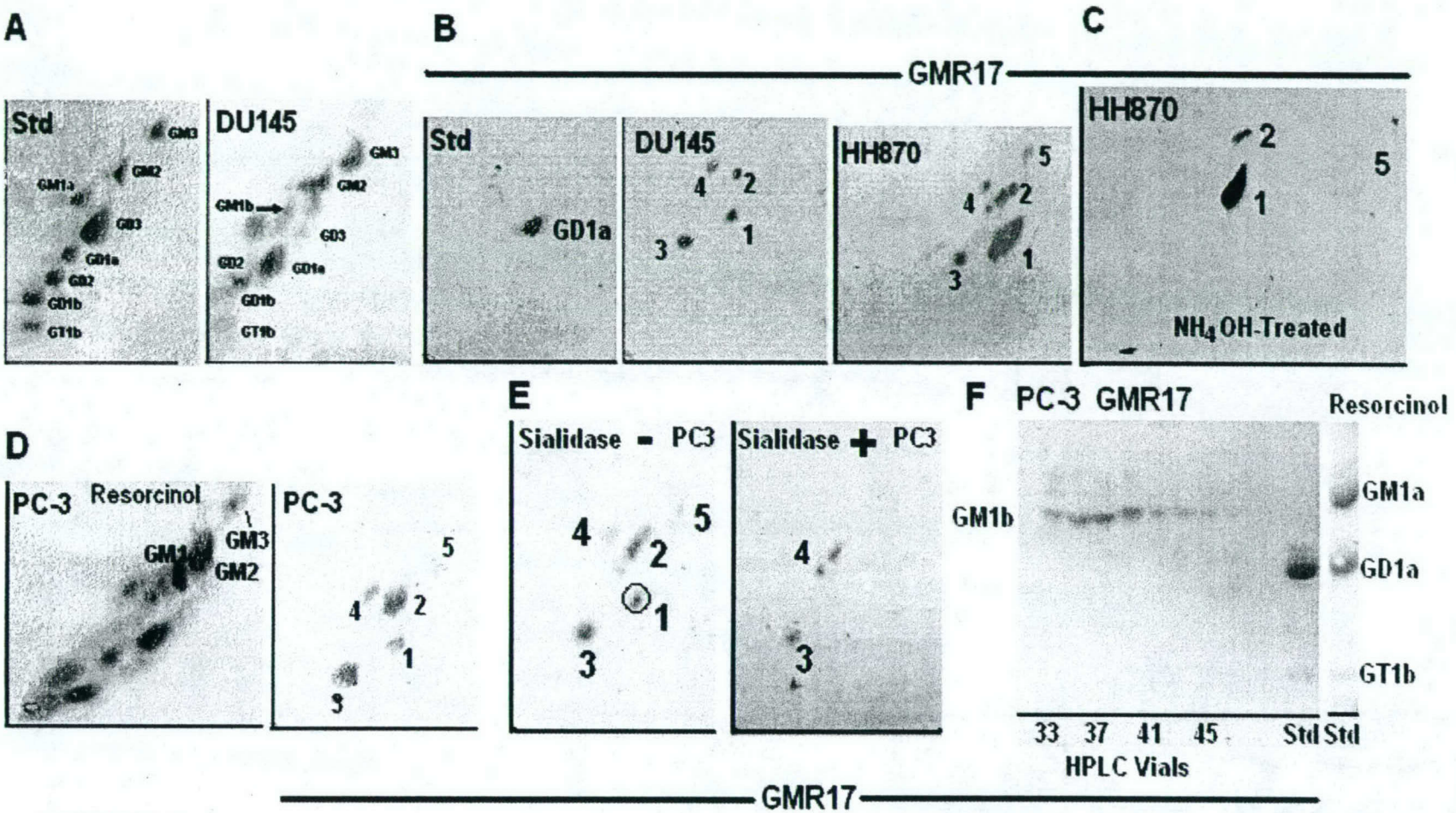
A
Std
GM3
GM2
GM1a
GD3
GD1a
GD2
GD1b
GT1b
DU145
GM3
GM2
GM1b
GD3
GD2
GD1a
GD1b
GT1b
B
GMR17
Std
GD1a
DU145
4
2
3
1
HH870
5
4
2
3
1
C
HH870
2
5
1
NH4 OH-Treated
D
PC-3 Resorcinol
GM3
GM1
GM2
PC-3
5
4
2
1
3
E
Sialidase - PC3
5
4
2
1
3
Sialidase + PC3
4
3
F
PC-3 GMR17
Resorcinol
GM1b
GM1a
GD1a
GT1b
33 37 41 45 Std Std
HPLC Vials
GMR17

FIGURE 6

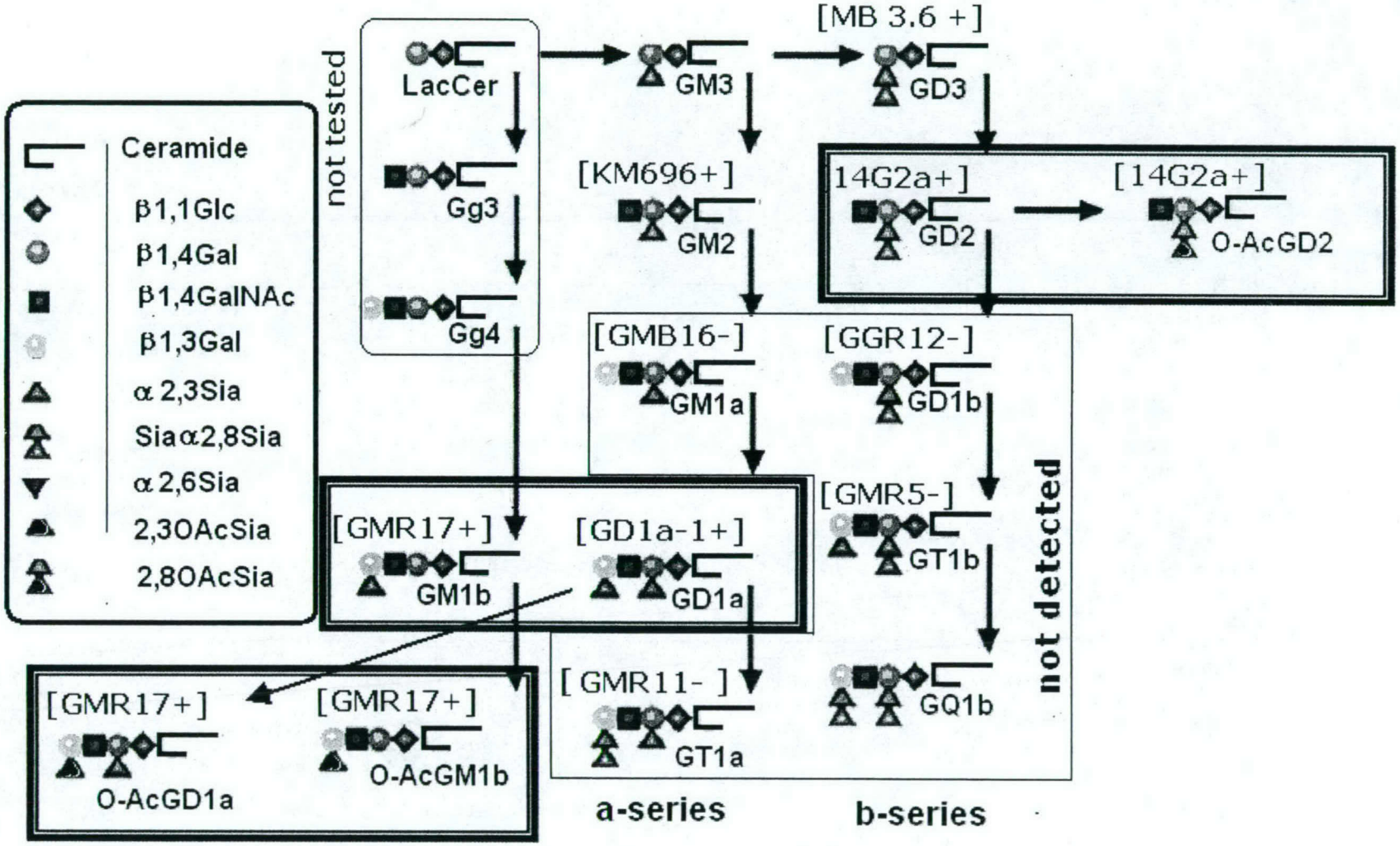
Ceramide
β1,1Glc
β1,4Gal
β1,4GalNAc
β1,3Gal
α 2,3Sia
Siaα2,8Sia
α 2,6Sia
2,3OAcSia
2,8OAcSia
not tested
LacCer
Gg3
Gg4
GM3
[MB 3.6 +]
GD3
[KM696+]
GM2
[14G2a+]
GD2
[14G2a+]
O-AcGD2
[GMB16-]
GM1a
[GGR12-]
GD1b
[GMR17+]
GM1b
[GD1a-1+]
GD1a
[GMR5-]
GT1b
not detected
[GMR17+]
O-AcGD1a
[GMR17+]
O-AcGM1b
[GMR11-]
GT1a
GQ1b
a-series
b-series

Figure 7.

