Complement Factor H or a Related Protein Is a Marker for Transitional Cell Cancer of the Bladder

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ABSTRACT

The BTAsstat and BTA TRAK tests are new immunoassays that detect and measure an antigen in the urine of individuals diagnosed with bladder cancer. As described in this report, the monoclonal antibodies used in these kits were developed by immunizing mice with partially purified protein preparations derived from the urine of patients with bladder cancer. The antigen that is recognized by the monoclonal antibodies was purified from the urine of bladder cancer patients by immunofinity chromatography and identified as being either complement factor H (FH) or a closely related protein (CFHrp) by partial amino acid sequence analysis. Like serum FH, the urine antigen was demonstrated to have a complement factor C3b binding site and to accelerate the degradation of C3b in the presence of complement factor I. The culture supernatants from several human bladder, cervical, and renal cancer cell lines contained antigen as determined by immunoassay, and antigen affinity-purified from HeLaS3 culture media was shown to have FH activity. Moreover, the cell lines were shown to make products of the expected sizes by reverse transcription-PCR using FH-specific primers. In contrast, normal human epithelial keratinocytes, a myeloid leukemia cell line, and the colon cancer line LS174T were negative for production of a FH-like protein (CFHrp). We propose that the expression of proteins with FH-like activities may confer a selective growth advantage to cancer cells in vivo by decreasing complement activity, thus aiding their escape from lysis by immune surveillance. Identification of these proteins as cancer products also suggests avenues of chemotherapy or immunotherapy of some cancers.

INTRODUCTION

Bladder cancer is the fifth most common cancer in the United States. The American Cancer Society estimated that a total of 52,900 new cases were detected in 1996, resulting in 11,700 deaths (1, 2). Bladder cancer is more common in men than in women by a ratio of approximately 3:1, and the incidence increases with age. Known risk factors include smoking; exposure to chemicals in the dye, textile, rubber, and leather industries; and chronic urinary infections. Symptoms frequently associated with the disease are hematuria, dysuria, and frequent urination.

About 90% of all bladder cancers are TCC,2 an epithelial malignancy that is often successfully treated when detected at an early stage. Included in the category of TCC is CIS. CIS is an uncommon, invasive bladder tumor that is difficult to detect and occurs as a flat and diffuse growth. The remaining cases are squamous cell carcinomas (7%), adenocarcinomas (2%), and undifferentiated carcinomas (1%).

The diagnosis and management of TCC is often performed as follows. A patient presents with symptoms such as hematuria or dysuria in the absence of infection. Cystoscopy is performed to determine whether a tumor is present in the bladder, at which time a biopsy may also be taken. Although this procedure is invasive and uncomfortable, it is highly accurate in detecting malignancy. It is thus considered to be the method of choice for confirmation of bladder cancer. In addition to cystoscopy, urine cytology, the identification of tumor cells in voided urine, is often performed. In some cases, cytology may allow the detection of tumors not visible during cystoscopy, as is often the case for CIS and for those carcinomas in the upper end of the bladder or the upper urinary tract.

As with most other forms of cancer, the key to successful treatment of TCC is early diagnosis. When the disease is detected early and treated, patients have a good survival rate. Metastatic spread of the disease is found in only 10% of those with superficial bladder tumors (stage Ta, T1, or Tis). However, up to 75% of bladder cancer patients recur within 5 years, in part because bladder cancer is multifocal. Thus, it is also important to have tests available that allow the disease to be easily monitored during and after treatment. Recently, noninvasive tests, which may be used in conjunction with standard methods to

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2 The abbreviations used are: TCC, transitional cell carcinoma; MAb, monoclonal antibody; RT-PCR, reverse transcription-PCR; AP, alkaline phosphatase; FBS, fetal bovine serum; sEIA, sandwich enzyme immunoassay; FH, complement factor H; CFHrp, FH-related protein; FI, complement factor I; CIS, carcinoma in situ; PVDF, polyvinylidene difluoride; NHEK, normal human epithelial keratinocyte; IMDM, Iscove’s modified Dulbecco’s medium; MWCO, molecular weight cutoff; CAPSO, (3-[Cyclohexylamino]-2-hydroxy-1-propanesulfonic acid); MW, molecular weight; PEG-DMSO, polyethylene glycol-DMSO.
manage and improve the care of patients with bladder cancer, have been developed.

