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PRINCIPAL INVESTIGATOR: Ann E. Stapleton, M.D.

CONTRACTING ORGANIZATION: University of Washington
Seattle, Washington 98105-6613

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Urinary tract infections (UTIs), generally caused by Escherichia coli or Staphylococcus saprophyticus, are extremely common among young women. Although UTIs can be treated, we currently lack effective means to prevent frequently UTIs, which occur in 25% of women with first UTI. A necessary prerequisite to UTI is adherence of uropathogens to the vaginal and bladder epithelium. This report describes the fourth year of progress in a project that defines uropathogenic E. coli and S. saprophyticus-binding glycosphingolipids (GSLs) in the vaginal and bladder epithelium, shown in preliminary studies to function as bacterial receptors, as a prerequisite to the rational design of new agents that will prevent colonization and infection in women. Key progress includes: (a) establishment of in vitro models of primary cultured bladder epithelial and human vaginal epithelial cells; (b) characterization of GSLs expressed by these epithelial cells and of bacterial adherence to them; (c) studies of the effects of exogenous estrogen on GSL and keratin expression and bacterial adherence; (d) cloning of a human α1-4Galactosyltransferase; and (e) enzymatic synthesis of globoseries based GSL compounds.
## 4. TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>ITEM</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRONT COVER</td>
<td>2</td>
</tr>
<tr>
<td>REPORT DOCUMENTATION PAGE</td>
<td>2</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>3</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>4</td>
</tr>
<tr>
<td>BODY</td>
<td>6</td>
</tr>
<tr>
<td>KEY RESEARCH ACCOMPLISHMENTS</td>
<td>20</td>
</tr>
<tr>
<td>REPORTABLE OUTCOMES</td>
<td>20</td>
</tr>
<tr>
<td>CONCLUSIONS</td>
<td>21</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>22</td>
</tr>
<tr>
<td>APPENDICES</td>
<td>23</td>
</tr>
</tbody>
</table>

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5. INTRODUCTION

a. Overview

The overall purpose of this project is to investigate interactions between bacteria which commonly cause urinary tract infection (UTI) and their cognate host cell receptors in the vaginal and bladder epithelium in order to design novel, non-antibiotic methods for preventing UTIs. The project is focused on studying the two most common uropathogens causing UTI in young women, *Escherichia coli* and *Staphylococcus saprophyticus*, as well as their interactions with glycosphingolipids (GSLs) on the cell surface of the bladder and vagina. In the first three years of this project, we will define the key GSLs on the eukaryotic cell surface that uropathogenic bacteria use for attachment and then in the last two years, we will take advantage of new biochemical techniques using carbohydrate mimetics to design UTI prevention methods that avoid the induction of antimicrobial resistance. This report describes progress made in the fourth year of this grant. It is an annual report because the project is presently in a no-cost extension period.

b. Background presented in original proposal

Acute uncomplicated UTIs caused by *E. coli* and *S. saprophyticus* occur in an estimated 7 million young women each year at an annual cost for diagnosis and treatment exceeding one billion dollars. Over half of all women have had a bacterial UTI by their late 20's and approximately 20% of women with UTI suffer very frequent (≥ 3/year) recurrences (1). Nonetheless, the only currently available preventive modality for these recurrent infections is antimicrobial prophylaxis. Though effective, antimicrobial prophylaxis may promote the emergence of drug-resistant strains (1). In addition, women typically revert to having frequent recurrences once prophylaxis ceases and little is known about why some women suffer frequent recurrences of UTI, since this phenomenon cannot usually be explained by underlying functional or anatomic abnormalities of the urinary tract (1). The interaction of infecting bacterial strains with the women's epithelial cells appears to be a critical point in the infectious process that determines host susceptibility, in particular the availability and nature of host cell bacterial ligands such as GSLs (2-4).

GSLs are important components of the glycocalyx surrounding mammalian cells and consist of an oligosaccharide moiety exposed on the cell surface, to which organisms attach, covalently linked to a lipid portion embedded in the outer leaflet of the plasma membrane. They serve as eukaryotic cell adhesion sites for many pathogens and their toxins, including *E. coli*, *Pseudomonas aeruginosa*, *Helicobacter pylori*, HIV, parovirus, rotavirus, cholera toxin, verotoxin of *E. coli* 0157, and others (5-9). Based on the structures of their carbohydrate components, they are grouped into families, such as the lacto- and neolacto-series, the globoseries, and the ganglioseries GSLs. GSLs are synthesized by the sequential action of glycosyltransferases, many of which are tissue-specific and/or genetically determined (10). Thus, GSLs on the cell surface play an important role both in determining tissue tropism and an individual host's susceptibility to specific infectious diseases.

Among uropathogenic *E. coli* isolates expressing the pap-encoded family of adhesins are significantly overrepresented among strains collected from patients with UTI as compared with fecal isolates from patients without UTI (11). The GSL receptors for these adhesins in the kidney are the globoseries GSL family that contain a minimal receptor consisting of a galactose -1-4 galactose moiety (11). Although *S. saprophyticus* is the second most common cause of UTI, to our knowledge, we are the first to actually investigate whether it binds to GSLs. In our grant proposal, we showed preliminary data demonstrating that the wild-type *S. saprophyticus* strain ST352 binds asialo GM1 (ASGM1), a neutral ganglioseries GSL and that other wild-type *S. saprophyticus* isolates bind to ASGM1 and/or structurally-related ganglioseries GSLs. Paradoxically, while globoseries GSLs have been identified in kidney tissue and vaginal epithelium, the bladder has been little studied with respect to GSLs, despite the fact that it is the most common site of UTI. Previous studies of GSLs in native bladder tissues have focused on oncogenesis or development and have not included GSLs that are directly involved in adhesion of uropathogens (ganglioseries and globoseries GSLs). Of note, other urogenital pathogens, including C.
albicans, C. trachomatis, N. gonorrhoeae, have also been reported to bind to ASGM-1 and it is possible
that a single class of inhibitors could prevent adherence and infection with all of these agents.

c. Culture and characterization of vaginal epithelial cells

The native vaginal epithelium in vivo and in vitro

As a mucosal surface, the vagina serves as a barrier to local infection, but since it is situated at the entrance
to the upper genital tract, it also serves protective functions. It is a non-keratinized, stratified squamous
epithelium lacking glands. Based on cellular morphology by light microscopy, the epithelium is divided
into several layers: the basal/proliferative, parabasal, intermediate, and superficial layers. A complex
ecosystem exists within the vagina of post-menarchal women, consisting of at least 50 species of bacteria,
but dominated by lactobacilli, which appear to have a protective effect in this microniche (2, 7, 18).
Conversely, increased vaginal colonization with E. coli in young, otherwise healthy women is associated
with increased risk of UTI (45), and during pregnancy, colonization with E. coli is associated with
preterm birth and other perinatal complications (18, 34).

Hormonal influences such as the onset of menopause or the use of hormone replacement therapy is
associated with changes in thickness of the vaginal mucosa and a alterations in the proportion of cells
demonstrating basal and parabasal morphologies. There is a loss of lactobacillus colonization and an
increase in colonization with potential pathogens such as E. coli, Group B streptococcus, and enterococci
(38) However, perhaps because of the paucity of in vitro model systems, there have been few studies of
the effects of exogenous sex hormones on nearly any aspect of the basic biology of the vaginal epithelium,
including markers of differentiation, bacterial attachment and colonization with normal flora. As an
extension of the original aims of this proposal, we have been developing and characterizing a model
system of vaginal epithelium. When we first proposed these studies, we planned to use vaginal epithelial
cell cultures established by a collaborator. For practical reasons, we established our own culture system
subsequently and we then found that the literature contained little information about the biology of vaginal
epithelial cells grown in culture. Our efforts to systematically characterize our culture system in a manner
analogous to procedures used by keratinocyte biologists have led us to new avenues of investigation of
hormonal and other effects, as described in detail below.

Keratins in vaginal tissue

Our initial approach to characterizing the cultured vaginal epithelial cells has been to define keratin
expression in our cultured cell. Keratins are key markers of cell origin and differentiation in stratified
squamous epithelium. Their expression has been more thoroughly studied in other stratified squamous
epithelium such as the mouth. A review of these studies as well as studies of native human and mouse
vaginal tissue suggests patterns of keratin expression correlating with specific cell layers and with
morphological degrees of differentiation have been developed. K19, generally associated with epithelial
proliferation, has been found in basal layers of human vaginal epithelium (14). K13 has been used as a
marker of suprabasal cell layers and thus of differentiation (42, 43). In the mouse, K14 is found in all
layers of the epithelium and K1 was not expressed in undifferentiated epithelium but was upregulated in
response to exogenous estrogen (15). Human vagina stained in all layers for K10, usually associated with
the epidermis in the skin (14).

d. Brief summary of preliminary data presented in original proposal

Our original proposal presented preliminary data demonstrating that primary cultures of human bladder
epithelial cells are a promising model system for the study of bladder GSLs in the pathogenesis of UTI
caused by E. coli or S. saprophyticus. Specifically, we showed that these cell cultures appear to express
globoseries GSLs, the host cell binding ligand for an important class of uropathogenic E. coli, those
expressing pap-encoded adhesins. We previously showed that SGG and other globoseries GSLs are
surface exposed in human kidney tissues and exfoliated vaginal epithelial cells (2). In our preliminary

(contains unpublished and/or proprietary information)
immunocytochemistry experiments with primary cultures of human uroepithelium, the cells demonstrated bright immunofluorescent staining with MAb ID4 directed against SGG, suggested that this epitope is also surface exposed on these bladder cells. We also showed that $S. saprophyticus$, the second most common cause of UTI in young women, binds to ganglioseries GSLs, especially ASGM1 and ASGM2. In addition, we demonstrated the presence of ASGM1 among GSLs extracted from human kidney and the surface exposure of this epitope in kidney sections in specific histological areas where bacteria also adhere. Another ganglioseries GSL, GM1, was identified among GSLs extracted from human kidney tissues and vaginal epithelial cells. ASGM1 appears to be surface exposed on both kidney and cultured primary bladder cell surfaces, as shown by positive immunofluorescent staining with MAb TKH-7, directed against ASGM1.

d. Originally proposed hypotheses

The original hypotheses of this project have been supported by data obtained during the first two years of the grant and thus remain unchanged, as listed below. The overall goal of this project remains to define the key eukaryotic cell surface GSLs that are used by uropathogenic bacteria for attachment and then to take advantage of new biochemical techniques utilizing carbohydrate mimetics to design novel means for preventing UTIs that avoid the use of antimicrobials.