PROSTATE SERA (Lab of GlycoImmunotherapy)

S. No.	Pat ID #	DOB	Age	Stage/Gr	Prior Rx	Bleed Date	PSA	Tot Gss	GM1	GM2	GM3	GD2	GD3	GD1a	GD1b	GT1b
23	8-02-89361 LB	7/12/1916	87	pT1a / 5/2	AA/CRx	8/1/2002	1.60	7.5	5000	700	100	700	100	2100	500	6400
1	7-02-23061 WK	1/15/1919	84	T1c / 4/4	untreated	7/15/2002	3.20	18.4	100	400	100	100	100	100	100	700
43	10-02-23061 WK	'		'	'	10/15/2002		12.8	100	100	100	200	100	100	100	300
54	11-02-23061 WK	'		'	'	11/12/2002			100	100	100	300	100	100	100	400
20	8-02-08231 EM	2/13/1936	67	T1c / 3/3	untreated	8/2/2002	8.75	13	1300	400	6400	6400	100	300	100	100
28	8-02-73572 CM	1/20/1943	60	T1c / 3/3	Post RP	8/21/2002	6.70	21.6	200	400	500	300	100	1600	100	1300
41	10-02-38071 JM	10/27/1931	72	T1c / 3/3	untreated	10/14/2002	2.43	15.8	100	100	100	400	100	300	300	600
79	1-03-38071 JM	'		'	'	1/13/2003		14.9	100	500	100	100	100	700	400	600
22	8-02-71831 RN	9/11/2029	74	T1c / 3/3	untreated	8/30/2002		10.6	100	100	100	100	100	100	100	2900
6	7-02-56351 CS	4/6/2023	80	T1c / 3/3	untreated	7/16/2002	3.70	13.4	100	100	100	100	100	800	100	400
31	8-02-56351 CS	'		'	'	8/22/2002		12.1	100	100	100	500	100	900	100	2600
49	10-02-56351 CS	'		'	'	10/22/2002		16.0	300	400	100	700	100	2400	1300	2700
61	11-02-56351 CS	'		'	'	11/18/2002		13	100	100	100	100	100	300	300	400
47	10-02-30941 SY			cT1c / 4/4	XRT TAB	10/22/2002		12.3	100	100	100	1000	100	200	300	800
27	8-02-98960 JJ	10/14/1929	74	T1c / 3/3	RP	8/21/2002	0.00	15.8	100	500	100	400	100	200	300	800
58	11-02-65791 JM	12/21/1934	69	cT1c 3/3	untreated	11/14/2002		19.2	100	600	300	300	3000	2600	6400	6400
73	01-03-63301 MR	11/21/1933	70	cT1c / 3/3	untreated	1/2/2003	5.70	11.6	200	100	100	100	100	100	100	100
99	2-03-63301 MR	'		'	'	2/10/2003		11.6	400	100	100	100	100	100	100	200
91	2-03-26672 GH	8/15/1939	64	T1c / 3/4	RP AA	2/3/2003	1.47	14	200	100	100	100	200	100	600	1500
93	2-03-90012 CS	12/15/1939	64	T1c / 3/4	AA / Casodex	2/3/2003	5.90	12.3	100	100	100	100	100	100	100	100
108	2-03-06723 RV	3/3/2017	86		AA	2/24/2003	2.91	18.7	100	300	200	100	400	100	100	2400
77	1-03-31513 JB	12/17/2029	74		RP	1/13/2003		13	100	100	100	100	100	100	100	100
78	1-03-50783 RN	11/14/1930	73		XRT/Pd	1/13/2003	0.09	14.9	100	100	100	100	200	200	100	200
29	8-02-39102 GS	1/11/1910	93	T2a / 3/3	XRT / AA	8/21/2002	0.09	90.6	100	100	300	700	100	1500	100	1200
64	11-02-29941 RC	7/17/1934	69	pT2a / 3/2	RP	11/26/2002	0.80	15.6	400	100	100	700	100	100	500	700

10	7-02-42041 GK	4/24/1927	76	T2a / 3/3	untreated	7/26/2002	3.95	17.4	100	100	100	900	100	200	100	700
50	10-02-42041 GK	"		"	"	10/23/2002		18.9	100	600	400	3600	100	200	2200	6400
33	8-02-63071 SM	11/20/1942	61	cT2a 3/3	untreated	8/29/2002	6.58	18.9	400	300	100	400	100	100	100	400
				pT3 4/3	RP											
53	10-02-63071 SM	'				10/30/2002		15.3	700	500	100	700	100	100	100	600
9	7-02-03432 JC	8/22/1939	64	T2b1 / 3/4	untreated	7/25/2002		16.5	100	100	100	400	100	300	100	400
14	7-02-62801 GO	6/16/2022	81	T2b / 3/3	RP	8/1/2002	0.38	6.2	100	400	100	1000	100	100	300	100
68	12-02-41311 JS	1/5/1936	67	T2b 3/3	none	12/20/2002		12.3	100	100	1400	100	100	100	800	200
103	2-03-42981 BC	8/30/2023	80	T2b / 3/3	RP 3/91	2/13/2003	2.10	16.8	900	4900		1400	1500	3800	2000	3300
52	10-02-65601 JH	10/24/1912	91	cT3 / 3/3	AA	10/28/2002		15.1	100	100	100	400	100	100	100	200
48	10-02-74081 GS			T3 / 3/4	untreated	10/22/2002		16.3	300	300	100	500	100	100	800	1300
17	8-02-60383 NS	1/1/2021	82	T3 / 3/4	XRT/AA	8/10/2002	21.80	10	100	100	400	700	100	100	100	500
34	8-02-64372 WB	4/18/1917	86	cT3 / 3/4	XRT	8/30/2002	5.31	18.3	700	200	100	500	100	100	600	2600
55	11-02-07372 RH	5/26/1922	81	pT3 / 3/4	RP AA	11/12/2002	1.47	14.4	100	100	100	1300	100	200	500	1300
100	2-03-07372 RH	'		'	'	2/11/2003		15.8								
56	11-02-27671 SB	4/16/1933	70	cT3 / 3/4	NONE	11/13/2002	4.73	16,6	100	100	100	100	100	100	100	700
46	10-02-06721 RV	3/3/2017	86	cT3 / 4/4	untreated	10/22/2002	2.91	18.1	300	700	100	500	100	1100	700	1900
36	9-02-83422 WO	7/27/1936	67	T3 / 4/4	untreated	9/24/2002	4.92	9.7	100	1400	500	1300	100	100	200	1400
65	12-02-83422					12/4/2002		14.2	100	700	100	1300	50	300	6400	5600
62	11-02-83422					11/19/2002		18.4	100	700	100	100	100	100	6400	400
59	11-02-27671 SB	4/16/1933	70	T3 / 3/4	untreated	11/15/2002	4.73	14.4	100	100	100	100	100	100	200	300
18	8-02-64373 WB	4/18/1917	86	T3 / 3/4	XRT	8/12/2002	5.31	12.3	100	600	100	700	100	100	100	1000
90	2-03-90992 GD	11/12/1926	77	T3 / 3/4	RP	2/3/2003	4.30	13.5	100	100	100	100	100	100	100	200
12	7-02-98862 RN	3/7/1921	82	T4 / 3/4	RP/XRT/ Crx	7/30/2002	200.00	17.8	100	100	100	900	100	100	100	900
13	7-02-89781 SA	12/17/1919	84	cT4/3/4	RP/AA	7/30/2002	7.50	18.6	100	900	400	100	100	1400	600	1200

37	9-02-90982 EG	8/11/1909	94	cT4 / 3/4	AA	9/30/2002	7.00	18.1	100	100	100	100	100	100	100	600
40	10-02-90982 EG	'		'	'	10/14/2002	28.00	17.9	300	100	100	100	100	100	100	400
72	01-03-90982 EG	'		cT4 / 3/4	AA	1/2/2003	30.00		200	100	100	100	100	100	100	200
8	7-02-07161 AK	9/3/1932	71	T4 / 3/5	untreated	7/18/2002	25.40	15.5	100	500	400	100	100	100	100	700
11	7-02-07161 AK	'		'	'	7/26/2002	25.40	17	100	100	100	100	100	200	100	700
74	1-03-07162 AK	'		'	post CRX	1/6/2003		21	100	100	100	100	100	100	100	400
66	12-02-09952 RI	6/10/1941	62	pT4 / 4/5	RP/AA	12/19/2002	0.10	16.6	100	100	100	100	100	300	1400	700
32	8-02-53692 LH	10/14/1929	74	T4 / 5/5	XRT / CRx	8/26/2002	0.70	20.6	100	200	100	400	100	400	100	1600
75	1-03-55822 LE	1/5/1932	71	T4 / 4/3	RP/XRT	1/6/2003	67.00	19.4	100	100	100	100	100	200	100	200

Appendix # 3. Table showing raw data of serum total PSA and total ganglioside levels in Cancer and BPH patients.

Age	Stage/Gr	PSA	Tot Gss
87	T1a / 5/2	1.60	7.5
66	T1c / 3/3	8.75	13
74	T1c / 3/3	0.00	15.8
60	T1c / 3/3	6.70	21.6
72	T1c / 3/3	2.43	15.8
80	T1c / 3/3	3.70	13.4
69	T1c / 3/3	5.70	13.1
84	T1c / 4/4	3.20	18.4
93	T2a / 3/3	0.09	90.6
76	T2a / 3/3	3.95	17.4
61	T2a / 3/3	6.58	18.9
81	T2b / 3/3	0.38	6.2
82	T3 / 3/4	21.80	10
85	T3 / 3/4	5.31	18.3
85	T3 / 4/4	2.91	18.1
67	T3 / 4/4	4.92	9.7
70	T3 / 3/4	4.73	17.3
85	T3 / 3/4	5.31	12.3
82	T4 / 3/4	200.00	17.8
84	T4/3/4	7.50	18.6
93	T4 / 3/4	7.00	18.1
70	T4 / 3/5	25.40	15.5
73	T4 / 5/5	0.70	20.6
46	BPH	0.36	12.8
53	BPH	0.68	13.4
75	BPH	4.30	12.6
73	BPH	1.50	10.4
72	BPH	5.19	8.7
83	BPH	15.00	16.2
64	BPH	4.57	15.4

APPENDIX # 4

Disease	n	Tot Gss
CaP T1	1	7.5
	2	18.4
	3	12.8
	4	13
	5	21.6
	6	15.8
	7	14.9
	8	10.6
	9	13.4
	10	12.1
	11	16.0
	12	13
	13	12.3
	14	15.8
	15	19.2
	16	11.6
	17	11.6
	18	14
	19	12.3
CaP T2	1	15.6
	2	17.4
	3	18.9
	4	18.9
	5	15.3
	6	16.5
	7	6.2
	8	12.3
	9	16.8
CaP T3	1	15.1
	2	16.3
	3	10
	4	18.3
	5	14.4
	6	16,6
	7	18.1
	8	9.7
	9	14.2
	10	18.4
	11	14.4
	12	12.3
	13	13.5
	14	17.8
CaP T4	1	18.6
	2	18.1
	3	17.9
	4	

	5	15.5
	6	17
	7	21
	8	16.6
	9	20.6
	10	19.4
BPH	1	12.8
	2	13.4
	3	10.8
	4	12.6
	5	12.8
	6	10.4
	7	8.7
	8	16.2
	9	8.7
	10	13.2
	11	15.1
	12	15.4
	13	14.2
	14	14.9
Prostatitis	1	14.2
	2	10.3
	3	17.2
	4	20.6

Total serum ganglioside level of healthy volunteers.

healthy#	sex	age	LASA-P
65	m	55	12.10
130	m	23	12.22
132	m	23	12.32
49	m	73	13.00
66	m	55	14.50
62	m	75	15.20
55	m	49	15.40
129	m	33	15.50
60	m	77	16.10
42	m	67	16.80
70	m	66	17.00
52	m	72	17.50
125	m	35	18.30
51	m	48	18.40
128	m	35	18.90
133	m	50	19.00
131	m	18	19.60
126	m	30	20.40
48	m	62	22.20
127	m	35	22.70

Endogenous Immune Response to Gangliosides in Patients with Confined Prostate Cancer

Running Title: Endogenous Immune response to Gangliosides in Prostate Cancer

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Abbreviations used:

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INTRODUCTION

Prostate cancer (CaP) progresses imperceptibly in its early stages and is potentially curable when organ confined. A better understanding of the tumor biology that distinguishes healthy prostatic cells from cancer cells, and organ-confined cancer from unconfined cancer would enable development of biomarkers for early detection and target antigens for novel therapies. Gangliosides, sally-oligosaccharides with ceramides, are overexpressed on tumor cell surfaces (1) and differ in their nature and distribution between normal and cancer cells and among different types of cancers (1-7). Although gangliosides of melanoma, colon cancer, and pancreatic cancer have been well characterized, there is little information on the ganglioside profile of CaP (8, 9, 10).