In an effort to develop a quantitative assay for use in managing bladder cancer patients, we embarked on a program to generate MAbs to protein isolates from bladder cancer patient urines. Over 100 MAbs of interest were identified by screening urine specimens from patients that were confirmed as having bladder cancer by cystoscopy and biopsy or cytology that were positive in the BTA test (3). The two MAbs with the greatest clinical utility were used to formulate a sEIA. The sEIA is the basis for two new bladder cancer diagnostic assays, BTAstat, a lateral flow chromatography immunoassay device for use in point of care testing, and BTA TRAK, a quantitative enzyme immunamaosay (4, 5). Both tests have received Food and Drug Administration clearance for use in conjunction with cystoscopy in the management of patients with bladder cancer. The results of a multicenter clinical trial have been reported for these products (4, 5). Mean levels of antigen in patient urines have been shown to correspond to tumor stage (CIS, 69 units/ml; Ta, 317 units/ml; T1, 851 units/ml; >T1, 1250 units/ml) and tumor grade (grade I, 212 units/ml; grade II, 543 units/ml; grade III, 914 units/ml). A receiver-operator characteristic plot was used to establish a normal range of 0-14 units/ml in the BTA TRAK assay, resulting in an assay sensitivity of 73% in a 216-patient clinical panel, with a specificity ranging from 75% in patients with a variety of nonmalignant genitourinary diseases to 97% in healthy individuals. There was no correlation between the levels of the tumor antigen and the presence or level of hematuria in any patient population tested (6).

During the course of assay development, we also used the two MAbs to isolate and identify the marker antigen. The antigen isolated has the biological and immunochemoical properties of FH. FH is a Mr 150,000 protein that is well described in the literature (7-9). The biological role of FH is the negative regulation of the alternative complement pathway. FH can bind C3b, displacing factor B (decay accelerator factor activity; Ref. 10), and in a separate reaction, it can serve as a cofactor for FI, a serine protease, in the degradation of C3b. These activities result in the inability of C3b to serve as the focal protein for the membrane attack complex of complement. Because C3b is capable of self-activation (11, 12), and because the alternative complement pathway is triggered by polyanions (13) that are found on the surfaces of some human cells, FH regulation is thought to be important in controlling autolysis by the alternative complement pathway (14).

In addition, experiments with human melanoma cell lines sensitized by pretreatment with MAb demonstrated that FH effectively inhibited the activity of C3b in the classical complement pathway of cell lysis (15).

MATERIALS AND METHODS

MAB Generation and Screening

Heparin-Agarose Chromatography. Antigen for immunization was partially purified by heparin-agarose chromatography from urine specimens collected from bladder cancer patients diagnosed as: (a) dysplasia (no diagnosis); (b) stage Ta, unknown grade; (c) stage Ta, grade I; and (d) stage T2, grade I. All patient materials were obtained after institutional review board approval. Urine samples, each collected over a 24-h period, were clarified by centrifugation at 6,000 X g for 20 min. Each sample was then concentrated 100 X on a stirred cell (Amicon, Beverly, MA) fitted with a YM30 membrane (MWCO Mr 30,000) or a hollow fiber concentrator (Microcon, Laguna Hills, CA; MWCO Mr 50,000). The concentrated sample was diluted 1:1 with 25 mm Tris-HCl (pH 7.4) and loaded onto a 50-m1 column of Affi-Gel heparin (Bio-Rad, Richmond, CA) equilibrated in 25 mm Tris-HCl (pH 7.4). The sample was followed with equilibration buffer until the A 280 nm elution profile returned to baseline. Bound material was eluted at a flow rate of 2 ml/min with a 50-ml linear NaCl gradient from 0-250 mm NaCl in 25 mm Tris-HCl (pH 7.4), followed by 50 ml of 250 mm NaCl, 25 mm Tris-HCl (pH 7.4), and, finally, 20 ml of a 10 X PBS (pH 7.4) solution. Fractions of 5 ml each were collected; fractions from the trailing half of the elution peak were pooled. Pooled fractions were concentrated with an Amicon stirred cell fitted with a YM30 membrane. A high molecular weight protein fraction was prepared by size exclusion chromatography on a 1.6 X 90-cm S-300 column (Pharmacia, Piscataway NJ), equilibrated, and eluted in PBS. The breakthrough fraction was used. Aliquots of pooled immunogen were stored at 2°C to 8°C until used.