(1) We hypothesize that globoseries and ganglioseries GSLs are present in primary cultures of bladder transitional epithelium and vaginal epithelium and serve as binding sites for $E. coli$ and $S. saprophyticus$, respectively.

(2) We hypothesize that the GSLs identified in the first hypothesis are surface exposed in primary cultures of bladder transitional epithelium and vaginal epithelium and are functionally relevant for $E. coli$ and $S. saprophyticus$ attachment and infection.

(3) We hypothesize that carbohydrate mimetic and synthesis techniques can be used to design high-affinity inhibitors of $E. coli$ and $S. saprophyticus$ binding to vaginal and bladder transitional epithelium.

e. Original technical objectives

During the third year of funding for this project, we did not alter any of our technical objectives, but we increased the emphasis on certain aspects of our objectives. We expanded previously established collaborations and developed new ones, some of which promise to open new areas of inquiry in the coming year. For some of the objectives, we used slightly different but more efficient technical approaches. These changes are discussed in greater detail below. Our technical objectives are as follows:

(1) We will extract and characterize GSLs that bind $E. coli$ or $S. saprophyticus$ from primary cultures of bladder transitional epithelium and vaginal epithelium, according to the following sequence: (a) purify the GSLs using high-performance liquid chromatography (HPLC); (b) identify bacteria-binding GSLs by overlaying radiolabeled isolates of $E. coli$ and $S. saprophyticus$ on these GSLs separated on high-performance thin-layer chromatography (HPTLC); (c) confirm the identities of these GSLs using specific monoclonal antibodies (MAbs) directed against the GSLs in HPTLC immunostaining assays; and (d) perform carbohydrate structural analysis on the bacteria-binding GSLs.

(2) To demonstrate that the GSLs identified in Hypothesis 1 are surface exposed in primary cultures of bladder transitional epithelium and vaginal epithelium and are functionally relevant for $E. coli$ and $S. saprophyticus$ attachment and infection, we will: (a) test representative isolates for adherence to primary bladder cell and vaginal epithelial cultures; (b) utilize immunofluorescence staining of the same cell cultures with MAbs directed against relevant GSLs; (c) repeat 2a and 2b after pretreatment of the cell cultures with an inhibitor of GSL receptor synthesis; and (d) repeat 2a after pretreatment of the cell cultures with the MAbs directed against relevant GSLs.

(contains unpublished and/or proprietary information)
(3) We will use carbohydrate mimetic techniques to design inhibitors of bacterial adherence, focusing initially on the interaction of \textit{E. coli} with sialosyl galactosyl globoside (SGG, a GSL to which \textit{E. coli} binds with high affinity; see preliminary data). We will test the inhibitory efficacy of the compounds in bacterial overlay assays and in bacterial adherence assays, as described in the second objective.

6. BODY OF REPORT

A. Overview

1. Review of changes and opportunities occurring in the first two years of funding:

(1) Establishment of a new collaboration with Dr. Steven Levery of the Complex Carbohydrate Research Center (CCRC) of The University of Georgia, Athens: We continue to work with Dr. Levery for assistance in structural characterization of GSLs (see results).

(2) Establishment of a collaboration with Drs. M. Juliana McElrath and Florian Hladik of the Fred Hutchison Cancer Research Institute and the University of Washington: Though this project has ended, the technical aspects and opportunities associated with this work has been replaced this new collaborations involving members of the Department of Obstetrics and Gynecology. (see below).

(3) Vaginal epithelial cell cultures: This aspect of the project has become a major focus of the project in the past year, through expansion of technical knowledge, development of new collaborations and protocols for tissue acquisition, and the development of new hypotheses. These are detailed below.

(4). New approaches taken by Dr. Stroud: Dr. Stroud has further expanded new approaches developed in the last funding period for accomplishing Technical Objective 3, detailed below.

2. New opportunities and approaches during the third year of funding:

1. Bladder cell cultures: we have begun collaborating with Dr. Richard Grady, Assistant Professor of Urology at the University of Washington and Children's Hospital and Regional Medical Center. Dr. Grady has assisted us in obtaining urothelial tissue and in augmenting our culture stocks.

2. Meetings with collaborators based outside Seattle:

a. Dr. Atala: Dr. Stapleton met with Dr. Atala in May 1999 and plans another meeting in November 1999.

b. Dr. Levery: Dr. Stroud met with Dr. Levery during several visits by Dr. Levery to Seattle.

c. Dr. Toyokuni: Dr. Stroud will meet with Dr. Toyokuni in November at a West Coast glycobiology meeting this fall.

3. New opportunities and approaches during the fourth year of funding:

1. We have initiated a collaborative effort with Dr Beverly Dale-Crunk, Professor of Oral Biology and Scientific Director of the Comprehensive Oral Research Center at the University of Washington to characterize keratin expression in cultured vaginal epithelial cells.

2. In collaboration with members of the Mass Spectrometry Laboratory in the Department of Medicinal Chemistry at the University of Washington, we have instituted the use of matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) technology to characterize GSLs isolated from bladder epithelial cells. The Mass Spectrometry Center is an instrument laboratory providing a wide variety of mass spectral services to the University, offering both analytical and instructional expertise in mass spectrometric

(contains unpublished and/or proprietary information)
techniques and instrumentation, with the primary aim of providing investigators with "hands-on" training in the use, as well as, ready access to the modern instrumentation necessary in carrying out their research goals.

4. Other issues: We are presently in a period of no-cost extension because of personnel issues that have left us with some of the objectives yet to be met in the coming year. Dr. Stroud's group moved its laboratory twice in the past year, a great inconvenience which caused him to lose considerable time. He is now housed in a newly renovated facility which will offer many advantages.

B. Original Statement of Work

The original technical objectives set for all four years of funding are listed below.

Technical Objective 1: Extract and characterize GSLs that bind E. coli or S. saprophyticus from primary cultures of bladder transitional and vaginal epithelium.

Task 1: Months 1 to 6: cultivation of primary cultures of bladder and vaginal epithelial cells
Task 2: Months 7 to 12: extraction of GSLs from bladder and vaginal cell cultures
Task 3: Months 7 to 12: bacterial overlay assays
Task 4: Months 7 to 12: immunostaining assays
Task 5: Months 13 to 24: carbohydrate structural analysis
Task 6: Months 25 to 36: data analysis and publication

Technical Objective 2: Demonstrate that the GSLs identified in Technical Objective 1 are surface exposed in primary cultures of bladder transitional epithelium and vaginal epithelium and are functionally relevant for E. coli and S. saprophyticus attachment and infection.

Task 1: Months 18 to 30: bacterial adherence assays to test representative isolates for adherence to primary bladder cell and vaginal epithelial cultures
Task 2: Months 18 to 30: immunocytology procedures utilizing immunofluorescence staining of the same cell cultures with MAbs directed against relevant GSLs
Task 3: Months 24 to 36: PDMP treatment of cell cultures, followed by GSL extraction and quantification and bacterial adherence assays
Task 4: Months 24 to 36: MAb pre-treatment, followed by GSL extraction and quantification and bacterial adherence assays
Task 5: Months 30 to 36: data analysis and publication

Technical Objective 3: Use carbohydrate mimetic techniques to design inhibitors of bacteria adherence, focusing initially on the interaction of E. coli with SGG; test the inhibitory efficacy of the compounds in bacterial overlay assays and in bacterial adherence assays.

Task 1: Months 24 to 42: synthesis of linear mimetic compounds
Task 2: Months 27 to 42: structural analysis of resulting compounds (procedures to begin as each new compound is prepared)
Task 3: Months 36 to 48: testing of inhibitory capacity of linear mimetic compounds in bacterial overlay assays, focusing on SGG as the target GSL to which binding should be inhibited

(contains unpublished and/or proprietary information)
Task 4: Months 36 to 48: testing of inhibitory capacity of linear mimetic compounds in bacterial adherence assays, using primary cultures of bladder and vaginal epithelial cells

Task 5: Months 30 to 48: synthesis of multivalent structures from linear structures that are effective in Tasks 3 and 4

Task 6: Months 40 to 48: repeating Tasks 3 and 4 using multivalent compounds

Task 7: Months 40 to 48: data analysis and publication

C. Details of Progress

1. Technical Objective 1

a. Task 1, Months 1 to 6: cultivation of primary cultures of bladder and vaginal epithelial cells

1. Experimental methods, assumptions and procedure

Primary cultures of human bladder epithelial cells were provided by Dr. Anthony Atala and maintained in serum free keratinocyte media using standard tissue culture techniques, as in our preliminary studies and as he has described (13). Briefly, cells were maintained in serum- and antibiotic-free keratinocyte medium and passed at 70% confluence in a ratio of 1:4 or 1:6 (13). Each time cells were passed, the number of flasks were maximally expanded to prepare cells for the purification of GSLs of interest. This procedure was continued with each cell sample until senescence was noted, usually at about passage 12.

In addition, we established primary cultures of bladder epithelial cells in our own facility, with the assistance of Dr. Richard Grady. To accomplish this, we adapted the methods developed for culture of primary vaginal epithelial cells, with one change. Specimens from bladder epithelium are generally processed in the operating room, rather than transporting them back to our facility first. Cultures are maintained as described above.

The collaboration we established with Drs. McElrath and Hladik during the first two years of funding resulted in our establishing a technique of maintaining primary cultures of vaginal epithelial cells in laboratory. The parent project for this collaboration has since ended, thus we replaced it with a project of our own. We added several new collaborators, listed below. Details of how these collaborations have assisted us in our investigations are noted in the results and recommendations sections for the relevant tasks below.