Tumor-associated gangliosides, when shed into the circulation (11-15), may elicit an endogenous IgM antibody response (16). In patients with advanced colon cancer, cryoablation (freezing and thawing) of hepatic metastases releases tumor-associated gangliosides into the circulation (16); these circulating gangliosides augment IgM antibodies without the need for exogenous adjuvants (16). Antiganglioside IgM antibodies occur naturally in healthy individuals (17) but in cancer patients the level of antiganglioside antibodies varies according to the stage of disease and tumor progression (15, 18).

We hypothesize that the endogenous IgM response to gangliosides might be an early immunological event of tumorigenesis. This preliminary study examines if specific antiganglioside IgM antibodies occur in the sera of patients with organ-confined CaP (stage T1/2) after screening the profile of tumor-associated gangliosides in androgen-receptor positive (LNCaP FGC & LNCaP FGC-10) and negative (PC-3 & DU 145) ATCC CaP cell lines.

MATERIAL AND METHODS

Cell lines. PrEC prostate epithelial cell line was obtained from Cambrex BioScience (Walkersville, Inc, MD) and cultured in growth media PrEGM (CC-4177, Cambrex). Two androgen receptor-negative CaP cell lines (PC-3 [ATCC CRL-1435] and DU 145 [ATCC HTB-81]) and two androgen receptor-positive CaP cell lines (LNCaP Clone FGC [ATCC CRL-1740] and LNCaP Clone FGC-10 [ATCC CRL-10995]) were obtained from the American Type Culture Collection repository. All four CaP cell lines had been established from metastases.

Cells were grown in RPMI 1640 with glutamine (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum, HEPES buffer, gentamycin (5 mg%) and fungizone (0.5 mg%), at 37°C in a humidified atmosphere of 5% CO_2. The cells were detached with sterile EDTA-dextrose (137 mM sodium chloride, 5.4 mM potassium chloride, 5.6 mM dextrose, 0.54 mM EDTA, 7.1 mM sodium bicarbonate) at 37° C for 5 minutes, recovered with cold RPMI 1640- 4% HSA (Grupo Grifols de America, Inc. Miami, FL), and resuspended in the same medium. Harvested cells were cryopreserved with 10% DMSO in RPMI-7.5% HSA, and stored under liquid nitrogen. When required, the cryopreserved cells were half-thawed at 37°C in a water bath for 15-30 sec and further thawed at room temperature. The cell suspension was washed with RPMI-4% HSA, centrifuged and resuspended again. Cell count was measured using a hemocytometer and the viability by trypan blue (0.2%) exclusion procedure.

Glycolipid extraction from cell pellets. Glycolipids were extracted from CaP cells following a protocol reported earlier (19). Freshly harvested or cryopreserved cells were resuspended in RPMI-4% HSA, counted and rewashed in PBS. Methanol was added to the pellets (v/v: 1/10), vortexed, mixed with equal volume of chloroform, and allowed to stand at room temperature for 5 hrs. After centrifugation, the supernatant was separated and evaporated to dryness under nitrogen. The dried moiety was resuspended in 2 ml of chloroform/methanol (v/v: 1/1). After the suspension was centrifuged, the supernatant was collected, dried over nitrogen and redissolved in 4 ml chloroform/methanol (v/v: 1/1). The final ratio of chloroform/methanol/PBS was adjusted to 1/1/0.7 (v/v/v). The extract was centrifuged, the upper phase containing gangliosides was recovered, and the lower phase was re-extracted with methanol/PBS. The upper phase was recovered thrice and pooled.

Isolation of Gangliosides. Gangliosides were isolated on columns (ENVI-Chrom P, Supelco, Bellefonte, PA) containing a resin made of small, non-ionic, highly cross-linked styrene-divinylbenzene beads (20). The columns were fixed in Visiprep solid-phase extraction vacuum manifold (Supelco). Initially the column was conditioned by adding methanol followed by PBS. The upper phase containing gangliosides was layered on the column, the flow rate adjusted in drops, and the solution was collected. The column was washed with distilled water twice in order to remove nonlipid contaminants such as salts, sugars, and amino acids. Water was removed completely. Gangliosides were eluted from the column by adding 3 ml of methanol and then 3 ml of chloroform/methanol (v/v: 2/1). Eluent was dried over nitrogen and the gangliosides were dissolved in chloroform/methanol (v/v: 2/1) and stored at -20°C.

High-performance Thin-layer Chromatography (HPTLC). Ganglioside signatures of CaP cells were analyzed by HPTLC as described earlier (21, 22). HPTLC plates (10x10 cm) precoated with silica gel 60 (glass or aluminum backing) (E. Merck, Darmstadt, Germany) were used. Two-dimensional HPTLC was performed using two different solvent systems. Solvent systems were equilibrated several hours before running the chromatogram. The sample was separated in the first dimension in chloroform/methanol/0.2%$CaCl_2$ (v/v/v: 55/45/10). The plate was dried in a vacuum desiccator overnight and run in the second dimension in chloroform/methanol/2.5M NH_4OH in 0.25% KCl (v/v/v: 50/40/10).

The following gangliosides were screened for purity and homogeneity: GM3 (Sigma: G 5642), GM2 (Sigma: G 8397), N-glycol-GM3 (gift from Dr. Adriana Carr, Center for Molecular Immunology, Habana, Cuba), GM1a (Sigma:G 7641), GD3 (Calbiochem: 345752), GD1a (Sigma: G 2392), GD2 (Advanced Immunochem, Long Beach, IG6), GD1b (Sigma:G 8146) and GT1b (Sigma: G 3767). These gangliosides were used as reference standards for HPTLC and as antigens for ELISA. The gangliosides were spotted onto the plates using Linomat (CAMAG Scientific Inc, Wilmington, NC). The plates were pre-run in chloroform to eliminate neutral lipid and other contaminants that may interfere with the mobility of gangliosides. Gangliosides were visualized by heating at 100°C after spraying resorcinol-HCl reagent (10 ml of 2% resorcinol in water, 40 ml concentrated HCl, 0.125 ml 0.1M copper sulphate). Each chromatogram represented ganglioside extract from 25 x 10^6 cells.

Immunostaining of Thin-layer Chromatograms. The following murine monoclonal antibodies against gangliosides were obtained from Seikagaku America (20, 21): GMB16 (anti-GM1a IgM; Cat No: 370685), GGR12 (anti-GD1b; Cat No: 370660), GD1a-1 (anti-GD1a IgG1; Cat No: 370706-1), GD1a-2 (anti-GD1a IgG2a, Cat No: 370706-2), GMR17 (anti-GD1a/GT1b IgM, Cat No: 370705) and GMR5 (anti-GT1b IgM; Cat No: 370675). Other monoclonal antibodies used in this study include KM696 (anti-GM2 IgM; Kyowa Hakko Kogyo Co, Tokyo), 14.G2a (anti-GD2 IgG2a, BD Biosciences, Cat No: 554272), MB3.6 (anti-GD3 IgG3, BD Biosciences, Cat No: 554274), 14F7 (anti-GM3 (NeuGc) IgG1; gift from Dr. Adriana Carr, Habana, Cuba). The specificity of each monoclonal antibody was tested with eight different gangliosides using an ELISA protocol described earlier (23).

Gangliosides were separated on aluminum-backed silica gel plates and the plates were dipped in 0.2% polyisobutyl-methacrylate in hexane for 1 minute. After drying, the plates were blocked with PBS-1% HSA for 30 minutes, washed with PBS and dried. Each plate was overlaid with a monoclonal antibody diluted in PBS-1% HSA for 2 h at 37°C. Primary antibodies were diluted as follows: GMB16 (1/400); KM696 (I/1000), 14.G2a (1/500); MB 3.6 (1/250) GGR 12 (1/200), GMR 5 (1/500), 14F7 (1/1000), GMR 17 (1/800), GD1a-1 (1/500). The plates were then washed three times with PBS (for 3 min), dried and incubated with biotinylated rabbit anti-mouse secondary antibody (Jackson Immunoresearch, rabbit anti-mouse IgG, Cat. No: 315-065-008; rabbit anti-mouse IgM Cat, No: 315-065-049) diluted at 1:500 for 1 h at 37°C. The plates were washed thrice in PBS, dried and incubated for 45 minutes in peroxidase conjugated streptavidin (Jackson Immunoresearch, Cat No: 016-030-084) diluted in PBS at 1:2500. The plates were developed with 4-chloro-1-naphthol solution (10 ml) and hydrogen peroxide (5 μl). A blue-grey color indicated specific binding of the antibody.

Patient population. This study involved the collection of sera from 10 patients with benign prostatic hyperplasia (BPH), 20 patients with untreated stage T1/T2 CaP (organ-confined) and 6 patients with untreated stage T3/T4 (unconfined) CaP. Tumor-related variables included clinical stage, biopsy Gleason score and serum PSA; for this study, total serum ganglioside level was also considered a tumor-related variable. Informed consent was obtained from each study participant in accordance with the Saint John's Health Center and John Wayne Cancer Institute Institutional Review Board (IRB) policy. Joint IRB committees of John Wayne Cancer Institute and Saint John's Health Center approved the study protocol.

Fifteen healthy male volunteers who had donated blood at Saint John's Health Center between January 1998 and November 2003 and had given consent for their blood samples to be used for research purposes were initially screened for serum IgM autoantibody titers against different gangliosides. Eleven of the 15 were selected as control subjects by computer matching of their ages with the ages of BPH and CaP patients. No control subject had active medical problems or was taking any medications.