Immunization and Screening. Five female 8-10-week-old BALB/c mice (Charles River Breeders, Cambridge, MA) were immunized i.p. with 0.2 ml of a 1:1 emulsion of antigen in Freund’s complete adjuvant (DIFCO, Detroit, MI). Three weeks later, booster immunizations of 10 µg of protein of an emulsion in incomplete Freund’s adjuvant were administered to the rear foot pads and peritoneum. Ten days later, each mouse was sampled for serum antibody response by ELISA (described below). The mouse showing the highest serum titer was chosen for fusion 4 days after boosting in the foot pads and peritoneum with 15 µg of pooled antigen in PBS. The popliteal and inguinal lymph nodes and the spleen were collected and used for fusion to the hybridoma line FO (American Type Culture Collection, Rockville, MD) as described by De St. Grath and Scheidegger (16) at a ratio of 1 lymphocyte:5 FO cells. PEG-DMSO fusogen was used, and cells were washed in HBSS and plated in IMDM-10% FBS with penicillin-streptomycin and a hypoxanthine/thymidine supplement at a density of 2 X 10^4 cells/well, with 2.6 X 10^3 peritoneal macrophages from nonimmunized BALB/C mice added as feeders. All reagents were from Sigma (St. Louis, MO).

Cloning was performed by successive rounds of limiting dilution in 96-well plates (Costar, Cambridge, MA). Hybridomas to be expanded were selected by a series of screening protocols. The first-level screen was for positive binding to the urine specimens from which the immunogen had been prepared and negative binding to two normal urine pools. In addition, hybridomas had to be negative for binding to pooled human erythrocyte preparations. The second-level screen was for differential binding to a panel of specimens from bladder cancer patients versus a panel of urine specimens from healthy, age-matched individuals. A third-level screen was performed, eliminating MAbs that bound to collagen, fibrinogen, fibronectin, and laminin. Hybridoma expansion was in Costar T-75 flasks in IMDM-10% FBS with penicillin-streptomycin. MAbs were purified by chromatography on immobilized Staphylococcus
protein A (Pharmacia) by standard methods, as described in the manufacturer's package insert.

Antigen Purification and Characterization

**MAb Affinity Chromatography.** Aliquots of 24-h urine specimens partially purified by chromatography on heparin-agarose were diluted 1:1 with 25 mM Tris-HCl and 250 mM NaCl (pH 7.4) and loaded onto 5-ml MAb affinity columns prepared from Bio-Rad Affi-Gel 10 derivatized according to the manufacturer's package insert with MAb X-13.2. A column was prepared by coupling normal mouse immunoglobulins to Affi-Gel 10 as a control for urine materials binding nonspecifically to IgG. Columns were derivatized with 3–5 mg antibody/ml gel. Samples were loaded at 0.5 ml/min and eluted with 25 mM Tris-HCl and 250 mM NaCl (pH 7.4) until the A_{280 nm} baseline was reached. Bound material was then eluted with 100 mM glycine-HCl (pH 3.0). The eluted fractions (5 ml each) were collected in tubes containing 1 ml of 1.0 mM Tris-HCl (pH 8.0). Conditioned media from confluent HeLaS3 cultures were pooled to provide 1 liter of starting material. Media were passed over a fresh X13.2 affinity column twice, and the remainder of the procedure was as described above. In control ELISAs and Western blots, neither MAb X13.2 nor X52.1 bound proteins from FBS.

**PAGE and Detection.** Purity was assessed by SDS-PAGE using 8–16% and/or 4–12% polyacrylamide gels (NOVEX, San Diego, CA) under reducing and nonreducing conditions along with NOVEX Mark XII molecular weight standards (M, 6,000–200,000). The gels were stained with Coomassie Blue R250 followed by silver staining and scanned using a Bio-Rad GS-700 densitometer. Molecular weights of individual bands were estimated by the Rf values of the molecular weight standards.