(1) Member of the Department of Obstetrics and Gynecology:

a. David Eschenbach, MD, Professor of Obstetrics and Gynecology, University of Washington, was a key contact person in the original project and has been a collaborator of ours on other projects for many years. He has continued to have provided discarded vaginal tissue specimens for use in our experiments. Along with Dr. Patton, he has consulted on issues regarding investigating estrogen stimulation of our primary vaginal epithelial cells lines, in order to better approximate the biology of the vaginal epithelium.

b. Dorothy Patton, PhD, Professor of Obstetrics and Gynecology and Adjunct Professor, Biological Structure and Ophthalmology, University of Washington. Dr. Patton has assisted us with issues specific to culturing vaginal epithelium and with immunohistostaining these tissues. Since she has years of prior experience with genital tissue-derived cultured cells, she has assisted us with assessing the effects of hormonally stimulating our vaginal epithelial cell cultures.

(contains unpublished and/or proprietary information)
c. Drs. Dee Fenner (Associate Professor) and Gretchen Lentz (Assistant Professor) have also assisted us with acquisition of appropriate discarded vaginal tissue for our studies and have also provided clinical correlation with hormone-related issues.

(2) Beverly Dale-Crunk, PhD, Professor of Oral Biology and Adjunct Professor, Medicine (Dermatology), Periodontics, and Biochemistry at the University of Washington. Dr. Dale has assisted us in determining markers of epithelial differentiation and in assessing epithelial morphological changes in response to estrogen during this past year of funding. We are in the early stages of preparing these data for publication.

2. Results and discussion

Primary bladder epithelial cells: We are continuing to culture primary bladder epithelial cells from samples obtained through Dr. Grady at Children's Hospital in Seattle, to augment our work with Dr. Atala. We have maintained our collaboration with Dr. Atala in order to compare the results obtained in our hands, using his techniques, with cell established in his laboratory, with his established methods. This funding year, we have processed 14 samples, with the following outcomes:

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<tr>
<td>5</td>
<td>Cell line established; pellets obtained for GSL extraction; stocks of these lines established in liquid nitrogen</td>
</tr>
<tr>
<td>5</td>
<td>Tissue never attached and was discarded</td>
</tr>
<tr>
<td>0</td>
<td>samples were combined with one another by accident and were discarded</td>
</tr>
<tr>
<td>2</td>
<td>Contaminated before establishment</td>
</tr>
<tr>
<td>2</td>
<td>recently established cultures; results pending</td>
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</table>

Primary vaginal epithelial cells: In the past year of funding, we processed 18 samples, with the following results:

<table>
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<th>Number of samples</th>
<th>Outcome</th>
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<tbody>
<tr>
<td>5</td>
<td>Cell line established; pellets obtained for GSL extraction; stocks of these lines established in liquid nitrogen</td>
</tr>
<tr>
<td>8</td>
<td>Cell line established, but insufficient material for storage of stocks</td>
</tr>
<tr>
<td>5</td>
<td>Contaminated before establishment</td>
</tr>
</tbody>
</table>

Further progress in the characterization of the largest vaginal epithelial cell pellet described last year is detailed below. We also describe characterization of GSLs extracted from a 6 ml pellet of bladder epithelial cells.

Details of technical progress in culture efforts are as follows:

a. General: Issues regarding seeding, avoidance of contamination, and eliminating fibroblast contamination: these techniques continue to be routine in the laboratory now, and no unusual problems have resulted this past year. We have also further adapted our methods for large surface area cultivation to obtain larger cell pellets of both types of cells for GSL characterization.

b. Cryopreservation: Our collection of cryopreserved cells has been steadily augmented, and methods for re-seeding frozen cultures have been maximized. We have been able to revive cultures preserved over one year ago.

(contains unpublished and/or proprietary information)
c. Characterization of cultured vaginal epithelial cells

**Immunohistostaining:** We used a standard immunohistostaining procedure to confirm the epithelial origin of our cultures. We tested cultures derived from five separate individuals at first to fifth passage by staining with a MAb (ascites) which stains all epithelial cells (PAN; Sigma) and with a MAb directed against fibroblasts (anti-fibroblast antibody; Sigma). We used HeLa cells and T24 bladder epithelial cells (ATCC) as positive controls for the PAN antibody staining. Pure cultures of fibroblasts from vaginal epithelium (derived from early, failed attempts at culturing VECs) as well as McCoy mouse fibroblast cells were used as positive controls in the anti-fibroblast antibody assays. Cultures of VEC cultures from all five individuals produced a strongly positive signal with the PAN MAb and no signal with the anti-fibroblast antibody.

**Characterization of differentiation markers:** To investigate the expression of keratins under the cell culture conditions previously investigated, we tested cells grown in SFM and PRF-SFM, with and without exogenous 17-β-estradiol, and differentiated by the addition of serum.

We have completed the first phase of our experiments performed in collaboration with Dr. Beverly Dale to characterize the expression of keratins in cultured vaginal epithelial cells; results are presented below.

**Keratins:** Based upon our discussions with Dr. Dale and our review of the literature, we investigated the expression of a panel of keratins that are predicted to stain differentially in cultured VECs or tissue sections, and/or their expression may be regulated by estrogen exposure: (a) K19, expected to be found expressed in basal cells; (b) K14, likely to be found in cells of varying degrees of differentiation; (c) K13, expected to be expressed in suprabasal cells; (d) K10, described as appearing in a mosaic pattern in native vaginal epithelium (28, 29); and (e) K1, a keratin whose expression is regulated by estrogen in vivo and which has also been seen in a mosaic pattern in native tissue (28, 29). We also have recently begun to study the expression of K6 and K16, usually associated with rapidly turning over epithelia, wound healing, growth in cell culture, and some hyperproliferative disorders of epidermis, and normally found in palatal and gingival epithelia.

**Protein extraction:** We extracted keratins from the V26 vaginal epithelial cell culture, using protein extraction methods designed to enrich for keratins to extract one of the VEC cell lines and performed Western blots using the PAN antibody. Briefly, equal volumes of cultured cells were harvested using trypsin, then cells were washed in PBS and extracted at 4°C in 6 M urea/10 mM Tris-HCl, pH 7.5, 1% Triton X-100, 5 mM EDTA, 5 mM dithiothreitol, and the protease inhibitor 1 mM phenylmethylsulfonyl fluoride (40). Extracts were stored at −20°C until use.

**SDS-Gel Electrophoresis and Immunoblotting:** One-dimensional SDS-polyacrylamide gradient gels (8-16%) were run according to Laemmli (41). Total protein concentration was determined using a commercial method (Biorad) and samples were normalized so that each lane was loaded with 15 µg protein. Samples were boiled for 5 min with 2% SDS and 10% 2-mercaptoethanol before loading for electrophoresis. Proteins were stained with Coomassie Brilliant Blue. Proteins from duplicate unstained gels were transferred electrophoretically to nitrocellulose membrane using a Bio-Rad Transblot apparatus (Bio-Rad Laboratories, for 1 h in Tris-glycine buffer, pH 7.5, with 20% methanol). Membranes were blocked for 1 h in Tris Buffered Saline with 0.05% Tween (TBST) containing 5% dried milk to block additional protein binding sites, then incubated sequentially in monoclonal antibody at 1: 100 to 1: 500 dilution overnight at +4°C, goat anti-mouse IgG and IgM (HRP conjugate, Pierce) at 1: 20 000 for 1 h at room temperature on a rocker platform, with buffer washes between each step. Immunoreactive proteins were visualized with enhanced chemiluminescence (ECL, Pierce, West Pico SuperSubstrate). The following antibodies were used:

(contains unpublished and/or proprietary information)
Antibodies directed against cytokeratins

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<th>Working dilution</th>
<th>Antigen Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone C-50</td>
<td>NeoMarkers</td>
<td>1:100</td>
<td>Cytokeratins 5,8 – simple and stratified epithelia, recognizes proteins of 58 kDa (5) and 52.5 kDa (8).</td>
</tr>
<tr>
<td>Clone KS-1A3</td>
<td>Novocastra</td>
<td>1:250</td>
<td>Cytokeratin 13-suprabasal marker (nonkeratinizing stratified squamous epithelial differentiation), acidic intermediate filament protein – 54 kDa.</td>
</tr>
<tr>
<td>RCK 108</td>
<td>ICN</td>
<td>1:100</td>
<td>Cytokeratin 19-simple epithelia as well as basal layer of nonkeratinizing stratified epithelia</td>
</tr>
<tr>
<td>Clone AE1/AE3</td>
<td>NeoMarkers</td>
<td>1:200</td>
<td>AE1- recognizes the 56.5, 50, 50, 48, and 40 kDa keratins of acidic subfamily. AE3- reacts with basic keratins of 65-67, 64, 59, 58, 56, and 52 kDa</td>
</tr>
<tr>
<td>LL001</td>
<td>Courtesy of Dr. B. A. Dale</td>
<td>1:200</td>
<td>Cytokeratin 14- distinguishes stratified epithelial cells from simple epithelial cells, acidic subfamily 50 kDa protein</td>
</tr>
</tbody>
</table>

The results of these preliminary data are represented in Figure 1. All of the cell extracts stained positively with the PAN antibody (Figure 1F), confirming the epithelial origin of the cells and validating our preliminary immunocytochemical staining. Results of staining for individual keratins suggest that the cell culture growth conditions have marked effects on the expression of the keratins tested to date. For example, though phenol red-containing media is typically used for keratinocytes and other similar cell types, growth in this medium produced cells with a completely different keratin phenotype, as compared with growth in media that was otherwise identical except for the absence of phenol red. Growth in phenol red produced pattern most consistent with a simple epithelium and/or the most basal layers of a stratified epithelium. Extracts of these cells stained strongly K19 positive and had almost no staining with MAb directed against K13 or K14. In contrast, the same cells grown in PRF showed a keratin pattern more consistent with a suprabasal or even superficial layer of stratified epithelium. Staining with MAb directed against K13 and K14 was strong, while staining with MAb directed against K19 was nearly absent. Addition of serum produced a keratin expression pattern most suggestive of suprabasal layers in intact stratified epithelium. Similar patterns but with lesser intensity of staining was seen in extracts from cells treated with 17-β-estradiol.