In all cases, serum was recovered within 6 hours after blood sampling, aliquoted, and stored at -70°C at JWCI serum bank. For antibody analyses, sera were thawed once, vortexed extensively for 45 seconds and aliquoted for further dilution. All analyses were carried out in a double-blinded fashion. The results obtained from

coded sera were sent to urologist (S.B.) who has deciphered the code and sent the results to statistian (X.Y.) for analyses.

Measurement of Total Serum Gangliosides (TSG). TSG levels in 100 µl of serum were measured by an assay for lipid-bound sialic acid (5). Briefly, the concentration of sialic acid in serum extract was determined in 100 µl of chloroform/methanol (v/v: 2/1) by the resorcinol-HCl method (24), after transferring the supernatant to a clean tube. The resorcinol/HCl reagent was mixed with supernatant and then heated at 100°C for 15 minutes. After cooling at room temperature, a butylacetate:n-butanol (v/v: 85/15) mixture was added and the organic layer containing the chromogen was read at an absorbancy of 580 nm. By using a standard curve obtained with commercial NeuAc (Sigma, St. Louis, MO), the TSG was expressed as mg/dL. No phosphotungstic acid was used for the measurement of lipid-bound sialic acids. For routine analyses, varying concentrations of HSA served as a negative control. HSA spiked with known amount of purified gangliosides served as a positive control. TSG levels were measured with an automated system at Dianon Systems, Inc. (Stratford, CT).

Approximately 30% of the sialic acids extracted with this protocol may be derived from sialoproteins or proteolipids with sialic acids. However, some of the sialic-acid containing proteins may include ganglioside-binding transport proteins or immunoglobulins complexed with gangliosides. The reliability and reproducibility of this assay was confirmed by a double-blind analysis. The coefficient of variation was always < 15%.

Quantitation of Cell-surface Antigens. Quantitation of cell-surface antigens was performed using methods described previously (25, 26). Trypsin was strictly avoided for harvesting the cells. All cells were washed once with RPMI-4% HSA and then suspended in cold RPMI-4% HSA. Cells were counted for viability using 0.2% trypan blue. Suspensions of 0.5×10^6 cells in microcentrifuge tubes containing 60 µl of solution were treated with secondary antibody (*background*), with primary and secondary antibody (*experimental*), or with class-matched isotypes of the primary antibody (*negative control*). The final dilution of the primary (120 µl) antibodies (determined based on box titration) varied with different monoclonals, as follows: 14F.7 (anti-GM3-N-glycolyl IgG1, 1/48,000), KM696 (anti-GM2 IgM, 1/10,000), 14.G2a (anti-GD2 IgG2a, 1/1500), MB3.6 (anti-GD3 IgG3, 1/2000), GGR12 (anti-GD1b IgG3, 1/200), GMR17 (anti-GD1a/GT1b IgM, 1/8000), GMR5 (anti-GT1b IgM, 1/10,000) and GMB16 (anti-GM1a IgM, 1/2000). These antibodies were assessed for their monospecificity in cs-ELISA using eight different gangliosides. All monoclonals showed remarkable specificity for their respective gangliosides except for GMR17, which stained for both GD1a and GT1b.

ELISA for Serum Antiganglioside Antibodies. Sera were screened for free IgM antibodies against commercially obtained, purified and homogenous gangliosides (GM1, GM2, GM3, GD1a, GD1b, GD2, GD3, and GT1b) by an ELISA developed and validated in our laboratory (23). Microtiter plates (Falcon Probind 3915) were coated with an ethanolic suspension of gangliosides (3 nmol/100 µL/well) (Sigma) and dried in a vacuum desiccator for two days. The plates were blocked with PBS-4% HSA, pH 7.2, for 90 minutes. Sera were diluted to 1/100 and incubated in a water bath for 30 minutes at 37°C. Sera were further serially diluted to 1/6400, overlaid on the plates

(Falcon 3915), and incubated at 37°C for 2 hours. The plates were washed 5 times with washing buffer (PBS-0.1% HSA- 0.1% Tween-20). Anti-human IgM coupled to peroxidase (Jackson Immunoresearch Laboratories, Inc., Pittsburgh, PA) was used as the second antibody at a dilution of 1/5000, and the plates were incubated with second antibody (100 µl/well) for 1 hour at 37°C. The plates were washed as before. The substrate, o-phenylenediamine hydrochloride (OPDE) (20 mg) dissolved in citrate-phosphate buffer (pH 5.0) and hydrogen peroxide (10.5 µL/25 ml of buffer), was added to the plates (100 µL/well) and incubated for 45 minutes in the dark at 25°C. The enzymatic oxidation was arrested with 120 µL of 6N H_2SO_4. The absorbancy difference at 490 nm and 650 nm was measured in an UV max kinetic microplate reader (Molecular Devices Corporation, Sunnyvale, CA). The values were corrected for background (wells without antigen) and expressed as titers. The reliability and reproducibility of this assay were previously validated. Because this assay detects IgM that can recognize and bind to gangliosides coated onto the plates, it measures the titer of free IgM but not IgM-ganglioside complexes.

Statistical analysis. Analyses of variance (ANOVA) were performed to compare antiganglioside IgM antibody titer among healthy volunteers, BPH patients, and patients with stage T1/T2 or stage T3/T4 CaP. Data were log-transformed for compatibility with the assumption of normal distribution and common variance. Fisher's least significant difference (LSD) method was used for pairwise comparisons of values significant at the 0.05 level.

RESULTS

Ganglioside Profiles of PrEC and CaP cells. Three different methodologies were used to characterize ganglioside profiles from the five cell lines. In the *first method*, one- and two-dimensional chromatograms of glycolipid extracts were stained with resorcinol-HCl; bovine brain gangliosides were used as reference standards. In the *second method*, one- and two-dimensional chromatograms were stained with specific murine monoclonal antibodies. In the *third method*, tumor cell-surface gangliosides were measured directly by cs-ELISA with murine monoclonal antibodies to gangliosides.

Resorcinol characterization of Gangliosides. Resorcinol staining provides approximate identification of the gangliosides in CaP cells in comparison with the mobility of the standard bovine brain gangliosides. **Figure 1A** shows the ganglioside profiles identified by resorcinol-HCl staining of one-dimensional chromatograms for normal and CaP cell lines. In general, gangliosides stained much less intensely in PrEC cells than in CaP cell lines. LNCaP-FGC and LNCaP-FGC-10 CaP cell lines stained less intensely than PC-3 and DU-145 CaP lines. Based on the staining intensity of one-dimensional chromatograms, the relative distribution of gangliosides in normal PrEC was as follows: GM3 > GD1a = GT1b > GM2 = GD3 = GD1b > GM1a (commonly known as GM1). The profile in PC-3 and DU 145 was as follows: GD1a > GM1 > GM2 > GD3 > GM3. GT1b and GD1b could not be identified. In LNCaP-FGC and LNCaP-FGC-10 cell lines, resorcinol staining revealed the presence of GM1> GM2 > GM3. Other gangliosides were not detectable in one-dimensional chromatograms.

Resorcinol staining of two-dimensional chromatograms identified GD1a as a common ganglioside in all CaP lines, including LNCaP-FGC (**Figure 1B**). GM1, GM2 and GM3 were also abundant, whereas disialo-gangliosides GD3 and GD2 were present in small amounts. A spot identical to GD1b was observed in PC-3 but not in DU 145 and LNCaP-FGC. A spot identical to GT1b was detected in all cell lines except LNCaP-FGC. Several additional spots could not be identified with standards, suggesting they may be unique to prostate cancer cells.

Immunochemical Characterization of Gangliosides. For specific identification of the gangliosides, one- and two-dimensional chromatograms were immunostained with murine monoclonal antibodies specific for each ganglioside except GM3, for which no antibody exhibited specificity. All the lots of the monoclonal antibodies were used after testing for their specificity by assessing their relative reactivity to all the gangliosides on ELISA (22)(data not shown).

KM696, the monoclonal specific for GM2, stained a single fraction in PrEC cells (**Figure 2A**) and two distinct spots in the 2-dimensional chromatograms of all four CaP lines (**Figure 2B,** *arrows*). The weaker of the two spots could be a GM2 with an altered fatty acid chain (change in length, double bonds, or hydroxyl groups) or altered sialic acid. Presence of GM2 in CaP cells is confirmed by both resorcinol and immunostaining. Using KM696 in immunohistochemistry, Zhang et al (10) have equally intensity in the immunostaining of GM2 in normal and neoplastic prostatic epithelial cells.

14.G2a (IgG2a), the monoclonal specific for GD2, stained 2-D chromatograms of all cell lines (**Figure 2C**). While 14.G2a stained only one spot in DU 145, it stained two distinct spots in PC-3 (one spot above GD2). The nature of the upper spot is not known. Presence of GD2 in CaP cells is confirmed by both resorcinol and immunostaining.

GD1a was identified by three monoclonal clones: GD1a-1, GD1a-2a, and GD1a-2b. All three antibodies stained one spot in 2-D chromatograms of DU 145 (**Figure 2D**). Only one of the clones was used to stain 2D-chromatograms of PC-3 and LNCaP-FGC. Results of resorcinol and immunostaining indicated that GD1a is a major ganglioside of all CaP cell lines (**Figure 2E**).

MB3.6, the monoclonal specific for GD3, was applied to one-dimensional chromatograms of all cell lines. Only DU 145 showed a faint staining. It appears that GD3 is not a major component of CaP cells.

Staining with murine monoclonal antibodies to GD1b (GGR12), GM1a (GMB16) and GT1b (GMR 5) failed to stain one-dimensional chromatograms of all cell lines (data not shown). Failure of GMB16 to stain GM1a is interesting because resorcinol-HCl stained GM1 intensely and the precursor of GD1a is GM1a. It is possible that GM1a may not occur in detectable quantity in CaP cells; instead there may be a variant of GM1 or neolacto- or Globo- series of a glycolipid that migrates similar to GM1, which remains to be identified.

Cell surface expression of Gangliosides. Gangliosides not identified in chromatograms of cell extracts may be detected by direct monitoring of the cell surface with ganglioside-specific monoclonal antibodies (26). **Figure 3** shows the expression of gangliosides on the surface of the four CaP cell lines. PrEC cells expressed low levels of gangliosides on the cell surface; the only detectable

ganglioside in PrEC cells was GM1a, which was also found in low levels on CaP cell lines. Cell-surface expression of GM2 varied as follows: LNCaP-FGC > DU 145 > PC-3 > LNCaP-FGC-10. GD1a was found prominently on the cell surface of DU 145, less on PC-3 and not detectable on the cell surface of other CaP cells. GD1b and GT1b, which were not detected by HPTLC immunostaining, were located on the surface of all CaP cell lines. GD2 was expressed on the cell surface of CaP cell lines. GD1a was the most abundant ganglioside in CaP cells, followed in decreasing order by GM2, GM1 variant, GD2, and GM3.