**Western Blot Analysis of Partially Purified Antigen Preparations.** The urine samples purified on heparin-agarose were diluted with SDS-PAGE 2× sample buffer in the presence of DTT, heated at 100°C in a boiling water bath for 5 min, and then allowed to cool to room temperature. Samples were loaded onto an 8–16% gradient Tris-glycine gel and electrophoresed at 125 V constant for 190 V-h. NOVEX SeeBlue molecular weight standards were loaded into a reference well. The SDS-PAGE bands were transferred to PVDF paper in transfer buffer at 125 mA constant for 60 min. The PVDF paper was blocked with PBS containing 2% nonfat dry milk solution for 60 min and washed with PBS containing 0.05% Tween 20 (PBS/Tween). MAb were diluted in PBS/Tween to 2 μg/ml, added to the PVDF paper for 2 h, washed with PBS/Tween, and incubated with an antimouse IgG AP conjugate (Kierkegaard and Perry, Gaithersburg, MD) for 1 h. PVDF was washed with 50 mM Tris containing 5 mM MgCl₂, and then a 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate solution was added in the 50 mM Tris-5 mM MgCl₂ solution, and the bands were developed.

**Partial Tryptic Digestion.** A volume of 100 μl of immobilized trypsin (Pierce Chemical, Rockford, IL) was added in a 1:10 enzyme:substrate ratio (w/w) to 300 μg of antigen at a working concentration of 0.5 mg/ml in PBS. The solution was gently mixed and placed on a rotator for 4 h at room temperature (21°C to 23°C). Digestion patterns were visualized by SDS-PAGE using a 4–12% gradient Tris-glycine precast gel with Tris-glycine-SDS running buffer with 1% DTT (NOVEX).

**Amino Acid Sequence Determination.** Samples of MAb affinity-purified antigen and fragments resulting from partial tryptic digestion of the native antigen were prepared for amino acid sequencing by SDS-PAGE. The sample was diluted 1:1 with SDS-PAGE 2× sample buffer (NOVEX) containing 2% DTT, heated at 100°C in a boiling water bath for 5 min, and then allowed to cool at room temperature. The sample was electrophoresed at 125 V constant for 190 V-h. The gel was removed and placed in a container of 10 mM CAPSO buffer (pH 9.0) containing 0.05% SDS on a rocker platform while the gel transfer sandwich was prepared. The SDS-PAGE bands were transferred to PVDF membrane (NOVEX) at 125 mA constant for 60 min. The PVDF membrane was removed and rinsed with deionized water and stained in a solution of 0.1% Coomassie Blue R-250 in 20% methanol for approximately 10 min. The stained PVDF was destained with several changes of 30% methanol until the background stain was minimal, followed by...
The stained bands of interest were excised with a clean razor blade and placed in capped tubes. Automated sequencing by Edman degradation was performed in the laboratory of Dr. Kenneth A. Walsh (Department of Biochemistry, University of Washington, Seattle, WA).

**Decay Accelerator Factor Activity of Complement.**

Assay of FH decay accelerator activity was measured by the method of Alsenz et al. (17). Five μg of affinity-purified antigen from either urine or serum, 5 μg of Fl (Sigma), and 50 μg of C3b prepared from C3 (Sigma) by the method of Sim et al. (18) were incubated with gentle mixing at 37°C for 90 min. Six μl of undiluted, dialyzed patient urine samples, 3 μl of Fl, and 30 μl of C3b were incubated as described above in a separate experiment. A small portion of each reaction mixture was boiled for 2 min with one-third volume of 4× load buffer and 4% DTT and electrophoresed and visualized as described above.

**C3b-MAB seIA.** Immunol 4 (Dynex) microtiter strip wells were coated with 50 μl/well of C3b, generated as described above, in 50 mM carbonate buffer (pH 9.6) either overnight at 4°C or for 2 h at 37°C. A control plate was coated with 50 μl/well of 2% BSA in PBS. After a single wash with wash buffer, the plates were blocked with 100 μl/well of a 2% BSA solution in PBS for 2 h at 37°C and washed four times. Antigen diluted in BTA TRAK kit diluent was added at 50 μl/well. After incubation for 1 h at 37°C, the plates were washed four times, and then the detection antibody was applied at 0.25 μg/ml (50 μl/well) and incubated at 37°C for 30 min. Detector MAb was AP-conjugated X13.2 (BION Diagnostics) or sheep antihuman FH (Binding Site, San Diego, CA) followed by a rabbit antisheep AP conjugate (Kierkegaard and Perry). After four washes, 50 μl/well of p-nitrophenyl phosphate (Sigma) at 1 mg/ml in 1 M diethanolamine buffer (pH 9.8) were added. Finally, the plate was incubated for 30 min at 37°C, the reaction was stopped with 25 μl/well of stop solution, and the plate was read at 405 nm on a Dynatech MR7000 reader.