(contains unpublished and/or proprietary information)
(contains unpublished and/or proprietary information)
Figure 1. Identification of human vaginal keratins by SDS-PAGE and Western blot analysis.

A. Coomassie Brilliant Blue staining of 8-16% gradient polyacrylamide gel. B: K19; C: K14; D: K8; E: K13. Western blots developed with enhanced chemiluminescence method.

Lane 1 ProSieve Protein Markers.
Lane 2 Epidermal extract (courtesy of B. Dale).
Lane 3 Extract of cultured human neural foreskin keratinocytes (courtesy of B. Dale).
Lane 4 Extract of keratins from vaginal cells (line V26) grown in keratinocyte-SFM media.
Lane 5 Extract of keratins from vaginal cells (V26) grown in keratinocyte-SFM media and treated with addition of fetal bovine serum for 48 hours prior to harvest.
Lane 6 Extract of keratins from vaginal cells (V26) grown in phenol red-free- (PRF)-SFM media.
Lane 7 Extract of keratins from vaginal cells (V26) grown in PRF-SFM media and stimulated with 250 pg/ml 17-β-estradiol for 24 hours.
Lane 8 SeeBlue pre-stained standards.

F: PAN; Western blot developed with a colorimetric alkaline phosphatase method.

Lane 1 ProSieve Protein Markers.
Lane 2 Epidermal extract (courtesy of B. Dale).
Lane 3 Extract of keratins from vaginal cells (line V26) grown in keratinocyte-SFM media.
Lane 4 Extract of keratins from vaginal cells (V26) grown in keratinocyte-SFM media and treated with addition of fetal bovine serum for 48 hours prior to harvest.
Lane 5 Extract of keratins from vaginal cells (V26) grown in phenol red-free- (PRF)-SFM media.
Lane 6 Extract of keratins from vaginal cells (V26) grown in PRF-SFM media and stimulated with 250 pg/ml 17-β-estradiol for 24 hours.
Lane 7 SeeBlue pre-stained standards.

(contains unpublished and/or proprietary information)
3. Recommendations in relation to the Statement of Work

1. Primary bladder epithelial cells: We will continue to collaborate with Drs. Atala and Grady to cultivate bladder cells, establishing additional frozen stocks and cell pellets for purification as needed.

2. Primary vaginal epithelial cells: In the next funding year, we will complete a few experiments to characterize VECs and complete a manuscript in preparation, describing these data. We have recently performed Western blots using antibodies directed against K6, K10, and K18 (not shown). The results of these experiments will be integrated with the data shown above in the manuscript.

b. Task 2, Months 7 to 12: extraction and purification of GSLs from bladder and vaginal cell cultures

1. Experimental methods, assumptions and procedure

A total of 10 bladder epithelial cell samples have been extracted and partially purified to date. The methods used to extract GSLs from primary cultures of human bladder epithelial cells and VECs as needed were unchanged from those described in our preliminary data: at each step, GSLs were chromatographed in various organic solvent systems and stained with orcinol (carbohydrate detection stain) to assess the purity of individual bands. In addition, HPTLC bacterial overlay procedures as described below were performed to identify and monitor the purification of GSLs of interest. Briefly, the GSL isolation and purification steps are as follows: cell cultures were maintained as described (13) then cells were trypsinized, pelleted, and washed, and the total GSLs were obtained by extracting the pellets with 10 volumes isopropanol:hexane:water (IHW; 55:25:20 by volume) with sonication in a warm bath and centrifugation at 2,500 RPM for 10 minutes. Samples were subjected to a modified Folch procedure to obtain the upper and lower phase GSLs (15), free salts and sugars removed with dialysis and/or reverse phase chromatography, and then contaminating phospholipids were removed using acetylation/deacetylation procedures. The packed cell volume of the largest pellet processed is 6 ml.

2. Results and discussion

Figure 2 (Appendix) shows a summary of the key cell pellets subjected to the procedures shown above. The samples have been extracted as described above and subjected to HPTLC and bacterial overlay assay with *E. coli* R45 to reveal globoseries GSLs.

3. Recommendations in relation to the Statement of Work

This stage of the GSL characterization is completed with respect to bladder epithelial cells. We will perform further extraction procedures with vaginal epithelial cells as needed.

c. Task 3, Months 7 to 12: bacterial overlay assays

1. Experimental methods, assumptions and procedure

This assay involves separating GSLs on HPTLC plates and overlaying the plates with radiolabeled bacteria (3, 18). *E. coli* organisms were metabolically labeled with $^{35}$S-methionine as previously described (3). *S. saprophyticus* organisms were also metabolically labeled with $^{35}$S-methionine using a gonococcal labeling method (Mandrell, unpublished data) that we adapted for *S. saprophyticus* (Stapleton et al., manuscript in preparation). The organisms have a final specific activity of 0.01 cpm/organism, similar to the specific activity achieved using comparable methods for *E. coli* (3). Radioactive bacterial overlay assays were performed as previously described, with a HPTLC plate run in parallel for orcinol staining. For the bacterial overlay assays with *E. coli*, we have continued to primarily use metabolically labeled wild

(contains unpublished and/or proprietary information)
type *E. coli* R45 (3), which expresses the pap-encoded class II adhesin (19) and thus specifically recognizes globo-series GSLs.

2. **Results and discussion**

**GSL expression in cultured vaginal epithelial cells**

Data described in last year’s report have been confirmed and are being incorporated into a manuscript in preparation.

**GSL expression in cultured bladder epithelial cells**

Data from experiments investigating the expression of GSLs that bind either *E. coli* or *S. saprophyticus* are being incorporated into two manuscripts in preparation.

3. **Recommendations in relation to the Statement of Work**

Manuscripts will be completed; experiments will be repeated only as needed.

d. **Task 4, Months 7 to 12: immunostaining assays**

1. **Experimental methods, assumptions and procedure**

Working with Dr. Hakomori, we attempted to harvest additional amounts of Mab ID4 from hybridoma cultures, using standard techniques. These are for use in immunostaining experiments using standard techniques, referenced in our earlier work [Stapleton, 1992 #40].

2. **Results and discussion**

After a considerable effort in concert with Dr. Hakomori’s lab, because of a labeling error, we found that we had harvested an antibody with a similar name, directed against Le^y^.

3. **Recommendations in relation to the Statement of Work**

1. We have made plans with Dr. Hakomori to again try to harvest Mab ID4 from the appropriate hybridoma cultures.

2. Additional immunostaining assays will be performed as needed to confirm presumptive identification of GSLs in assay described above.

e. **Task 5, Months 13 to 24: carbohydrate structural analysis; and**

f. **Task 6, Months 25 to 36: data analysis and publication**

1. **Experimental methods, assumptions and procedure**

a. **Definitive carbohydrate structural analysis:** Full details of the methods described in previous reports for purifying and performing definitive carbohydrate structural analysis of compounds mentioned below are referenced in Dr. Stroud’s publication. Methods newly adopted are described below.

b. **New methods using exoglycosidases:** Last year’s report, we described pilot studies of using a new method of deducing carbohydrate structures among mixtures. The principle behind this method is as follows: when we are working with a mixture of structures purified from a source such as VECs, often we have tools for definitively identifying some of the bands seen when the mixture is separated on HPTLC,
such as reactivity with specific MAbs directed against that carbohydrate structure. In addition, some of the structures of interest are members of families of GSLs, such as the globoseries, which differ from one another in structure only by one or two terminal sugar(s). Thus, we can identify some compounds for which we have specific MAbs by cleaving the relevant terminal sugar(s) with specific exoglycosidases and then using staining with MAbs, bacterial overlay assays, and assessment of relative mobilities on HPTLC to deduce the structures(s). An example of how we can take advantage of these structural relationships is shown below in Table 1. We piloted this approach for identifying the globoseries GSLs that bind pap-adhesin expressing E. coli in our bladder cell extracts. Using Gb5 synthetically produced by Dr. Stroud we used specific exoglycosidases to first cleave the terminal galactose to obtain Gb4 (globoside), followed by another enzyme to cleave terminal N-acetylgalactosamine from Gb4 to obtain Gb3, an epitope for which we have a specific MAb.

<table>
<thead>
<tr>
<th>Globoseries</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSLs core:</td>
<td>Galβ1-4 Galβ1-R</td>
</tr>
<tr>
<td>CTH (Gb3)</td>
<td>Galβ1-4 Galβ1-4 Glcβ1-1cer</td>
</tr>
<tr>
<td>Globoside (Gb4)</td>
<td>GalNAcβ1-3 Galβ1-4 Galβ1-4 Glcβ1-1cer</td>
</tr>
<tr>
<td>Galactosyl</td>
<td>Galβ1-3GalNAcβ1-3 Galβ1-4 Galβ1-4 Glcβ1-1cer</td>
</tr>
<tr>
<td>globoside (Gb5)</td>
<td></td>
</tr>
</tbody>
</table>

2. Results and discussion

Expansion of our studies with exoglycosidase treatments using purified globoseries compounds proved more expensive and less efficacious than anticipated when applied to mixtures of materials. Thus, to investigate the chemistry of the glycolipids found in cultured cells of human primary bladder cell lines, a sensitive mass spectrometric technique was developed for native GSLs found in complex mixtures. Various purified neutral and monosialylated GSLs were examined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) in the reflector mode. Native globo-series GSLs, Gb4 and Gb5, and the monosialyl ganglioside GM1 were found to give good spectra in the positive ion mode (detection limits of 10-100 pmol; see Appendix). MALDI-TOF mass spectrometric analysis of a total glycolipid fraction isolated from a human primary bladder cell line also gave a good spectrum of glycolipid derived molecular ions (see Appendix). Post source decay experiments are underway on these mass-selected ions to obtain further structural information.

3. Recommendations in relation to the Statement of Work

1. As needed, we will continue MALDI-TOF experiments and work with Dr. Levery to perform carbohydrate structural analysis as appropriate.

2. Technical Objective 2

a. Task 1, Months 18 to 30: bacterial adherence assays to test representative isolates for adherence to primary bladder cell and vaginal epithelial cultures

1. Experimental methods, assumptions and procedure

Methods described in our preliminary data and prior reports.