Endogenous IgM response to Gangliosides. Having established the ganglioside profile of Cap cells, we have studied the profile of antiganglioside IgM antibodies in the sera of untreated CaP (T stage) patients to find out whether there is any evidence of endogenous immune response to tumor-associated gangliosides. Age of patients, biopsy Gleason score, and serum levels of PSA and TG are presented in **Table 1**. To compare the profile of antiganglioside IgM antibodies in sera of healthy controls and patients with BPH, confined CaP (substages T1 and T2) or unconfined CaP (substages T3 and T4), titers derived from ELISA analyses were converted to natural log titers; mean and median values were calculated (**Table 2A**). Since these values were compatible with the assumptions of the normal distribution and common variances, ANOVA was done. Two-tailed p values showed that anti-GM1, anti-GM2, anti-GM3, anti-GD1b and GT1b titers did not vary among the four groups; however there were significant differences in anti-GD2 and, to a lesser extent, anti-GD1a and anti-GD3 (**Table 2A**). The LSD method used for pair-wise comparison showed that the log titers of anti-GD3 IgM were significantly lower in BPH and CaP groups than in controls. Log titers of anti-GD2 and anti-GD1a IgM were similar between control and BPH groups. Log titers of anti-GD1a is significantly higher in confined CaP group than in control ($p = 0.018$), BPH ($p = 0.021$) and unconfined CaP group ($p = 0.019$). Log titers of anti-GD2 in BPH did not differ from control, however the titers in CaP groups (both confined, $p = 0.001$; and unconfined, $p = 0.006$) are significantly higher than that of the control (**Table 2B**).

DISCUSSION

This is a preliminary effort to identify the endogenous immune response to gangliosides in patients with CaP and relate it with the CaP-associated gangliosides. This study, if validated, may help to develop a reliable immunologic marker for early detection of confined CaP. The strength and limitations of this study are discussed as follows. This study relies on two broad and basic tenets. First, the tumorigenesis may involve the biosynthesis of ceramides (27), which mediate apoptosis. The tumor cells escape from ceramide-mediated apoptosis by glycosylating ceramide (27-30) and storing them as sialyllactosylceramides or gangliosides. Second, the nature and distribution of gangliosides differ between normal and neoplastic cells, and also varies according to the type of cancer (1). For example, GM3 is the dominant ganglioside in melanocytes, whereas GD3 is the dominant ganglioside in melanoma cells (1-4). In contrast to melanoma, the pancreatic adenocarcinoma and hepatic metastases of colorectal carcinomas are devoid of GD3 but overexpresses GM2, GD1b and GT1b (5, 6 16).

Ganglioside profiles of normal and neoplastic prostate epithelial cells.

This investigation adds additional information to the previous knowledge of ganglioside profile of normal and neoplastic prostatic epithelial cells. Shiraishi et al (8) analyzed gangliosides from surgical samples of benign human prostate tissue by chemical, enzymatic and immunostaining procedures. The monosialoganglioside fraction contained GM3 and GM1 plus multiple species of monosialylated lactosamine. The disialoganglioside fraction contained GD3 as the major component plus GD1a, GD2 and GD1b. GT1b was the major trisialoganglioside. Satoh et al (9) reported that the expression patterns of glycolipid from prostatic hyperplasia, prostatic cancer and normal prostate tissues. In cancer tissue, both of the lactosyl and globoside series glycolipids were found to be generally reduced, although in the ganglioside series, GM3 and GD3 were not reduced and only the glycolipids with longer chains than GD2 were found to be reduced. These results indicated that the inhibition of sugar chain elongation, but not sialylation, was the main synthetic change occurring with carcinogenesis of the human prostate. The present investigation confirms that gangliosides GM1a, GD1b and GT1b are not detectable in the CaP cells. However, GD1a and GD2 are found in the CaP cell lines. Previous studies on the analyses prostate tissue ganglioside expression have been restricted to the use of resorcinol-HCl staining of one-dimensional chromatograms and/or selected immunostaining (8, 9). However, one-dimensional chromatograms cannot identify several long-chain gangliosides including GD1a. The present study identified GD1a in CaP cell lines by staining two-dimensional chromatograms with resorcinol-HCl and several monospecific monoclonal antibodies. Although resorcinol-staining showed that all CaP cell lines contained abundant GM3, GM2 and GM1 (monosialogangliosides), immunostaining with GMB16 revealed that the intense resorcinol-positive GM1 fraction was not GM1a; Since it is resorcinol positive it could be GM1b or ganglioside belonging to non-ganglioside series such as globo series or neolacto series as reported earlier (8, 9). Further characterization of this fraction is required.

The most abundant gangliosides in CaP cells were GM3, GM2, GD2 and GD1a. High expression of GM2 in cancer cell lines is not surprising, since Zhang et al (1998) have shown that GM2 is found in abundance (4+ in immunohistochemically with KM696) both in normal prostatic epithelial cells and in primary and metastatic prostate cancer cells. Earlier investigators have noted that N-acetyl GalNAc-transferase (GM2-synthase) activity is more pronounced under tissue culture conditions (31) and may contribute to overexpression of GM2 in cell grown in culture. Work of Zhang et al (10) has clearly eliminated that possibility by showing that the normal and prostate epithelial cells obtained from biopsies, intensely stained with KM696.

Endogenous immune response to gangliosides.

Additional strength for this investigation emanates form the finding on CaP patients elicits endogenous antiganglioside IgM response. The foundation for this observation is based on our previous reports (5, 16-18). Gangliosides are T-cell independent antigens that do not require T-cell help to produce antibodies (34). Antiganglioside antibodies are invariably IgM in human and they occur at low levels in healthy individuals (5, 17). Cryosurgical ablation of liver metastases in patients with colon carcinomas (16) increased the serum level of total gangliosides. Interestingly,

this increase was followed by an increase in IgM titers against GM2, GD1b and GT1b (gangliosides found on colorectal cancer cells that metastasize to the liver) but not against GM3 and GM1 (gangliosides found on normal liver cells). Observations in patients with early sarcoma (18) further confirm that the gangliosides released from tumor cells can elicit endogenous IgM production without exogenous adjuvants.

Several reports support the contention that the gangliosides in the cytoplasm and on the cell surface are released into the tumor microenvironment and circulation (11-15). In pancreatic cancer, melanoma, and sarcoma, the level of serum gangliosides correlates with tumor burden (5, 15, 18). Circulating tumor-derived gangliosides can suppress a variety of cellular immune functions (32), possibly by interfering with cytokine function. For example, melanoma-associated GD1b binds to IL-2, an antitumor cytokine (33). In this study, we have observed that patients with confined CaP had elevated serum levels of IgM antibodies against GD1a, a prominent ganglioside of CaP cells with ability to induce T cells to produce IL10 (37), the cytokine-inhibiting cytokine. Possibly the augmentation of anti-GD1a IgM may represent the host's attempt to stop the immunosuppressive effects of GD1a in the tumor microenvironment and circulation. Matzinger (35, 36) proposed that self-antigens may act as danger or distress signals and induce an endogenous immune response.

The strength of this investigation lies in documentation of specific IgM antibodies against gangliosides in patients with organ-confined CaP. The double blinded nature of this investigation adds further strength to our observation that augmented response to gangliosides GD2 and GD1a are observed only in patient with CaP and IgM response to GD1a is restricted to patients with organ-confined CaP. Another noteworthy and significant finding of this investigation is the reduction in anti-GD3 antibody response in CaP patients, possibly reflecting either a decline in the level of expression of GD3 or decline the level of shedding of the ganglioside or lack of immunogenicity for tumor-associated GD3, as has been reported earlier (39, 40). Furthermore we observe that GM3 and GM2 the major gangliosides of CaP cells failed induced IgM response in patients with CaP. Probably the sample size of patients is too small to detect any significant differences in the endogenous immune response to GM3 or GM2. GM2 was prevalent in both normal and neoplastic CaP cells (see ref. 10) and is reportedly very immunogenic *in vivo* (38-41). Absence of a significant endogenous immune response to GM2 in CaP patients could be due to overexpression of GM2 both in normal cells and in CaP cells (10).

The titers of anti-GD2 IgM were increased in CaP patients with both confined and unconfined disease but not in healthy volunteers or in patients with BPH. The presence of anti-GD2 antibodies in CaP patients is not surprising because both resorcinol-HCl staining and immunostaining of one- and two-dimensional chromatograms with 14.G2a identified GD2 in CaP cells but not in normal epithelial cells. Moreover, GD2 is highly immunogenic in human cancers (40) and it is a target of both passive (42-44) and active specific immunotherapies (45). Whether the endogenous immune response to GD2 in CaP patients is protective remains to be investigated with a larger sample size including all stages of CaP.

Endogenous immune response to GD1a in patients with confined CaP.

The most interesting finding of this investigation is the increased production of endogenous IgM antibody against CaP-associated GD1a in the sera from patients with organ-confined CaP but not in sera from patients with unconfined CaP. It is possible that the expression of GD1a observed in stage T1 and T2 may be altered at subsequent stages of disease progression. Supportingly, Satoh et al (8) have observed the murine monoclonal antibody that binds to sialoglycosphinoglipids, APG1 showed intense reaction observed in the frozen tissue sections of human prostate decreased with the increasing grade of cancer, suggesting a change in the pattern of gangliosides during early phases of neoplastic differentiation and disease progression. Although the exact nature of the ganglioside that has decreased with increasing grade is not known, the observations on change in the anti-GD1a IgM response between confined and unconfined CaP suggests that GD1a may be the most prevalent ganglioside of the confined CaP and expression of GD1a would have declined with tumor progression. One the other hand anti-GD2 IgM response is observed both in confined and unconfined CaP suggesting that GD2 expression in confined and unconfined CaP might have remained unaltered during tumor progression.

IgM response in CaP patients: Limitations.