**Synthesis of CFHp by Human Cells**

**Cell Culture.** Human cancer-derived cell lines were purchased from the American Type Culture Collection. NHEKs were purchased from Clonetics (San Diego, CA). Cell culture media and supplements were from Sigma or Clonetics. Cells were grown in plastic culture flasks (Costar) in water-jacketed CO₂ incubators (Forma Scientific, Marietta, OH).

**Immunoassay of Conditioned Media.** Conditioned media derived from tumor cell lines was clarified by centrifugation at 5000 × g for 30 min at 4°C. Antigen levels were estimated using the Bard BTA TRAK kit (Bard Diagnostic Sciences, Redmond, WA) according to the package insert. One unit of activity in the kit is equivalent to 4.7 ng of FH.

**RT-PCR.** RT-PCR was performed with Perkin-Elmer (Foster City, CA) kits. Total cellular RNA was isolated from cancer cell lines by the method of Sambrook et al. (19). cDNA was synthesized from mRNA present in preparations of 2 μg of total cellular RNA from cancer cell lines using reverse transcriptase plus 5 μM random hexamer primers. The cDNA reaction was allowed to proceed for 90 min at 42°C. PCR of FH and CFHp mRNA was performed on a Perkin-Elmer 2400 thermal cycler with the primer pairs (at 0.5–1 μM) listed in Table 1. Primers were designed using the FH sequence reported in GenBank Accession number Y00716 as the template. Thermal cycling protocols were as follows: (a) for the 2910/3610 primer set, 40 cycles with annealing and extension at 72°C for 60 s and

**Table 2 Sequences of urine antigen tryptic fragments**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fragment (Mr)</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M₁, 128,000</td>
<td>GYPFPVAVGKY Y (Y) Y?D</td>
</tr>
<tr>
<td>2</td>
<td>M₁, 79,000</td>
<td>[RYPFPVAVGKYYS Y YCD]</td>
</tr>
<tr>
<td>3</td>
<td>M₁, 46,000</td>
<td>RSSQESYAHGTK</td>
</tr>
<tr>
<td>4</td>
<td>M₁, 37,000</td>
<td>[EDCNELPPRTNLITGSW-D]</td>
</tr>
<tr>
<td>5</td>
<td>M₁, 66,000</td>
<td>RYPFPVAVGKYSYIY?DEFE?P</td>
</tr>
<tr>
<td>6</td>
<td>M₁, 33,000</td>
<td>SLGNIMVY?RGXGLVNLPRK</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[RYPFPVAVGKY]</td>
</tr>
</tbody>
</table>

* Amino acid residue numbers refer to the mature FH molecule. AA, amino acid.

* Major protein sequence.

* Minor protein sequence.

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RESULTS

MAb Generation and Screening. Production of hybridomas from mice immunized with the partially purified high molecular weight protein fraction of patient urine samples yielded hundreds of MAbs. These MAbs were tested by use of the following fusion screen. Antibody binding was tested with: (a) two urines from patients with clinically diagnosed active bladder cancer, stages T2III and T3III, respectively; (b) two pools of normal human urines; (c) human type IV collagen; and (d) pooled human RBCs diluted and bound to polylysine-coated plates. Criteria for selection were high binding to cancer urine-coated plates and low binding to normal urine-coated plates and other test antigens. Some MAbs that showed high antibody binding levels in other patterns with respect to the test antigens were also selected for further research uses. From the 9312 wells plated in the fusion, a total of 880 wells showing growth were screened, with a total of 118 clones selected for further work.

The selected clones were further screened on the basis of high MAb binding to positive specimens, low binding to negative specimens, and retention of high signal levels upon dilution of the culture supernatants. This additional testing for antibody sensitivity, specificity, and production quantities narrowed the selection to a total of 32 subclones. Finally, after the preparation of AP conjugates from 14 of the selected MAbs and extensive testing with a panel of 120 urine specimens, the MAb pair selected for the detection of TCC of the bladder was X52.1 (capture MAb) and X13.2 (detector MAb, an AP conjugate). These two MAbs were formulated into a sEIA (19).

Immunoaffinity Purification of Antigen from Patient Urines. Based on Western blots, we selected MAb X13.2 for production of an affinity column. A 450-ml pool of three urines was used for immunoaffinity purification using anti-factor H/anti-sheep Conjugate.