2. Results and discussion

(contains unpublished and/or proprietary information)
3. Recommendations in relation to the Statement of Work

These data are completed and are incorporated in prior reports and in manuscripts in preparation.

b. Task 2, Months 18 to 30: immunocytology procedures utilizing immunofluorescence staining of the same cell cultures with MAbs directed against relevant GSLs

1. Experimental methods, assumptions and procedure

Methods used were as described in our preliminary data.

2. Results and discussion; and

3. Recommendations in relation to the Statement of Work

These data are completed and are incorporated in prior reports and in manuscripts in preparation.

c. Task 3, Months 24 to 36: PDMP treatment of cell cultures, followed by GSL extraction and quantification and bacterial adherence assays

1. Experimental methods, assumptions and procedure

Methods used were as described in our prior reports and preliminary data.

2. Results and discussion; and

3. Recommendations in relation to the Statement of Work

These data are completed and are incorporated in prior reports and in manuscripts in preparation.

3. Technical Objective 3

a. Task 1, Months 24 to 42: synthesis of linear mimetic compounds

1. Experimental methods, assumptions and procedure; and

2. Results and discussion

As noted in last year's report, we pursued a slightly different approach to generate the parent compounds for synthesis of the linear mimetics, namely enzymatic synthesis of the parent GSLs. Details of the progress in this area are as follows:

Enzymatic synthesis of globoseries based GSL receptor analogs

An underlying goal of the experiments described in this report is to chemically modify sialosyl galactosyl globoside (SGG) to identify the functional groups that give rise to its high affinity characteristics as a receptor for uropathogenic E. coli. To generate relatively large quantities of SGG for our chemical modification studies, we extracted chicken pectoral muscle GSLs, separated them on HPTLC as described above, and performed bacterial overlay assays with representative E. coli strains described above. We detected an E. coli-binding GSL that co-migrates with SGG in ganglioside fractions of chicken muscle (data not shown).

We have also continued to pursue the partial enzymatic synthesis of galactosyl globoside (Gb₃), the immediate precursor to SGG. We detected relatively high levels of β1-3 galactosyltransferase activity capable of catalyzing the biosynthesis of Gb₃ using globoside (Gb₄) as an acceptor substrate in two cell lines. While pursuing these strategies a recently cloned β1→3Galactosyltransferase was obtained from

(contains unpublished and/or proprietary information)
Dr. Henrik Clausen at the University of Copenhagen and assayed using \( \text{Gb}_4 \) as an acceptor substrate. The enzyme efficiently converted \( \text{Gb}_4 \) in a reaction mixture containing UDP-galactose and \( \text{MnCl}_2 \) to a slower migrating glycolipid that co-migrated with a \( \text{Gb}_5 \) standard when assessed by thin-layer chromatography. The enzyme was expressed as a soluble construct in insect cells (baculovirus system) purified to homogeneity and used to biosynthesize the putative \( \text{Gb}_5 \). A large-scale purification of \( \text{Gb}_5 \) from human erythrocytes was completed and used as the acceptor substrate. \( \text{Gb}_5 \) was synthesized, purified and sent to Dr. Steve Levery at the University of Georgia for structural analysis. The structure of \( \text{Gb}_5 \) was confirmed by \( ^1\text{H}-\text{NMR} \) as follows:

\[
\text{Gal}^{\beta 1-3}\text{GalNAc}^{\beta 1-3}\text{Gal}^{\alpha 1}\text{Gal}^{\beta 1-4}\text{Glc}^{\beta 1-1}\text{Ceramide}
\]

Since a sufficient amount of \( \text{Gb}_5 \) has now been synthesized, we will sialylate the terminal galactose of \( \text{Gb}_5 \) using CMP-sialic acid as the donor substrate and a commercially available \( \alpha 2,3 \) sialyltransferase to generate SGG for further studies.

In parallel with these studies a rat liver \( \alpha 1\rightarrow2 \text{fucosyltransferase} \) previously thought to only utilize the ganglioside \( \text{GM}_1 \) as an acceptor substrate was assayed using \( \text{Gb}_5 \) as a substrate. Preliminary data suggests that this enzyme actually has a lower \( K_m \) for \( \text{Gb}_5 \) than \( \text{GM}_1 \) and catalyzes the biosynthesis of Globo-\( \text{H} \). The enzyme was expressed in COS-7 cells and a detergent extract of the cells was used as the enzyme source in a reaction mixture containing GDP-fucose, \( \text{MnCl}_2 \) and the detergent Triton CF-54. The final product has been confirmed by NMR. Although this \( \alpha 1\rightarrow2 \text{fucosyltransferase} \) was identified and characterized over 15 years ago the substrate \( \text{Gb}_5 \) was not easily available until now. Sufficient quantities of SGG and globo-\( \text{H} \) will soon be available and we will have a complete panel of globo-series glycolipids to perform additional \( \text{E. coli} \) binding studies.

### Cloning of Human \( \alpha 1\rightarrow4 \text{Galactosyltransferases} \)

The Gal\( \alpha 1\rightarrow4 \text{Gal} \) sequence found in all globo-series glycolipids is the minimum structural element required for binding of uropathogenic strains of \( \text{E. coli} \) expressing a Class II \( \text{pap} \)-encoded adhesin. This structure also forms the basis for the human P histo-blood group system. The gene encoding the glycosyltransferase responsible for catalyzing the synthesis of this linkage has now been identified. The sequence of a recently cloned \( \alpha 4\text{GlcNAc-transferase} \) was used in a BLAST search to identify a novel homologous gene. Expression of full coding constructs of the gene, designated \( \alpha 4\text{Gal-T}1 \), in insect cells revealed \( \text{P}_k \) synthase activity, but no \( \text{P}_1 \) activity (see Appendix) The LgtC gene product from \( \text{N. gonorrhea} \) is involved in the biosynthesis of lipidoligosaccharide (LOS) and encodes an \( \alpha 1\rightarrow4 \text{Galactosyltransferase} \). Expressed sequence tags (ESTs) based on sequence similarity to the LgtC gene product were retrieved using the tBLASTn algorithm against a human EST database. Blast analysis of gene databases resulted in identification of two human genes encoding putative type II transmembrane proteins. The genes have open reading frames encoding 349 and 371 amino acid residues. To date, expression of full coding constructs of each gene in insect cells failed to demonstrate \( \alpha 4\text{Gal-T} \) activity.

### 3. Recommendations in relation to the Statement of Work

1. **SGG synthesis:** We now have sufficient amounts of \( \text{Gb}_5 \) are synthesized to sialylate the terminal galactose of \( \text{Gb}_5 \) using CMP-sialic acid as the donor substrate and a commercially available \( \alpha 2,3 \) sialyltransferase to generate SGG for further studies. The final product will be confirmed by \( ^1\text{H}-\text{NMR} \) by Dr. Levery.
7. KEY RESEARCH ACCOMPLISHMENTS

- Establishment of in vitro models of primary cultured bladder epithelial and human vaginal epithelial cells
- Characterization of glycosphingolipids (GSLs) expressed by these epithelial cells
- Characterization of bacterial adherence to these cells by organisms that are key uropathogens for healthy young women
- Establishment of the principle that GSLs are essential for the adherence of pap-adhesin-expressing uropathogenic E. coli in these systems
- Studies of the effects of exogenous estrogen on the expression of GSLs and keratins in vaginal epithelial cells and characterization of the effects of this hormonal stimulation on bacterial adherence
- Cloning of a human α1-4Galactosyltransferase
- Enzymatic synthesis of globoseries based GSL receptors
- Obtaining interpretable MALDI spectra on a complex mixture of native GSLs in small quantities, not requiring prior permethylation

8. REPORTABLE OUTCOMES

a. Manuscripts, abstracts and presentations

Manuscripts


(contains unpublished and/or proprietary information)


Abstracts

1. Carriage of *Escherichia coli* and expression of virulence factors in urine and periurethra of patients with neurogenic bladder on intermittent catheterization. International Bladder Symposium, November 4-7, 1999, Washington DC

Manuscripts in press


Presentations and Meetings

1. Moderator and Scientific Committee, 2001 International Bladder Symposium

2. Invited speaker, University of Washington Science in Medicine New Investigator series, 1999-2000 (also received award in connection with this lecture series)


Development of cell lines

Primary cultures of vaginal epithelial cells derived from individuals have been developed for the purposes of these studies. However, these cells have not been provided to other investigators as a "reagent" and are not suitable for an application of that nature, such as submission to a cell banking facility.

9. CONCLUSIONS

Our initial years of funding were focused on refining the techniques we developed for culturing primary vaginal epithelial cells and on adapting Dr. Atala's methods for culturing primary human urothelial cells to our model system. We have now established these in vitro models of primary cultured bladder epithelial and human vaginal epithelial cells for the identification, purification and structural characterization of *E. coli*- and *S. saprophyticus*-binding glycosphingolipid moieties. We have completed studies characterizing bacterial adherence to these cells by organisms that are key uropathogens for healthy young women, *E. coli* and *S. saprophyticus*. We have also established that GSLs are essential for the adherence of pap- adhesin-expressing uropathogenic *E. coli* in these systems. We have completed the first phase of studies of the effects of exogenous estrogen on the expression of GSLs in vaginal epithelial cells and characterization of the effects of this hormonal stimulation on GSL and keratin expression and bacterial adherence. We have established and now developed several new collaborations that will allow us to expand and advance our studies related to epithelial biology, especially those of the vaginal epithelium. Establishing our model of the vaginal epithelium opens opportunities for studying numerous aspects of women's urogenital health, including testing vaginal products, probiotics, and contraceptives;

(contains unpublished and/or proprietary information)
understanding protective roles of organisms in the normal flora; and studying the cellular effects of hormone replacement therapy on a key target tissue, the vagina.

The globo-series GLSs have not been thoroughly studied in normal adult tissues. Thus, the new approaches taken by Dr. Stroud in undertaking the enzymatic synthesis of globo-series GSLs and cloning of a human α1-4Galactosyltransferase have yielded basic findings that are important in the field of glycobiology, apart from their relevance to this work.

