Characterization of a Novel Human Corneal Endothelial Antigen

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The antigenic composition of the human corneal endothelium, a cellular layer essential for maintaining corneal function, has not been well characterized. A novel corneal endothelial antigen was identified by generating a monoclonal antibody (MAb) against normal human corneal endothelial cells. This MAb, designated 2B4.14.1, reacted strongly by immunoperoxidase staining with the endothelium of corneas from all human donors tested but not with other corneal components, including epithelium and stroma. Positive immunohistologic reactions of 2B4.14.1 with several other human tissues, including kidney (parietal epithelium of Bowman's capsule, proximal convoluted tubule, ascending limb of Henle's loop, and distal convoluted tubule), glandular epithelia of numerous organs, and mesothelial linings of several thoracic and abdominal viscera, also were observed. One of the renal antigens recognized by 2B4.14.1 was identified as Tamm-Horsfall glycoprotein (THGP), based on the ability of the antibody to recognize THGP in western immunoblots and the abrogation of immunohistologic reactivity of the antibody by preincubation with purified THGP. These findings raise the possibility that the human cornea expresses a molecule with homeostatic properties similar to those ascribed to THGP. However, it is unlikely that the corneal antigen recognized by 2B4.14.1 is conventional THGP; a MAb specific for THGP did not react with corneal endothelium. Invest Ophthalmol Vis Sci 32:2473-2482, 1991

The corneal endothelium, a cellular monolayer lining the posterior corneal surface, plays a crucial role in the osmotic homeostasis of the cornea. Under normal circumstances, ion pumps located in the endothelial cell membrane and specialized intercellular junctions prevent the accumulation of excessive fluid in the corneal stroma. Failure of the endothelium (as a result of intrinsic corneal disorders such as Fuchs' dystrophy, trauma secondary to intraocular lens implants, or corneal allograft rejection) can lead to corneal edema and opacification. The endothelium of the human cornea has limited regenerative capacity, and these changes often are irreversible.

Despite the importance of the corneal endothelium in maintaining corneal clarity, its structure and function currently are not completely understood. In particular, the antigenic makeup of this cellular layer has not been characterized in detail. A valuable tool for such antigenic discrimination is provided by the monoclonal antibody (MAb) technique; this has provided substantial information regarding the structure and function of molecules expressed by such diverse tissues as hematopoietic cells, thymic stromal cells, vascular endothelium, and renal parenchyma. Although MAbs reactive with corneal epithelium and stromal collagen have been generated, monoclonal reagents directed against corneal endothelium have not been available.

To examine antigen expression of human corneal endothelium, we generated MAbs against normal human corneal endothelial cells. One MAb, designated 2B4.14.1, reacted with all human corneal endothelia tested. Initial characterization of the antigen recognized by this MAb was done using immunohistologic and western blotting techniques.

Materials and Methods

Tissues

Human corneas were obtained from the Carolina Organ Procurement Organization and the Duke University Medical Center Autopsy Service. For histologic examination, they were either snap frozen for cryostat sectioning or fixed in 10% phosphate-buffered formalin or Omnifix (Xenetics, Tustin, CA) and processed to paraffin for routine sectioning. Other formalin-fixed, paraffin-embedded human tissues were provided by the Kathleen Price Bryan Brain Bank, Joseph and Kathleen Bryan Alzheimer's Dis-
ease Research Center, and the Division of Surgical Pathology, Department of Pathology, Duke University Medical Center.

Monoclonal Antibody Production

BALB/c mice were immunized by subcutaneous injection of pooled endothelial cells mechanically stripped from two human corneas and emulsified in complete Freund's adjuvant. Splenocytes were hybridized with the SP2/0 mouse myeloma by routine methods. Several hundred hybrid supernatants were screened by indirect immunofluorescence on frozen sections of human cornea. Strong endothelial staining with minimal staining of other corneal layers was obtained with supernatant from one well from the fusion, designated 2B4. Cells from this well were cloned and subcloned at limiting dilution, and a reactive subclone, designated 2B4.14.1, was selected for further study. This MAb is of the immunoglobulin M kappa subclass, as assessed by enzyme-linked immunosorbent assay.