Fig. 2  Immunoaffinity-purified antigen, commercially obtained human FH, immunoaffinity-purified FH from human serum, or three urine specimens (Normal Urine, PES, and CHD) were tested in a sEIA format. The capture molecule was C3b, and the detector molecule was either AP-conjugated MAb X13.2.1.1 or sheep antihuman FH, followed by a rabbit antisheep AP conjugate.
was initially chromatographed on a heparin-Affi-Gel column (2.5 × 16 cm). Immunologically active protein fractions were eluted with 100 mM NaCl in 25 mM Tris-HCl buffer (pH 7.4). Fractions were pooled, and 50 ml of the first-step product at a protein concentration of 265 µg/ml were loaded directly onto a 2-ml MAb column. The bound antigen was eluted with 100 mM sodium citrate (pH 2.5) and immediately neutralized with 1.0 M Tris-HCl buffer (pH 8.0).

The affinity-purified fractions contained predominant bands at Mr 151,000, Mr 130,000, and Mr 40,000, with very minor contaminant bands at Mr 77,000, Mr 60,000, and Mr 25,000 by SDS-PAGE under reducing conditions (Fig. 1). Under nonreducing conditions, the MAb-specific bands exhibited apparent molecular weights of approximately Mr 138,000, Mr 121,000, and Mr 40,000, with the Mr 138,000 band being typically the most intense. The shift in apparent molecular weights, after reduction, of the dominant band from Mr 138,000 to Mr 121,000, and a band from Mr 121,000 to Mr 130,000 suggested the presence of a large number of intrachain disulfide bonds in these molecules. The appearance of additional bands in the Mr 66,000 to Mr 116,000 region of the gel and the presence of a significant protein band at Mr 138,000 after reduction with 50 mM DTT indicate that some of the FH and some of the urine antigen are partially degraded, more than one protein resides in the very dense Mr 138,000 band, or both.

**Amino Acid Sequence.** Samples of the MAb affinity-purified antigen were transferred to a PVDF membrane and sequenced by automated Edman degradation. The single amino acid sequence obtained was EDCN?LPPR?NT, in which the question mark indicates a residue that could not be identified. The sequence was consistent with FH as reported in GenBank File Accession number Y00716. The size of the antigen by SDS-PAGE was consistent with that of FH but also suggested that the generation of smaller peptide fragments by proteolysis and sequencing those was warranted.

Samples of affinity-purified antigen (300 µg) were digested with 100 µl of immobilized trypsin at a working concentration of 0.5 mg/ml at an enzyme:substrate ratio of 1:10. Seven fragments were observed (data not shown). Fragments were transferred to PVDF for amino acid sequence determination. The results are shown in Table 2. Residues for which only tentative assignments could be made are shown within parentheses, whereas those that could not be assigned are indicated with a question mark. Hyphens are used to indicate gaps inserted in sequence to improve alignments. A comparison of the partial sequences of the seven tryptic fragments to the corresponding amino acid sequence of human FH revealed very strong similarities. Sequences from the patient antigen fragments yielded 72 residues of a possible 78 residues that matched cognate FH. Of the remaining six residues, five could not be assigned, and one corresponds to a gap inserted in the sequence of fragment four. Additionally, in one of four similar fragments (samples 1, 2, 5 and 6-minor), a residue has been identified as glycine in one of the fragments instead of the expected arginine found in the other three fragments. This discrepancy may be a consequence of using urine antigen preparations from different patients. Finally, in one case (sample 5), a gap has been inserted in the FH sequence to facilitate alignment with the fragment. The insertion of gaps and the identification of discrepant residues should be considered tentative.

**Reactivity of MAb with Components of Human Complement.** Putative identification of the affinity-purified antigen from urine of cancer patients as FH or a closely related protein suggested a number of additional experiments. Reactiv-
ity of MAbs X13.2 and X52.1 was tested by sandwich EIA and Western blot analysis against commercially obtained FH. Using either technique, both antibodies bound to FH. Additional experiments determined that the MAbs did not react with C3/C3b, factor B/Bb, or properdin by either method (data not shown).