10. REFERENCES


(contains unpublished and/or proprietary information)

11. APPENDICES


Appendix B: MALDI-TOF spectra for Gb₄, Gb₅, GM-1, and the complex mixture of GSLs described in the text.


(contains unpublished and/or proprietary information)
Figure 2. Extraction of glycosphingolipids from primary bladder epithelial cells. As described in the text, primary bladder epithelial cells were grown to near confluence, harvested by scraping, and subjected to GSL isolation procedures. Partially purified GSLs were then separated on HPTLC and overlaid with $^{35}$-S metabolically labeled *E. coli* R45 to reveal globoseries GSLs. CTH (ceramide trihexosyl; globotriaosyl ceramide; GLOB, globoside, glotetraosylceramide); SGG (sialosyl galactosyl globoside); and GG (glactosyl globoside) are GSL standards.
Appendix B: MALDI-TOF spectra for Gb₄, Gb₅, GM-1, and the complex mixture of GSLs described in the text.

(contains unpublished and/or proprietary information)
Cloning and Expression of the Histo-blood Group P<sup>k</sup> UDP-galactose: Galβ1-4Glcβ1-Cer α1,4-Galactosyltransferase

MOLECULAR GENETIC BASIS OF THE P PHENOTYPE

Rudi Steffensen‡‡, Karine Carlier‡, Joelle Wiels‡, Steven B. Laverly, Mark Stroud‡**, Bertil Cedergren‡, Birgitta Nilsson Sojka‡, Eric P. Bennett‡, Casper Jersild§, and Henrik Clausen††‡‡

From the ‡Department of Cell Surface Biochemistry, Northwest Hospital, Seattle, Washington 98125, and the ††Department of Transfusion Medicine, Umed University Hospital, S-901 85 Umed, Sweden.

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The molecular genetic basis of the P histo-blood group system has eluded characterization despite extensive studies of the biosynthesis of the P, P<sup>k</sup>, and P<sup>+</sup> glycolipids. The main controversy has been whether a single or two distinct UDP-Gal:Galβ1-R 4-α-galactosyltransferases catalyze the synthesis of the structurally related P<sub>1</sub> and P<sub>1</sub> ants. The P<sub>1</sub> polymorphism is linked to 22q11.3-ter. Data base searches with the coding region of an αGalNAc-transferase identified a novel homologous gene at 22q13.2 designated αGal-T1. Expression of full coding constructs of αGal-T1 in insect cells revealed it encoded P<sup>k</sup> but not P<sub>1</sub> synthase activity. Northern analysis showed expression of the transcript correlating with P<sup>k</sup> synthase activity and antigen expression in human B cell lines. Transfection of P<sup>k</sup>-negative Namalwa cells with αGal-T1 resulted in strong P<sup>k</sup> expression. A single homozygous missense mutation, M183K, was found in six Swedish individuals of the rare P phenotype, confirming that αGal-T1 represents the P<sub>k</sub> gene. Sequence analysis of the coding region of αGal-T1 in P<sub>1</sub> and P<sup>k</sup>-individuals did not reveal polymorphisms correlating with P<sub>1</sub> and P<sup>k</sup> typing.

The P histo-blood group system is the last of the known carbohydrate defined blood group systems for which the molecular genetic basis has not yet been clarified. The P blood group system involves two major blood group phenotypes, P<sup>+</sup> and P<sup>−</sup>, with approximate frequencies of 80% and 20%, respectively (1, 2). P<sup>−</sup> individuals normally express the P antigen (P<sup>−</sup> is designated P<sub>2</sub> when P antigen expression is demonstrated), but the rare P<sup>k</sup> phenotype lacks both P and P<sup>+</sup> antigens (for reviews, see Refs. 3–7). The P<sup>+</sup> phenotype is defined by expression of the neolecto-series glycosphingolipid P<sub>2</sub> (for structures, see Table I) (8). In contrast, the P, P<sup>k</sup>, and P antigens constitute intermediate steps in biosynthesis of globo-series glycolipids and give rise to P<sub>1</sub>k, P<sub>1</sub>, and P phenotypes (9). Although the rare P<sup>k</sup> phenotype shows the same frequency of P<sub>1</sub> antigen expression as individuals expressing the P antigen, the P phenotype is always associated with lack of P<sub>1</sub> antigen expression. Extensive studies of the chemistry, biosynthesis, and genetics of the P blood group system identified the antigens as being exclusively found on glycolipids, with the blood group specificity being synthesized by at least two distinct glycosyltransferase activities; UDP-galactose:β-D-galactosyl-β1-R 4-α-galactosyltransferase activity(ies) for P<sup>k</sup> and P<sub>1</sub> synthases and UDP-GalNAcG β<sub>3</sub>-N-acetylgalactosaminyltransferase activity (EC 2.4.1.79) for P<sup>+</sup> synthesis (for reviews, see Refs. 6 and 7). At least two independent gene loci, P and P<sub>1</sub>, are involved in defining these antigens. The P blood group-associated LKE antigen, shown to be the extended sialylated Gal-globoside structure (10), may involve polymorphism in an α2,3-sialyltransferase activity. A long-standing controversy has been whether a single or two independent α1,4-galactosyltransferases catalyze the synthesis of the P<sub>1</sub> neolecto-series glycolipid antigen and the P<sup>+</sup> globo-series structure (3–7). Several hypotheses have been proposed, including: (i) a model with two distinct functional genes being allelic or non-allelic, where the P<sub>1</sub> gene encodes a broadly active αGal-T, the P<sup>k</sup> gene encodes a restricted αGal-T, and a null allele encodes a non-functional protein; (ii) a model with two distinct non-allelic genes, where P<sub>1</sub> encodes an αGal-T that can only synthesize P<sub>1</sub>k and P<sub>1</sub>, and the P<sup>k</sup> encodes an αGal-T that only synthesizes the P<sup>k</sup> structure; and (iii) a model where one gene locus encodes an αGal-T that is modulated by an independent polymorphic gene product to synthesize both P<sub>1</sub> and P<sub>1</sub>k structures. Bailly et al. (11) reported that kidney microsomal αGal-T activity from P<sub>1</sub> individuals does not compete for the two substrates used by P<sub>1</sub> and P<sup>k</sup> αGal-T activities, and no accumulative effect in P<sub>1</sub> synthase activity was observed.

† The abbreviations used are: αGal-T, UDP-galactose:β-D-galactosyl-β1-R 4-α-galactosyltransferase; UTR, untranslated region; EST, expressed sequence tag; PCR, polymerase chain reaction; bp, base pair(s); nt, nucleotide(s); EBV, Epstein-Barr virus; CDH, ceramide dihexoside; CTH, ceramide trihexoside; Gb<sub>3</sub>, globotriaosylceramide.
observed when mixing microsomal fractions from individuals of P1 and Pk groups. Based on this, Bailey and colleagues suggested the existence of two distinct genes, coding for one P1 α4Gal-T with exclusive activity for neolacto-series substrates and one Pk α4Gal-T with exclusive activity for the globo-series substrate. Since individuals lack the Pk antigen, this model inferred that two independent genetic events inactivating both genes was responsible for the P phenotype.

Several approaches to gain insight into the P blood group α4Gal-T gene(s) have been attempted. Purification of the mammalian enzymes has not been successful, but identification and cloning of a bacterial α4Gal-T involved in lipopolysaccharide biosynthesis (12, 13) potentially provided a strategy to clone the mammalian genes using sequence similarity. Previously, a bacterial α3-fucosyltransferase was identified in *Helicobacter pylori* using a short sequence motif conserved among mammalian α3-fucosyltransferases (14). BLAST analysis of gene data bases with the coding region of the α4Gal-T gene from *Neisseria meningitides* resulted in identification of two human genes encoding putative type II transmembrane proteins with low sequence similarity to the bacterial gene. The genes have open reading frames encoding 349 (EST cluster Hs.251809) and 300 amino acid residues and are located at 1p21.1 and 3p21.1, respectively. Previously, we established Epstein-Barr virus (EBV)-transformed B cells from two individuals (15). Only the gene at 3p21.1 was found to be expressed in the EBV-transformed p cells, as well as in Ramos cells known to have high P1 α4Gal-T activity. Sequencing of the coding region of the gene showed no mutations in p phenotypes or truncated, secreted constructs of either gene in insect cells failed to demonstrate glycosyltransferase activity with a large panel of substrates, prepared by genomic PCR using primer pair HCRS126, HCRS1 (5’-ATCTCACTTCTGAGCTGC), and HCRS4 (5’- CATGAAAATGTACTT-CAAGCCCCCCGACCTC) and HCRS125 (5’-CATGAAAATGTACTT-CAAGCCCCCCGACCTC) and HCRS6 (5’-ACCAAGCATGAAAGTGCTCCGCGCCGGAATGTT) (30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 45 s), and a total of 31 P1 + and 51 P1 − phenotyped individuals was typed. RcaI digestion cleaves the expected product (319 bp) of A109 in two fragments of 182 and 137 bp. The RcaI digestion of PCR products for 312 of 312 individuals was confirmed by Southern analysis on 3 biological replicates of the translational start and stop sites, respectively. The PCR products were sequenced in both directions using the primers HCRS122, HCRS123, HCRS124, HCRS125, and HCRS126, respectively.

Expression of α4Gal-T1 in Insect Cells—Full coding constructs were prepared by genomic PCR using primer pair HCRS131 (5’-ACCAAGCATGAAAGTGCTCCGCGCCGGAATGTT) and HCRS126 (5’-ACCAAGCATGAAAGTGCTCCGCGCCGGAATGTT) (30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 45 s), and a total of 31 P1 + and 51 P1 − phenotyped individuals was typed. α4Gal-T expression was monitored by Western analysis on P1 + and P1 − individuals. Full-length α4Gal-T variants were expressed in insect cells with exclusive activity for the globo-series substrate. Since P1 individuals lack the α4Gal-T activity, CAAGCCCCCCGACCTC and HCRS125 (5’-CATGAAAATGTACTT-CAAGCCCCCCGACCTC) and HCRS6 (5’-ACCAAGCATGAAAGTGCTCCGCGCCGGAATGTT) (30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 45 s), and a total of 31 P1 + and 51 P1 − phenotyped individuals was typed. RcaI digestion cleaves the expected product (319 bp) of A109 in two fragments of 182 and 137 bp. The RcaI digestion of PCR products for 312 of 312 individuals was confirmed by Southern analysis on 3 biological replicates of the translational start and stop sites, respectively. The PCR products were sequenced in both directions using the primers HCRS122, HCRS123, HCRS124, HCRS125, and HCRS126, respectively.