The significance of the augmentation of the antibodies to GD1a and GD2 can be better realized when considering the double-blind nature of this study, particularly in the absence of any difference in the log titers for other antiganglioside IgM antibodies. However, a larger sample size of organ-confined and unconfined patients who have not received any treatment is critical to derive solid conclusions. If the endogenous IgM response to CaP-associated GD1a is an early immunological event of tumorigenesis, this may represent an early humoral response to cancer cells still contained within the prostate. The antiganglioside IgM antibody response is intriguing because the levels of antiganglioside antibodies in healthy individuals tend to decline with age and remain low after the age of 50 (17). One of the major limitations of this study is the availability of cell lines from organ-confined CaP. Although we have relied on ganglioside profiles of ATCC cell lines, there is a need for a detailed immunohistochemical characterization of gangliosides in CaP cells from tumor biopsies to validate our observations. Comparing the profiles of gangliosides from tumor biopsies and antiganglioside IgM antibodies between primary and metastatic CaP may provide a better understanding of the host response to tumorigenesis.

Conclusion

This preliminary observations point out that a humoral immune response to a tumor-ganglioside represents a valuable tool to understand the biological changes associated with tumor progression particularly since it is not possible to detect the quantity of the specific tumor-gangliosides shed into the circulation. We have used a sensitive ELISA protocol for antiganglioside IgM in a blinded fashion, which has been validated for screening sera of melanoma patients receiving a cancer vaccine in a Phase III multicenter clinical trial conducted at JWCI (45). Using the same assay, we have observed a significantly ($p<0.025$) higher level of anti-GD1a IgM in the sera from patients with organ-confined CaP than from controls and patients with BPH and those with unconfined patients. The unique endogenous immune response to GD1a and

GD2, in the absence of IgM response for other tumor-associated gangliosides, suggests that it could possibly serve as immunologic marker biologically relevant to neoplastic transformation of prostate. A similar study is undertaken in a larger patient population to validate the findings.

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Table 1 Age, diagnosis, stage and grade of CaP, PSA and total Gangliosides in the cohorts of patients.

Serial Number – Age /ID number	Diagnosis or Stage/ Grade	Serum PSA (ng/ml)	Serum total Ggs (mg/dL)
1-46 /38	BPH	0.36	12.8
2-53 /02	BPH	0.68	13.4
3-76 /21	BPH	n/a	10.8
4-75 /42	BPH	4.30	12.6
5-73 /03	BPH	1.50	10.4
6-72 /15	BPH	5.19	8.7
7-75 /109	BPH	1.40	13.2
8-82 /84	BPH	2.40	15.1
9-64 /24	BPH	4.57	15.4
10-67 /110	BPH	7.70	14.9
11-68 /20	T1c/ 3/3	8.75	13.0
12-74 /22	T1c/ 3/3	n/a	10.6
13-60 /28	T1c/ 3/3	6.70	21.6
14-73 /41	T1c/ 3/3	2.43	15.8
15-80 /06	T1c/ 3/3	3.70	13.4
16-68 /58	T1c/ 3/3	0.003	18.1
17-71 /73	T1c/ 3/3	5.70	13.1
18-78 /118	T1c/ 3/3	19.1	15.0
19-49 /131	T1c/ 3/3	3.2	16.5
20-80 /147	T1c/ 3/3	11.8	17.1
21-55 /193	T1c/ 3/3	5.8	24.3
22-69 /47	T1c/ 4/4	n/a	12.3
23-85 /01	T1c/ 4/4	3.20	18.4
24-77 /10	T2a/ 3/3	3.95	17.4
25-62 /33	T2a/ 3/3	6.58	18.9
26-62 /117	T2a/ 3/3	6.7	20.2
27-68 /194	T2a/ 3/4	2.1	18.1
28-77 /178	T2a/ 4/4	5.5	15.8
29-67 /68	T2b/ 3/3	n/a	12.3
30-64 /09	T2b/ 3/4	n/a	16.5
31-80 /48	T3/ 3/4	16.3	16.3
32-71 /59	T3/ 3/4	4.73	17.3
33-87 /46	T3/ 4/4	18.1	18.1
34-68 /36	T3/ 4/4	9.7	9.7
35-93 /187	T3/ 4/4	130	19.5
36-44 /11	T4/ 4/3	151	35.4
37-71 /124	T4/ 3/5	17.0	16.0

Table 2A. ANOVA assessment of p values shows significant differences in log titers of anti-GD3, anti-GD2 and anti-GD1a IgM antibodies in sera from healthy controls and patients with benign prostatic hyperplasia (BPH) or prostate cancer (CaP).

Log Titer	Healthy (n = 11)		BPH (n = 10)		T1/2 CaP (n = 20)		T3/4 CaP (n = 7)		ANOVA
	Mean ± SD	Med	Mean ± SD	Med	Mean ± SD	Med	Mean ± SD	Med	p value
GM1	4.48 ± 1.26	4.61	4.61 ± 0.00	4.61	5.28 ± 0.99	4.61	5.46 ± 1.17	5.70	0.065
GM2	5.93 ± 0.65	5.97	5.16 ± 0.99	4.61	5.16 ± 0.98	4.61	6.07 ± 1.55	5.99	0.073
GM3	4.43 ± 0.22	4.61	4.68 ± 0.22	4.61	5.19 ± 1.19	4.61	5.15 ± 1.25	4.61	0.181
GD3	5.45 ± 0.91	5.19	4.61 ± 0.00	4.61	4.78 ± 0.86	4.61	4.51 ± 0.27	4.61	**0.022**
GD2	4.18 ± 1.01	4.61	5.06 ± 0.77	4.61	5.62 ± 1.19	5.70	5.74 ± 1.37	6.21	**<0.007**
GD1a	4.90 ± 1.09	4.61	4.90 ± 0.63	4.61	5.74 ± 1.18	5.70	4.76 ± 0.58	4.61	**0.02**
GD1b	5.19 ± 1.09	5.60	4.88 ± 0.58	4.61	5.04 ± 1.07	4.61	5.74 ± 1.04	5.30	0.638
GT1b	5.91 ± 1.04	6.27	5.07 ± 0.83	4.61	5.75 ± 1.29	5.65	6.20 ± 1.24	6.55	0.181

Table 2B. Pairwise comparison by LSD method reveals that anti-GD1a IgM log titers distinguish between T1/2 and T3/4 CaP.

Pairwise Comparisons	P values		
	Anti-GD3	**Anti-GD2**	**Anti-GD1a**
Healthy *vs* BPH	**0.01***	0.076	0.992
Healthy *vs* T1/2 CaP	**0.016***	**0.001**	**0.018**
Healthy *vs* T3/4 CaP	**0.009***	**0.006**	0.756
BPH *vs* T1/2 CaP	0.546	0.205	**0.021**
BPH *vs* T3/4 CaP	0.757	0.224	0.767
T1/2 CaP *vs* T3/4 CaP	0.398	0.804	**0.019**

*lower than healthy volunteers

FIGURE LEGENDS

Fig. 1. Ganglioside profiles in normal PrEC and CaP cell lines stained by resorcinol-HCl. **A.** Unidimensional chromatograms shows the gangliosides profile of normal PrEC (Lane 1), PC-3 (Lane 2), DU 145 (Lane 3), LNCaP FGC-10 (Lane 4), and LNCaP FGC (Lane 5). The standards were bovine brain GM2 and GD1a (Lane 6) and bovine brain gangliosides (Lane 7). Each lane contains gangliosides-extract obtained from 25 million cells. The concentration of each ganglioside in the bovine brain standard is 3 nm. Upper horizontal line demarcates GM1 and lower horizontal line refers to the position of GD1a. The bottom line represents point of application. The solvent system is chloroform/methanol/0.2% $CaCl_2$ (v/v/v: 55/45/10). **B.** Two-dimensional chromatograms compare all cell lines except LNCaP FGC with the standard bovine brain gangliosides run simultaneously. Each lane contains gangliosides extract obtained from 25 million cells. The concentration of each ganglioside in the bovine brain standard is 3 nm. Vertical line represents direction of flow of the first solvent system, chloroform/methanol/0.2% $CaCl_2$ (55/45/10, v/v/v). Horizontal line represents the direction of flow of the second solvent system, chloroform/methanol/2.5M NH_4OH in 0.25% KCl 50/40/10,v/v/v). GD1a and GM2 are the most prevalent gangliosides in all cell lines.

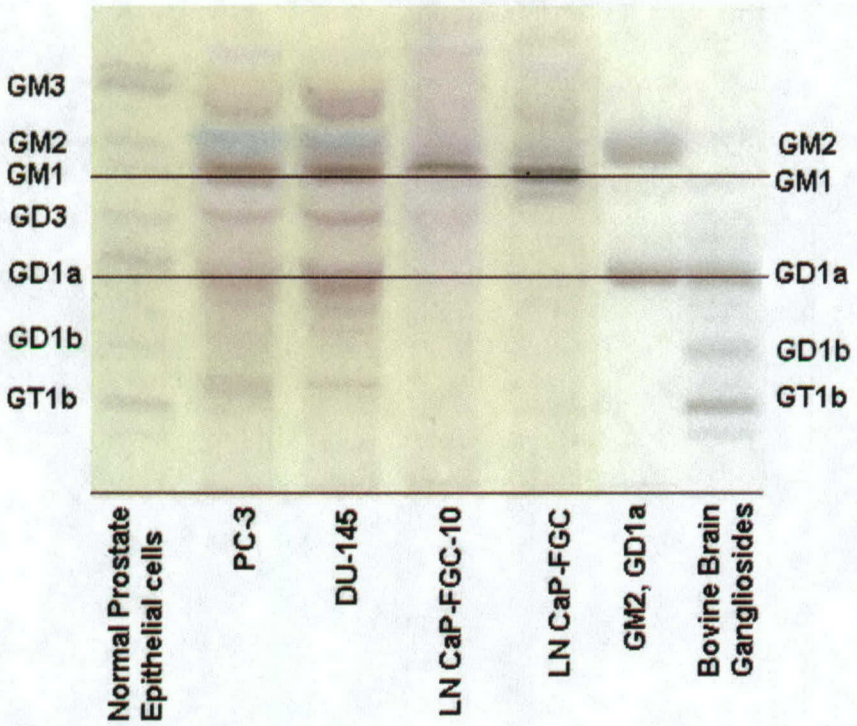
Fig. 2. Immunochemical characterization of ganglioside extracts from CaP cell lines and normal PrEC. **A.** Unidimensional chromatogram shows that PrEC extracts reacted with KM696 (murine monoclonal antibody specific for GM2) but not with GMB 16 (specific for GM1) or GMR 5 (specific for GT1b). Bovine brain standards, GM2, GM1 and GT1b reacted with all the three antibodies. **B.** Two-dimensional chromatogram shows that **KM696** recognized only GM2 in all cell lines. The two fractions of GM2 are indicated by a larger band (*right arrow)* and a weak band (*left arrow*). The solvent systems used for first and second runs are reported under Materials and Methods. **C.** Two-dimensional chromatogram identifies GD2 and 14.G2a-reactive gangliosides. Standard bovine gangliosides (3 nmol each) stained in resorcinol-HCl. Two fractions in PC-3, LNCaP FGC and one fraction in DU 145 were stained with 14.G2a. **D.** Two-dimensional chromatogram identifies GD1a. Standard bovine gangliosides (3 nmol each) and DU 145 stained in resorcinol-HCl. The three monoclonal antibodies (Seikagau America: Clones, GD1a-1, GD1a-2a and GD1a-2b) to GD1a stained GD1a distinctly in DU 145. The low reactivity of clone IgG2a suggests that the concentration of antibody may be low. **E.** Two-dimensional chromatogram identifies GD1a with Clone GD1a-1 (IgG1) in both PC-3 and LNCaP cell lines.