Preparation of Purified Tamm-Horsfall Glycoprotein

Tamm-Horsfall glycoprotein (THGP) was purified by standard methods. Briefly, crystalline NaCl was added to pooled urine from healthy volunteers to a final concentration of 0.58 M. After a 12-hr incubation at 4°C, precipitated material was collected by centrifugation at 2000 g for 15 min. The pellet was washed four times with cold 0.58 M NaCl, dissolved in distilled H₂O, and dialyzed at 4°C against four changes of distilled H₂O. A second cycle of NaCl precipitation and dialysis then was done. The purity of the resulting preparation was greater than 95% as assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and staining with Coomassie brilliant blue (Fig. 1).

Immunohistology

Indirect immunofluorescence staining was done on cryostat sections of snap-frozen tissue by standard methods, with fluoresceinated goat anti-mouse immunoglobulin as a secondary reagent. Sections stained with normal murine serum (NMS) as a primary reagent served as controls for nonspecific binding. Stained sections were examined with a fluorescence microscope (Zeiss, West Germany).

Immunoperoxidase staining of sections of frozen and fixed tissues was done using the avidin-biotinylated peroxidase complex (ABC) method. Briefly, sections of paraffin-embedded tissues were prepared and deparaffinized by standard methods. In some experiments, sections of tissues initially fixed in formalin were incubated with 0.1% porcine pancreatic trypsin (type II; Sigma, St. Louis, MO) in 0.1% CaCl₂, pH 7.8, for 30 min at 37°C. The sections then were immunostained by capillary action using a Code-On automated stainer (Instrumentation Laboratory, Lexington, MA). In addition to 2B4.14.1, monoclonal anti-THGP (clone 10.32; Accurate Chemical and Scientific, Westbury, NY) was used as a primary antibody in some experiments. Biotinylated goat antimouse immunoglobulin M or goat anti-mouse immunoglobulin G (Vector, Burlingame, CA) were used as
secondary reagents and avidin-DH-biotinylated horse-
radish peroxidase (ABC, Vector) as a tertiary reagent. 
In all experiments, sections stained with NMS as a 
primary reagent were included as negative controls.

For blocking studies, MAb were incubated for 12 hr 
at 4°C with serial twofold dilutions of purified 
THGP. Immunoperoxidase staining then was done as 
described.

Western Blotting

Western blotting was done by standard methods. 
Samples to be analyzed were precipitated with eight 
volumes of cold absolute ethanol, dried in a vacuum 
centrifuge (Hetovac VR-1; Heto, Birkerod, Den-
mark), resuspended in reducing sample buffer, and 
subjected to SDS-PAGE on 10% gels. The gels then 
were blotted onto nitrocellulose membranes 
(Schleicher and Schuell, Keene, NH) using an electro-
phoretic blotting apparatus (Transfer TE 50; Hoefer, 
San Francisco, CA). The blots were preincubated for 
12 hr at 25°C with a dry milk powder-based buffer 
solution (BLOTTO), followed by a 12-hr incubation 
at 4°C with an appropriate dilution of MAb in the 
buffer. After five washes with BLOTTO, the blots 
were incubated for 2 hr with OX20 (rat anti-mouse 
immunoglobulin kappa chain; Bioproducts for 
Science, Indianapolis, IN) labeled with NaI25I (New 
England Nuclear, Boston, MA) using Iodobeads 
(Pierce, Rockford, IL). After five additional washes 
with BLOTTO, the blots underwent autoradiography 
on XAR-5 X-ray film (Eastman Kodak, Rochester, 
NY) with intensifying screens (Lightening Plus; E. I. 
du Pont de Nemours, Wilmington, DE).