Biochemical Activity of Affinity-purified Urine Antigen. FH is known to have three C3b binding sites (9). We tested urine specimens from TCC patients that were positive in the EIA (Fig. 2. PES and CHD) and affinity-purified antigen from serum (putative FH) or urine in an sEIA variant using C3b as the capture molecule (Fig. 2). A signal indicated the formation of a sandwich between C3b, the affinity-purified urine antigen or patient specimens, and the MAb conjugate, demonstrating that the antigen contained a C3b binding site. We confirmed this result by using a commercially obtained sheep antihuman FH immunoglobulin.

A second assay for biological activity of FH was used. FH is known to serve as a cofactor in the Fl-mediated proteolysis of C3b to fragments ineffective in complement fixation. Electrophoretic analysis, under reducing conditions, was performed on incubation mixtures containing C3b and Fl to which had been added either FH or antigen purified from urine or serum (Fig. 3A, Lanes 8–10, respectively). Each reaction mixture showed the same Fl-mediated proteolysis of the C3b α chain, which was characterized by the disappearance of a Mr 108,000 band and the concurrent appearance of degradation products at Mr 67,000 and Mr 43,000 (21). The control containing neither FH nor the antigen (Lane 5) demonstrated the requirement for exogenous decay accelerator factor for proteolysis of C3b to occur. Similarly, the requirement for exogenous Fl in this system is shown in Lane 6.

Human Cancer-derived Cell Lines Produce FH. The next group of experiments was designed to test whether epithelial cancer cells could synthesize and secrete FH. We used two methods of analysis. Cell culture supernatants from several cancer cell lines including TCC of the bladder, prostate adenocarcinoma, cervical adenocarcinoma, colon adenocarcinoma, and renal clear cell carcinoma as well as lymphomas and NHEKs were tested for presence of human FH. Most of the bladder, renal, and cervical cancer cell supernatants contained the antigen (Table 3). The quantity of secreted CFHrp varied with confluence of the culture, increasing when confluence was greater than 90%. In contrast, the prostate cancer cell lines (DU145, PC3, and LNCaP), the colon cancer cell line LS174T, the lymphoma cell line Raji, the promyelocytic leukemia cell line HL60, and NHEKs produced no detectable FH or CFHrp by this method. Control cell culture media containing 10% FBS was unreactive in the immunoassay.

In addition, RT-PCR was used to detect the presence of FH or CFHrp mRNA in the cells used to condition the culture media. A cell line was considered positive for the FH transcript when more than one primer set from different regions of the FH transcript amplified cDNA of the expected size from total cellular RNA that had been reverse-transcribed with random hexamer primers. In general, the presence of transcript by this approach corresponded to the presence of CFHrp by sEIA in the cell culture media.

Fig. 4 shows data obtained from optimized primer sets [described in “Materials and Methods” (Table 1)] on four of the cell lines listed in Table 3. The CFHrp-positive cell lines selected for more detailed analysis were HeLaS3 and HTB9. Both produced FH-related mRNA, based on the expected amplicon size after RT-PCR with primers to three different regions of the predicted FH mRNA. All of the obtained amplicons from HTB9 and HeLaS3 were confirmed as FH by DNA sequencing (data not shown), although numerous single-nucleotide differences were observed, the significance of which is unknown at present. NHEKs and LS174T cells were negative for the three corresponding regions.

Biochemical Activity of Cancer Cell Line-derived FH. We then tested whether MAb affinity-purified HeLaS3 cell culture antigen had decay accelerator activity using the same SDS-PAGE format assay used in testing patient urine and normal serum antigen. The results shown in Fig. 3B demonstrated that the HeLa antigen was active as a cofactor for Fl in the degradation of C3b.

DISCUSSION

This work was undertaken in an attempt to identify new analytes that could be of utility in the management of patients with TCC of the bladder. MAbs were generated to partially purified proteins from urine specimens collected from patients who had been clinically confirmed to have active bladder cancers. Extensive screening of the MAbs yielded an antibody pair with apparent utility in discriminating urine specimens from cancer patients from urine specimens from healthy individuals. Because the immunogen was complex and was only partially
characterized, the identity of the tumor-associated antigen was unknown. Analysis of MAb reactivity with positive specimens from cancer patients indicated that both MAbs bound to a protein with an apparent molecular mass of 138 kDa on SDS-PAGE under nonreducing conditions and an apparent molecular mass of 151 kDa under reducing conditions. Purification of this protein by heparin-agarose and MAb affinity chromatography yielded sufficient quantities to be sequenced by Edman degradation. The eight amino acids identified at the amino terminus were identical to the corresponding residues in the published sequence of human FH, suggesting that the protein was FH or a closely related protein.