Fig. 3. Cell-surface expression of gangliosides on normal PrEC and CaP cell lines. The cell surface density of gangliosides (density of expression in Cs-ELISA) was comparable with total fluorescence intensity (TFI) (mean FI x number of events) (25). For each cell line, analyses were done in triplicate and negative controls included treatment of cells with non-specific isotype of each monoclonal antibody (IgM, IgG1, IgG2a, IgG3). Reactivity of the isotypes with cell surface is negligible. GM3 was not tested since no specific antibody is commercially available. Normal epithelial cells

failed to express gangliosides other than GM1a, but GM1a expression was highly negligible in all cell lines. All CaP lines expressed GM2 on the cell surface; GM2 expression was as follows: LNCaP FGC > DU 145 > PC-3 > LNCaP FGC-10. N-glycolyl GM3 and GD3 were negligible. GD1a was the most prominent ganglioside in DU 145 > PC-3. Cell-surface expression of GD1a was low in other cell lines, particularly in LNCaP-FGC and LNCaP-FGC10. All CaP cells expressed low levels of GD1b and GT1b on the cell surface.

FIGURE 1

A


GM3
GM2
GM1
GD3
GD1a
GD1b
GT1b
GM2
GM1
GD1a
GD1b
GT1b
Normal Prostate Epithelial cells
PC-3
DU-145
LN CaP-FGC-10
LN CaP-FGC
GM2, GD1a
Bovine Brain Gangliosides

B

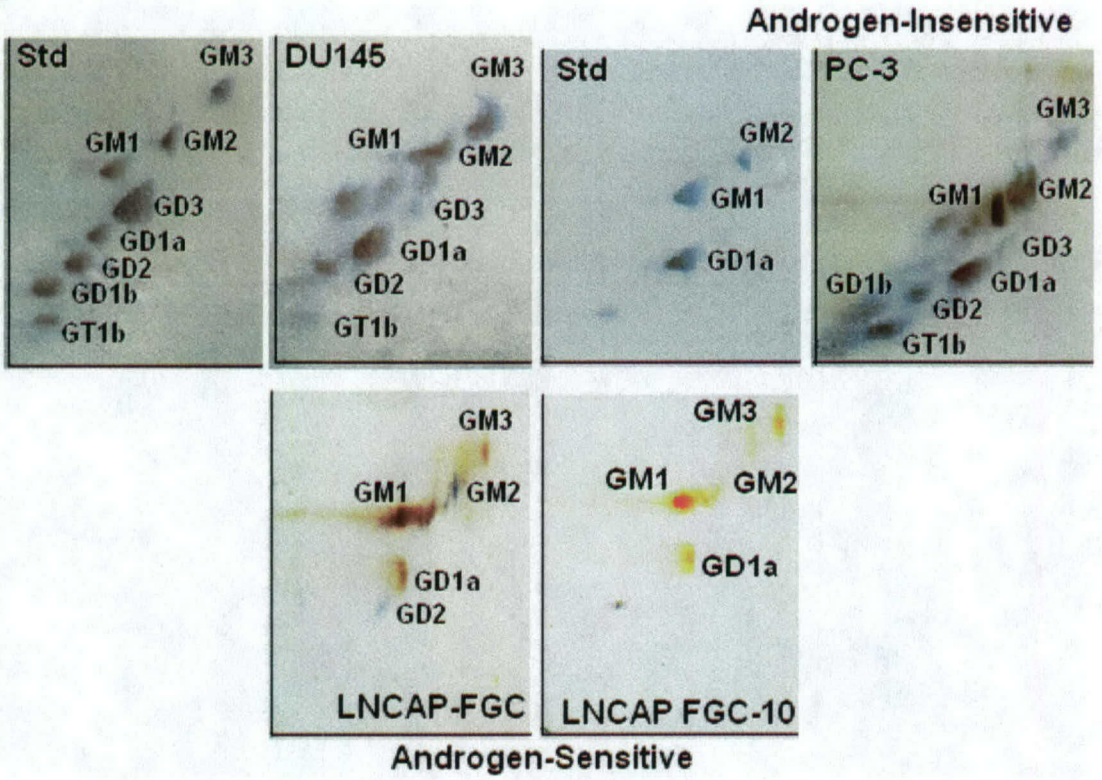

Androgen-Insensitive
Std
GM3
GM1
GM2
GD3
GD1a
GD2
GD1b
GT1b
DU145
GM3
GM1
GM2
GD3
GD1a
GD2
GT1b
Std
GM2
GM1
GD1a
PC-3
GM3
GM1
GM2
GD3
GD1b
GD1a
GD2
GT1b
GM3
GM1
GM2
GD1a
GD2
LNCAP-FGC
GM3
GM1
GM2
GD1a
LNCAP FGC-10
Androgen-Sensitive

FIGURE 2

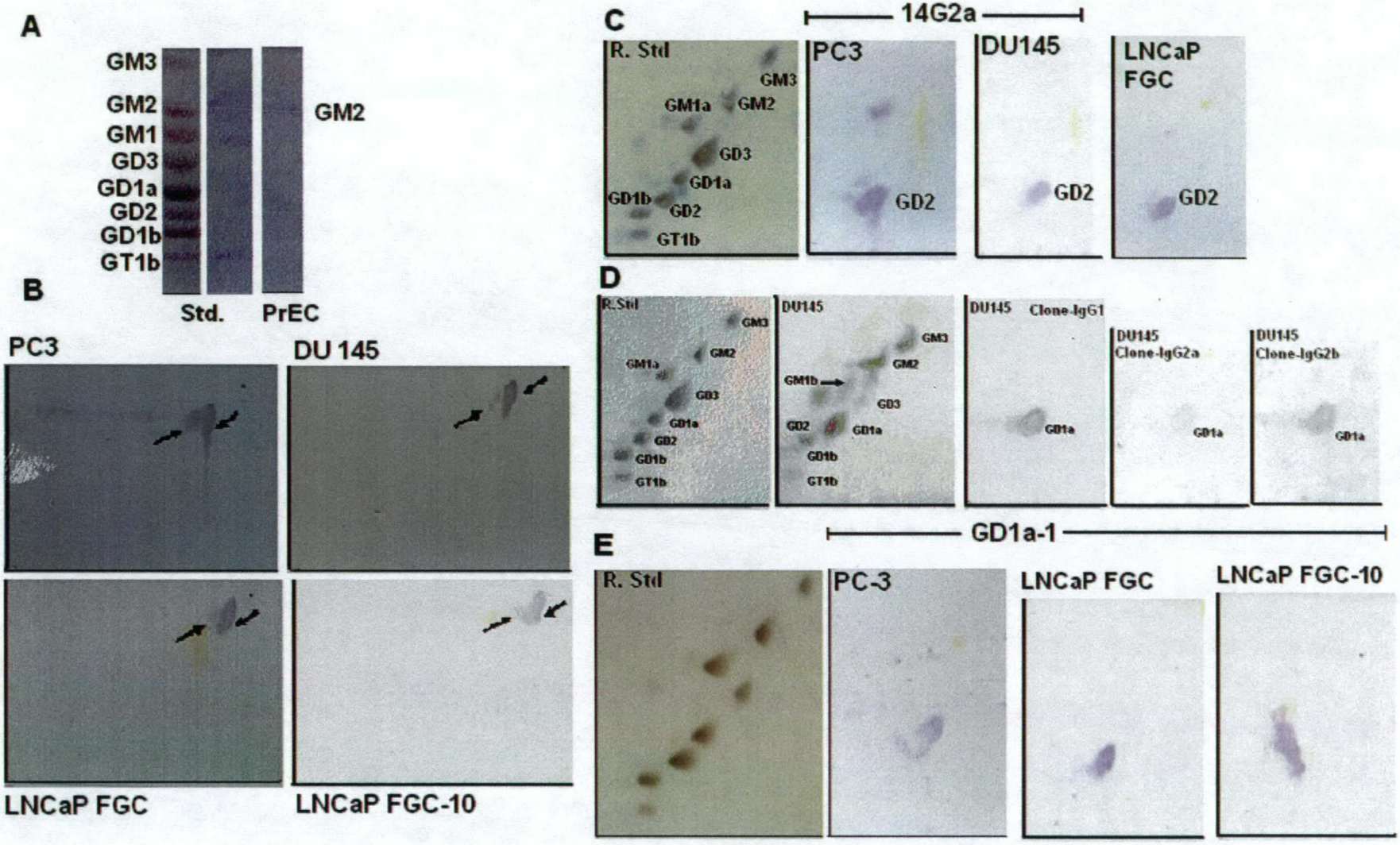
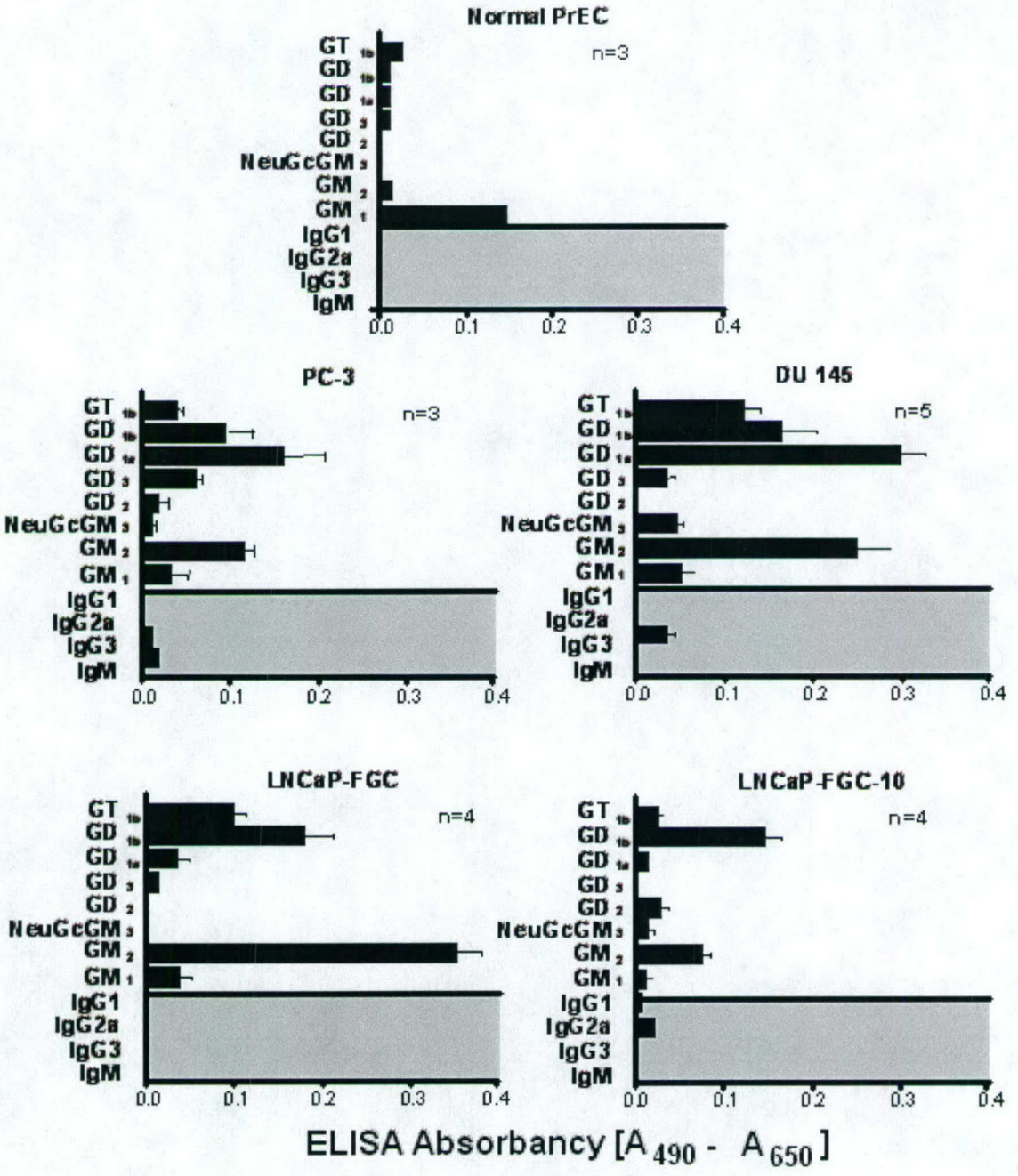