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EXPERIMENTAL PROCEDURES

Identification and Cloning of α4Gal-T1—BLASTn analysis of the human genome survey sequences, unfinished high throughput genomic sequences, and dbEST data bases at the National Center for Biotechnology Information (National Institutes of Health, Bethesda, MD) with the coding sequence of a human α4GlcNAc-transferase recently cloned by Nakayama et al. (17), produced a novel open reading frame of 1059 bp with significant similarity. The full coding sequence was available from BAC clone SC22CB-33BT on chromosome 22 (GenBank™ accession no. Z82176) in a single exon. With the release of the sequence of chromosome 22, the mapping data are c23BT (2,6275 × 106 × 107) and 2,6044 × 107 flanked by diaphorase (NADH) and an unknown protein. Linkage analysis of the P1 polymorphism was originally performed with NADH-cytochrome b5 reductase (16). Few ESTs cover the coding region (e.g. B48889), but the 3’-UTR is covered by EST Unigene cluster Ha.168956. Available ESTs are mainly derived from tonsil, prostate, and germ cell tumors.

Identification of Sequence Polymorphisms in the Coding Region of α4Gal-T1—the sequence analysis was performed in three steps. Initially, the coding region of α4Gal-T1 from seven P1 +, five P1 −, and six P phenotype individuals were sequenced in full by direct sequencing of a genomic fragment of 1295 bp derived by PCR with primer pair HCRS122 (5’-CCAGCTGCTGGGCTGGTCAAGTGC) and HCRS126 (5’-CCCGTGTCAGGGCTGGCTGGTCAAGTGC) located downstream and upstream of the translational start and stop sites, respectively. The PCR products were sequenced in both directions using the primers HCRS122, HCRS123, HCRS124, HCRS125, and HCRS126, respectively.

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hypothesized that it would represent one member of a family of observed (Table III). The PCR-based RcaI restriction enzyme of homologous glycosyltransferase genes is that different individuals (Fig. 3). The more common allele of the missense homologous glycosyltransferase genes. A characteristic feature analysis was confirmed by Southern blot analysis of P,+/-. 

The gene was mapped to chromosome 3p14.3. The human a4GlcNAc-transferase responsible for the synthesis of human a4GlcNAc-transferase was extracted from cell lines using the RNeasy Midi kit (Qiagen SA). The probe was random primer-labeled using residue 183 is not invariant among a4Gal-T1 and the nature of the linkage formed is the same chromosomal region (22q13.2) where the synthase activity and antigen expression revealed one missense homozygous mutation and this was not present in 0.03% tetramethylsilane (containing 0.03% tetramethylsilane) precedes a sequence encoding a potential hydrophobic transmembrane segment (Fig. 1). 

**Identification and Cloning of Human P ST a4Gal-T1**—Sequence analysis of the a4Gal-T1 gene from six p phenotype individuals from northern Sweden revealed only one single homozygous missense mutation T548A leading to the change of residue 183 from methionine to lysine. This substitution is a few amino acid changes away from previously published data for which a somewhat different temperature (65 °C) was employed (20, 21).

**RESULTS**

**Identification and Cloning of Human P ST a4Gal-T1**—Na-kayama et al. (17) recently reported the cloning of a novel human a4GlcNAc-transferase responsible for the synthesis of the structures GlcNAcα1-4Galβ1-4GlcNacβ1-R and GlcNAcα1-4Galβ1-3GlnAca1-R. The gene was mapped to chromosome 3p14.3. Since this is the first mammalian glycosyltransferase gene available that forms an α-4 linkage, we hypothesized that it would represent one member of a family of homologous glycosyltransferase genes. A characteristic feature of homologous glycosyltransferase genes is that different members may encode enzymes which have different donor or acceptor sugar specificities, but the nature of the linkage formed is often retained (24). BLAST analysis of data bases using the coding region of the αGlcNAc-transferase identified a sequence BAC clone containing an open reading frame of 1059 bp. The coding region depicts a type II transmembrane protein of 353 amino acids with 35% overall sequence similarity to human αGlcNAc-T (Figs. 1 and 2). The two genes show conservation of a DXD motif (25), and spacings of five cysteine residues. The predicted coding region of αGal-T1 has a single initiation codon in agreement with Kozak's rule (26), which precedes a sequence encoding a potential hydrophobic transmembrane segment (Fig. 1).
Fig. 2. Multiple sequence analysis (ClustalW) of human α4Gal-T1 and α4GlcNAc-T. Introduced gaps are shown as dashes, and aligned identical residues are black boxed. The two amino acid substitutions (M37V and M183K) are indicated above the α4Gal-T1 sequence. Conserved cysteine residues are shown by asterisks.

**TABLE II**

Sequence polymorphisms identified in the coding region of α4Gal-T1 in P₁⁺, P₁⁻, and p blood group individuals.

<table>
<thead>
<tr>
<th>Donor no.</th>
<th>Phenotype nt 109 (Met⁴⁷ → Val)</th>
<th>Phenotype nt 548 (Met¹⁴⁷ → Lys)</th>
<th>Phenotype nt 903 (silent Pro³⁸⁵)</th>
<th>Phenotype nt 987 (silent Thr¹³³)</th>
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<tbody>
<tr>
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<td>P₁⁺</td>
<td>T</td>
<td>G</td>
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<td>A/G</td>
</tr>
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<td>A</td>
<td>G/C</td>
<td>T</td>
</tr>
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<td>G/C</td>
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<td>A</td>
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<td>G</td>
</tr>
<tr>
<td>6</td>
<td>p</td>
<td>A</td>
<td>A</td>
<td>G</td>
</tr>
</tbody>
</table>

* Indicates that the sequence obtained by direct sequencing of PCR products were confirmed on cloned products.

**TABLE III**

Correlation of the missense polymorphism with P₁⁺/⁻ blood group phenotype.

Genotyping was performed by RcaI restriction analysis of PCR products.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Genotype (nt 109)</th>
<th>Cases</th>
<th>Allele frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td>P₁⁺</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AA</td>
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<td>0.63</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>P₁⁻</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>32</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

mutation at A109G encodes a methionine at residue 37 in the C-terminal part of the putative hydrophobic signal sequence (Figs. 1 and 2). The conservative substitution of residue 37 to valine is not predicted to change the catalytic activity or affect retention in the Golgi.

α4Gal-T1 Encodes Exclusive P₆ α4Gal-T Activity—Expression of full coding constructs of α4Gal-T1²⁷⁷⁸⁶ and α4Gal-T1²⁷⁷⁹ in insect cells resulted in marked increase in galactosyltransferase activity with CDH, compared with uninfected cells or cells infected with a control construct (Fig. 4). In contrast, no activity was found with the α4Gal-T1²⁷⁷⁸⁶ gene from p individuals. Importantly, neither α4Gal-T1²⁷⁷⁸⁶ nor α4Gal-T1²⁷⁷⁹ constructs conferred α4Gal-T activity with the neolacto-series (paragloboside) glycolipid acceptor for P₁ synthesis activity (Fig. 4). The assay conditions for measuring P₆ and P₁ synthesis activity was the same except substitution of the acceptor substrate, and these conditions were previously used to demonstrate both activities in kidney extracts from P₁⁺ and P₁⁻ individuals (11). The soluble, secreted construct encoding residues 47–353 did not result in active α4Gal-T activity (data not shown). Attempts to obtain complete conversion of CDH to CTH were unsuccessful, but a one-dimensional ²H NMR spectrum of the purified reaction mixture (data not shown) clearly exhibited H-1 resonances diagnostic for CTH at levels approximately 30% of those of the CDH acceptor substrate. Thus, in addition to major resonances at 4.35 ppm (J₁,₂ = 7.2 Hz) and 4.16 ppm (J₁,₂ = 6.9 Hz), corresponding to H-1 of Galβ4 and Glcα1of CDH, minor resonances were observed at 4.78 ppm (J₁,₂ = 3.7 Hz) and 4.25 ppm (J₁,₂ = 6.9 Hz), corresponding to H-1 of Galα4 and Galβ4 of CTH (the chemical shift of Glcβ1 H-1 is not affected by the addition of the terminal Galα4 residue). The chemical shift and ²H coupling of the downfield H-1 resonance are particularly characteristic for Galα4 of CTH and other globo-series glycosphingolipids (20, 21). Analysis with a number of saccharide acceptors including lactose, lactosamine, and benzyl β-lactoside revealed no significant activity over background values (data not shown).

Expression Pattern of α4Gal-T1—Northern analysis with mRNA from 12 human organs revealed a ubiquitous expression pattern with high expression in kidney and heart and low
Cloning of the \(P^k\) \(\alpha_4\)-Galactosyltransferase

**FIG. 3.** RcaI genotyping of position A109G by Southern analysis. DNA from five phenotyped donors was digested with restriction enzymes as indicated, and the blot probed with the full coding \(\alpha_4\)Gal-T1 (67) construct. The RcaI digestion confirmed the PCR-based genotyping presented in Table II. The EcoRI polymorphism found in individuals 165 and 183 is outside the coding region of \(\alpha_4\)Gal-T1 and is unrelated to the \(P^k\) phenotype.