The purified antigen was digested with trypsin, and seven major fragments were isolated for amino acid sequencing. These fragments yielded four unique sequences, all of which were consistent with the reported sequence of FH. A major limitation of these results is that these four partial sequences represent less than 6% of the whole molecule (72 of 1231 amino acids, if that molecule is FH). Moreover, identification of the isolated antigen is complicated by the existence of a number of related proteins coded for by alternate mRNA splicing (22). In addition, there are additional mRNA species isolated from the human liver that are related to but not identical to FH (23). In a review by Zipfel and Skerka (24), these putative FH family members have been named FH-like and FH-related. Because of these complications, we have elected to call the urinary antigen FH-like protein rather than FH.

The functional similarity of the urine antigen to FH was assessed in experiments designed to determine whether the isolated analyte had biochemical activities similar to those of FH. We chose to test for the presence of two properties of FH: (a) complement factor C3b binding; and (b) the ability to serve as a cofactor for FI in the proteolysis of C3b. The experimental results confirmed that affinity-purified antigens from urine possessed both biological activities. The control experiments in which the MAbs were used to purify serum FH showed that the purified product had both biological activities.

We chose to begin our investigations into the source of the antigen with a model system, specifically, cancer cell lines in vitro. RT-PCR of total cellular RNA using PCR primers designed from the reported liver FH cDNA sequence demonstrated the expression of FH-like mRNA in many cancer cell lines. The product was confirmed as FH-like by DNA sequencing (data not shown). Analysis of matching cell culture supernatants for many of the cell lines by sEIA demonstrated the presence of antigen. Finally, MAb affinity-purified antigen from HeLaS3 cell cultures had the decay accelerator activity of FH and the appropriate size by SDS-PAGE. This activity could not be attributed to FBS in the cell culture media, because the MAbs do not bind to any proteins found in FBS (Table 3) or mouse serum and seem to be species specific for human FH. The HeLaS3 and HTB-9 RT-PCR products were cloned and sequenced. Riboprobes were generated to allow further investigation of the nature of the expressed antigen and to allow future testing for FH expression in normal and/or cancerous bladder epithelia.4

Although these studies suggest that bladder tumor cells are a source of the FH or FH-like antigen in the urine of bladder

cancer patients, other sources may also exist. Microhematuria has long been used as a diagnostic aid in detection of bladder cancer (25), suggesting that bleeding resulting from the tumor invasion process is also a source of FH. However, the presence of analyte in the urine of patients in whom there is no evidence of hematuria, such as those with CIS, and the direct correlation of urine analyte concentrations with tumor stage and grade (5) point toward a different source of the antigen. Inflammation is a general hallmark of cancer, and inflammatory processes are mediated by complement. It could be that macrophages, which have been shown to produce complement proteins (including FH; Ref. 26), are the source of the antigen, although the concentrations in urine specimens from advanced-stage cancer patients (5) are clearly too high for a macrophage source. It is also known that mesangial cells of the kidney express alternative pathway complement proteins, including FH (27). However, FH was not found in significant concentrations in the urine of normal individuals, and its size is more than double the filtration cutoff size for normal kidney, taken at M₅₀ 65,000 based on albumin (28).

FH production has also been reported in cultured glioma cells (29). Finally, it is possible that bladder epithelia are a source of FH and related proteins.

The biological significance of the expression of FH in cancer cell lines is unknown at this time. However, the complement regulatory role of FH suggests a model for a growth advantage of cancer cells expressing FH or FH-like proteins. FH prevents target cell lysis in the alternative (nonantibody-requiring) complement pathway by promoting the dissociation of the C3-activating convertase (decay accelerating factor activity) and by promoting the proteolytic degradation of C3b by FI, thus preventing complement fixation. The ability of soluble FH to promote the proteolytic degradation of C3 to iC3b on human melanoma cell lines in vitro has been reported, and this effect of FH was apparent although the alternative complement pathway was inactivated, and cell lysis was mediated by the classical complement pathway in the presence of MAb to a cell surface antigen (15). Taken together, these data suggest that synthesis of FH by cancer cells could be a very effective method of evading immune surveillance. Recent reports (30–33) of other complement regulatory proteins expressed on cancer cell membranes would seem to add credence to the hypothesis that cancer cells synthesize complement regulators to evade lysis by the immune system.

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