A
GM3
GM2
GM1
GD3
GD1a
GD2
GD1b
GT1b
GM2
Std.
PrEC
B
PC3
DU 145
LNCaP FGC
LNCaP FGC-10
C
14G2a
R. Std
GM3
GM1a
GM2
GD3
GD1a
GD1b
GD2
GT1b
PC3
GD2
DU145
GD2
LNCaP FGC
GD2
D
R.Std
DU145
DU145 Clone-IgG1
GD1a
DU145 Clone-IgG2a
GD1a
DU145 Clone-IgG2b
GD1a
E
GD1a-1
R. Std
PC-3
LNCaP FGC
LNCaP FGC-10

FIGURE 3


Normal PrEC
n=3
PC-3
n=3
DU 145
n=5
LNCaP-FGC
n=4
LNCaP-FGC-10
n=4
GT 1b
GD 1b
GD 1a
GD 3
GD 2
NeuGcGM 3
GM 2
GM 1
IgG1
IgG2a
IgG3
IgM
0.0
0.1
0.2
0.3
0.4
ELISA Absorbancy [A 490 - A 650]

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Evidence for IgM response to GD1a and GT1b in patients with early stage Prostate carcinoma and Melanoma

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Using specific monoclonal antibodies to measure cell surface density of different gangliosides in human prostate carcinoma cell lines with ELISA system, we have examined the expression of gangliosides in five prostate cancer cell lines. Most of the cell lines expressed high density of GM2, GD1a, GD1b and GT1b on the tumor cell surface. We have screened the sera of 14 prostate cancer patients (TNM stage T1c) for antibodies against GM3, GM2, GM1, GD3, GD2, GD1b, GD1a and GT1b using a sensitive ELISA. None of the patients showed IgG antibodies to any of the gangliosides. While the IgM titers of GD1a and GT1b were very high and ranged between 400 and 6400, the IgM titers against other gangliosides remained low, suggesting that the major prostate carcinoma-associated gangliosides GD1a and GT1b signaled antibody production in these patients. Although GM2 and GD1b are found on tumor cell surface, the serum titers against these gangliosides were low in most of the patients. Sera of Stage III melanoma patients were used as positive controls, which showed high titers of IgM to GD3, GD2, GM2 and GD1b.

While IgG antibodies to gangliosides are low or negligible, the profiles of IgM antibodies are most prevalent in the sera of melanoma patients (TNM stages T1a/b & T2a/b). The titers were high only for anti-GD1a and anti-GT1b IgM antibodies. Although GD1a and GT1b were reported in melanoma tumor biopsies and cell lines, the antibody response in early stage of the disease was intriguing. We have compared the serum anti-GD1a and GT1b IgM titers of the patients who had recurrent disease and expired (EXP) after surgical resection of the primary (median survival time 23.9 months) with those who are alive and have no evidence of disease (NED) (median follow up time 203.4 months) within 6 months after surgery. We found that the titers of anti-GD1a IgM ($p < 0.01$) and anti-GT1b IgM ($p< 0.01$) were significantly higher in patients who expired due to recurrent disease as compared to those with NED, suggesting that anti-GD1a and anti-GT1b IgM antibodies are poor prognosticators of stage T1 &T2 melanoma with or without ulceration.

In this study, we have identified, for the first time, the gangliosides GD1a and GT1b as important immunogenic gangliosides of early stages of prostate cancer and melanoma. GD1a and GT1b are known as potent immunosuppressive gangliosides, the former induces production of IL-10. Probably, IgM antibodies are produced during early stages of the disease to clear them from circulation to prevent immunosuppression.
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Neoplastic transformation changes the ganglioside profile of prostatic epithelial cells.

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Neoplastic transformation changes the expression of cell-surface gangliosides in melanoma and possibly in other cancers of epithelial origin, such as prostate cancer. We compared the ganglioside profiles of a normal prostatic epithelial cell line with five prostate cancer cell lines. All tumor cell lines were grown in RPMI with 10% fetal calf serum. The density of gangliosides in methanol/chloroform extracts of these cells was assessed by colorimetric resorcinol testing and by immunostaining of uni- and bi-dimensional thin-layer chromatograms. The density of gangliosides on the surface of these cells was assessed by cell-suspension enzyme-linked immunosorbent assay. In normal prostate epithelial cells, GM1a, GM2 and GM3 were the predominant gangliosides in cell extracts and on the cell surface, but their levels were highly negligible. Thin layer chromatography and immunoassaying of the cell extracts of prostate cancer cell lines PC-3, DU-145 and HH-870 indicated the presence of gangliosides comparable in their mobilities to GM1b, GD1a, GD1c, GD1-alpha (?), GD3, GM2, GM3, and GD2, listed in order of decreasing concentration. The surface of these cell lines expressed GD1a, GM2, GD1b, and GT1b, listed in order of decreasing density. The surface of HH-870 cells also expressed GM1a and GD2. The ganglioside profile of LNCaP-FGC/FGC-10 prostate cancer cell line was more limited; GM1b, GM2, GD1a and GD1b are found in the extract, whereas only GD1b and GM2 were predominant on the cell surface. These findings indicate that the major gangliosides of prostate cancer are GM1b, GD1a, GD1c, GD1-alpha (?), GD2, GM2, and GD1b. Since the expression of gangliosides GM2 and GD2 increased when biopsied melanoma cells are grown in culture, the high level of GM2 and GD2 in our cultured prostate cancer cell lines could be an *in-vitro* effect. Thus it appears that GM1b, GD1a, GD1c, GD1α, GD1b and GT1b are the gangliosides most likely to be found in prostate cancer cells. Presence of GM1b in prostate cancer cell lines suggest that GM1b may be the precursor of GD1c and GD1a, which in turn is the probable precursor of GD1b. Analysis of ganglioside profiles of prostate biopsy is needed to confirm these findings and identify those ganglioside antigens that are most important for diagnosis and/or immunotherapy of prostate cancer. This study is supported by Department of the US Army Grant No. DAMD17-01-1-0062.

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Endogenous IgM Response to Tumor-associated Ganglioside GD1a in Patients with Prostate Cancer or Melanoma

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Gangliosides are T-cell independent self-antigens found on normal cells; the serum of healthy volunteers contains low levels of antiganglioside IgM antibodies. Because gangliosides are overexpressed on tumor cells, an elevated endogenous antiganglioside response could signal subclinical metastatic progression of neoplastic disease. To test this hypothesis, a sensitive ELISA was used to screen the sera of patients with prostate cancer (CaP) or melanoma. The CaP study included 10 patients with benign prostatic hyperplasia (BPH), 11 with confined CaP (stages T1/2), 15 with unconfined CaP (stage T3), and 11 age-frequency matched healthy volunteers. The melanoma study included 75 patients with clinically localized, histopathlogically node negative melanoma (Breslow 1-2 mm) and 33 age- and sex-matched healthy controls. Log titers of IgM against eight gangliosides were assessed in a blinded fashion. In CaP patients, ANOVA showed significantly elevated IgM titers against GD2, GD1a and GT1b but not against other gangliosides. Pairwise comparison identified a significant anti-GD1a IgM response in patients with confined CaP; because this response was absent or weak in all other groups, anti-GD1a IgM might be a marker for confined CaP. In the melanoma study, GD1a IgM titer was significantly higher ($p<0.02$) when Breslow thickness was <1.5 mm. The reverse was true for GD3 ($p<0.02$). Thus tumor-derived GD1a might be the first alarm signal to the immune system; as the primary melanoma metastasizes, the signal might shift to GD3. Findings of both studies indicate that the immune system recognizes tumor gangliosides as danger signals and generates an endogenous immune response. Supported by DAMD17-01-1-0062, NIH CA 107831, NCI CA 29605.