**FIG. 4.** Expression of full coding \(\alpha_4\)Gal-T1 variants in High Five cells. Assays were performed with microsomal fractions, and controls included constructs encoding polypeptide GalNAc-T3 and -T4 (52), as well as a \(\beta_3\)GalNAc-T (24). Autoradiography of high performance thin layer chromatography of reaction products (4 h) purified by SepPack C-18 columns. Panel A, \(P^k\) assay using 25 \(\mu\)g of CDH as substrate. Plate was run in chloroform-methanol-water (60/35/8, v/v/v). Constructs from the two different alleles identified from \(P^+,+/-\) individuals (45 and 67) resulted in \(\alpha_4\)Gal-T activity toward CDH, while the construct derived from \(P^k\) showed no activity above background found with control constructs. Panel B, \(P^k\) assay using 20 \(\mu\)g of paragloboside (PG) as substrate. Plate was run in chloroform-methanol-water (60/40/10, v/v/v). No specific product was formed with UDP-Gal donor substrate, whereas the \(\beta_3\)GalNAc-T transferred GlcNAc into paragloboside with UDP-GlcNAc. Considerable GlcNAc-T activity was observed in both 67 and \(\beta_3\)GT microsomal fractions, yielding a GlcNAc-CTH-related product.

**FIG. 5.** Northern blot analysis with human organs. Multiple human Northern blot (MTN-H12) was probed with \(\alpha_4\)Gal-T1 probe. kb, kilobases.

**FIG. 6.** Northern blot analysis with eight human B cell lines. Transcript sizes are approximately 2 and 3 kilobases (kb).

DISCUSSION

The \(\alpha_4\)Gal-T1 gene characterized in this report provides a molecular genetic basis for the rare \(P^k\) histo-blood group phenotype found in Västerbotten County, in the northern part of Sweden (28). A single inactivating homozygous nonsense mutation in the catalytic domain of the enzyme was found in all six \(P^k\) phenotype individuals studied. We have previously characterized erythrocyte \(P^k\) antigen expression and \(\alpha_4\)Gal-T activity in EBV-transformed cells from two of these individuals (15), and found a complete deficiency of \(P^k\) antigen and \(\alpha_4\)Gal-T activity. Iizuka et al. (29), reporting essentially the same experiment, suggested that a catalytically active \(P^k\) transferase was indeed expressed in \(P^k\) individuals, as evidenced by \(P^k\) synthase activity in EBV-transformed cells; however, in accordance with the proposed \(P^k\) phenotype of the individual studied, the transformed cells did not express \(P^k\) antigen. This led Iizuka et al. (29) to suggest that \(P^k\) phenotype individuals carry a functionally active \(P^k\) \(\alpha_4\)Gal-T gene, and that the \(P^k\) phenotype was a result of an yet unknown epigenetic mechanism. The data presented here are not in agreement with this, and support a simple allelic model with an active \(P^k\) and an inactive \(P^k\) allele. It is, however, possible that the \(P^k\) phenotype in different populations has a different molecular genetic basis. The molecular genetics of all other characterized histo-blood group systems defined by carbohydrate antigens, i.e. ABO (30), Hh (31), Sese (32), and Lewis (33, 34), have been shown to adhere to a model with simple inactivating mutations of glycosyltransferase genes.

The presented data, however, do not explain the molecular genetic basis of the \(P^k\) blood group polymorphism. Although, the \(P^k\) polymorphism is linked to the same chromosomal localization as \(\alpha_4\)Gal-T1, we found no genetic polymorphisms in the \(\alpha_4\)Gal-T1 gene associated with the \(P^k\) phenotype, and recombinant \(\alpha_4\)Gal-T1 variants did not express \(P^k\) synthase activity in vitro (Tables II and III, Fig. 4). Searching the available chromosomes 22 sequence did not reveal additional homologous genes. Therefore, the following possibilities exist: (i) \(\alpha_4\)Gal-T1 can be activated by another non-homologous polymorphic gene or gene product and function as a \(P^k\) synthase; (ii) a second polymorphic \(\alpha_4\)Gal-T gene, which is non-homologous to \(\alpha_4\)Gal-T1, exists; or (iii) an alternatively spliced version of \(\alpha_4\)Gal-T1 encodes a form capable of functioning as a \(P^k\) synthase.
Cloning of the Pk α4-Galactosyltransferase

Fig. 7. Cell surface expression of Pk/CD77 antigen in Namalwa cells after transient transfection of α4Gal-T1. Constructs p5, 45, and 67 as well as empty pDR2 vector were electroporated into Namalwa cells, and expression of Pk/CD77 antigen was tested after 48 h. Cells were labeled with 1A4 monoclonal antibody and goat anti-mouse-fluorescein isothiocyanate (gray histograms) or with goat anti-mouse-fluorescein isothiocyanate alone (empty histograms) and analyzed with a FACSCalibur flow cytometer. Strong labeling with 1A4 was found only with constructs 45 and 67.

that the first possibility has a precedent in two members of the βGal-T gene family, β4Gal-T1 and -T2, both of which are modulated by α-lactalbumin to change their function from N-acetyllactosamine synthases to lactose synthases (35–37). Binding of α-lactalbumin to these galactosyltransferases changes the acceptor substrate specificity from GlcNAc to Glc, but also to some degree affects the donor substrate specificity to include UDP-GalNAc (38). The induction of β4Gal-T1 by α-lactalbumin to enable it to function as a lactose synthase is combined with a complex regulatory mechanism by which the β4Gal-T1 synthase is 100-fold up-regulated in mammary glands (39). As lactose is the major nutrient in milk, this complex model for its synthesis appears to be in accordance with the biological function. The P1 antigen has only been detected as a minor glycosphingolipid component, and no biological function for this polymorphic antigen has been identified. At present, therefore, it may seem less likely that a unique modulator of the α4Gal-T1 gene has evolved. The second possibility of the existence of another polymorphic non-homologous α4Gal-T gene located in the same chromosomal region implies that the encoded α4Gal-T functions as both Pk and P1 synthases. This is based on the findings that p individuals do not produce P1 antigens, and it is supported by the finding that erythrocytes of P1 individuals contain relative less LacCer and more Gb3 than P2 individuals (40). Generally, glycosyltransferases with similar functions are encoded by homologous glycosyltransferase gene families (24); however, recently two non-homologous β3GlcNAc-transferases both functioning as polyn-acetyllactosamine synthases have been identified (41, 42). The third possibility is unlikely, as the coding region of α4Gal-T1 was found in a single exon. However, there are precedents for multiple protein forms originating from such genes by differential usage of splice donor sites of introns placed in 3′-UTR (43). Although the Northern analysis with mRNA from human organs only revealed one transcript of 2.3 kilobases (Fig. 5), the Northern analysis with total RNA from human cell lines may suggest the presence of additional larger transcripts (Fig. 6). The nature of these transcripts required further analysis to explore the possibility that they may encode a novel form of the α4Gal-T1 protein with P1 synthase activity. A possible mechanism for the P1 blood group polymorphism would include sequence polymorphisms in the 3′-UTR directing different donor splice sites in the 3′ coding region of the characterized α4Gal-T1 coding sequence. It is important to note, however, that a total of three polymorphisms were identified in P1+/− individuals (Table II), and none of these correlated with the phenotype. This finding argues against the existence of another polymorphism in this gene (3′-UTR) responsible for the P1+/− polymorphism, because it would be expected that at least some of these would have arisen after the P1+/− polymorphism and hence co-segregate with this as found e.g. for the polymorphisms in the ABO gene locus (30).

α4Gal-T1 is homologous to an α4GlcNAc-T located at 3p14.3 (17). The α4GlcNAc-T forms the linkage GlcNAcα1-4Galβ1-3/4R, where R can be GalNac, GlcNac, or less effectively, glucose. Preference for mucin oligosaccharides of the core 2 structure was found, and the gene was shown to control expression of Con-A-binding class-III mucins in stomach and pancreas. Genetic polymorphisms in expression of the α4GlcNAc-T structures have not been reported. The sequence similarity with α4Gal-T1 (35% overall amino acid sequence similarity) is similar to that found among other analogous glycosyltransferases with similar functions, and the characteristic feature of conserved spacings of cysteine residues (five cysteine residues align, Fig. 2) is also found. Both enzymes transfer to galactose, but, whereas the acceptor disaccharide specificity of the α4GlcNAc-T appears to be broad, α4Gal-T1 is apparently highly specific for the glycolipid, lactosylceramide. Lopes et al. (44) recently characterized an α4Gal-T activity in insect cells and found it had preferred acceptor substrate specificity for Galβ1-3GalNAcα1-R rather than lacto-series structures. Thus, the acceptor substrate specificity is similar to that of the α4GlcNAc-T and different from α4Gal-T1.

The N. gonorrhoeae lgtC α4Gal-T (12) exhibits no significant sequence similarity to the α4Gal-T1 reported here. However, two human genes homologous to lgtC were identified with significant sequence similarities and conserved DXD motif (data not shown). We have been unable to demonstrate glycosyltransferase activity with these two human genes using full coding and secreted recombinant constructs expressed in insect cells. Nevertheless, they may represent glycosyltransferase genes and encode α4Gal-Ts, but they are unlikely to be involved in the P1 polymorphism as they are not located on chromosome 8. One of the genes is located at Bj24 distal to the c-myc oncogene, and hence part of region of chromosome 8, which is translocated in Burkitt’s lymphoma. Since Burkitt’s lymphoma is associated with high level expression of Pk (45), this represented a candidate for the Pk gene. However, North-
ern analysis of various B cell lines (Fig. 6) shows that α4Gal-T1 expression clearly correlates with Pk synthase activity and antigen expression in Burkitt's lymphoma cell lines (22, 23).

The P blood group system is implicated in important biological phenomena. Blood group P individuals have strong anti-P, PkP Pk antibodies and these are implicated in high incidence of spontaneous abortions (46). The globo-series glycolipids constitute major receptors for microbial pathogens with the Galα1-4Gal linkage being an essential part of the receptor site (for a review, see Ref. 47). The Pk glycolipid is the CD77 antigen, a B cell differentiation antigen, which is able to transduce a signal leading to apoptosis of the cells (48). Furthermore, the association of this glycolipid with the type I interferon receptor or with the human immunodeficiency virus type 1 co-receptor, CXCXR4, seems to be crucial for the functions of these receptors (49, 50). Cloning of the Pk synthase is an important step toward understanding the biological roles of the globo-series of glycolipids, and a first step in elucidating the molecular genetics of the P blood group system.

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REFERENCES

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

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