Results

Immunostaining of Cornea With 2B4.14.1

Immunoperoxidase labeling of a panel of eight nor-
mal human corneas was done with 2B4.14.1 and its 
parent clone. Endothelial staining was observed with 
all eight specimens. Antibody reactivity was confined 
to the endothelial cells themselves; staining of the 
underlying basement membrane (Descemet's mem-
brane) and other corneal constituents (stroma and epi-
thelium) was absent. Although 2B4 and its clones ini-
tially were screened on frozen tissue sections, 
2B4.14.1 also reacted strongly with tissue fixed with 
Omnifix or formalin and embedded in paraffin. A 
section of human cornea stained by the immunopero-
xidase technique with 2B4.14.1 is shown in Figure 2.

Whole-mount sections of five human globes also 
were stained with 2B4.14.1 by the immunoperoxidase 
method. In addition to corneal endothelial staining, a 
variable amount of staining of ciliary body epithelium 
was noted in all specimens examined, particularly in 
the apical portions of cells. No other ocular structures 
were stained by the MAb.

Corneas and globes from various animal sources, 
including rabbit, guinea pig, rat (PVG), and mouse 
(C3H and BALB/c) also were stained with 2B4.14.1.
No endothelial 2B4.14.1 reactivity was noted in frozen or fixed specimens from these species, although guinea pig ciliary body epithelium was stained by the MAb.

**Immunostaining of Other Tissues With 2B4.14.1**

Immunoperoxidase staining of sections of a panel of formalin-fixed human tissues was done to identify other possible tissue reactivities; these results are shown in Table 1. Reactivity was observed for several tissue types.

Staining of columnar epithelia from various sources, including gastrointestinal (colon, gallbladder, and stomach), respiratory (pharynx and trachea), and female genital (fallopian tube and cervix) organs, was observed. Representative sections of colon, gallbladder, and fallopian tube stained with 2B4.14.1 are shown in Figures 3A–C. In most cases, the staining was in a coarse, granular pattern concentrated at the apical poles of the epithelial cells; more basal staining was noted in some sections. Although 2B4.14.1-reactive epithelia were distributed widely, nonreactive epithelia also were observed for organs of each system examined.

A second group of 2B4.14.1-reactive tissues consisted of mesothelial linings, including epicardium (Fig. 3D), visceral pleura (eg, visceral peritoneum of ovary, liver, and spleen), and tunica vaginalis of testis. The mesothelial reactivity of 2B4.14.1 probably is more general than indicated by Table 1 because meso-

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**Table 1. Tissue distribution of 2B4.14.1 reactivity**

<table>
<thead>
<tr>
<th>System</th>
<th>Tissue</th>
<th>Reactivity</th>
<th>Comments</th>
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<tbody>
<tr>
<td>Digestive</td>
<td>Colon</td>
<td>+</td>
<td>Diffuse epithelial reactivity</td>
</tr>
<tr>
<td></td>
<td>Gallbladder</td>
<td>+</td>
<td>Diffuse epithelial reactivity</td>
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<tr>
<td></td>
<td>Stomach</td>
<td>+</td>
<td>Focal epithelial reactivity</td>
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<td></td>
<td>Small bowel</td>
<td>+</td>
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<td></td>
<td>Esophagus</td>
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<tr>
<td></td>
<td>Pancreas</td>
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<tr>
<td></td>
<td>Liver</td>
<td>−</td>
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<tr>
<td>Genitourinary</td>
<td>Kidney</td>
<td>+</td>
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<td></td>
<td>Fallopian tube</td>
<td>+</td>
<td>Tubules and Bowman’s capsule</td>
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<td></td>
<td>Endocervix</td>
<td>+</td>
<td>Focal epithelial reactivity</td>
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<td></td>
<td>Uterus</td>
<td>−</td>
<td>Focal epithelial reactivity</td>
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<td></td>
<td>Prostate</td>
<td>−</td>
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<td></td>
<td>Exocervix</td>
<td>−</td>
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<td></td>
<td>Ovary</td>
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<td></td>
<td>Testis</td>
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<tr>
<td>Respiratory</td>
<td>Pharynx</td>
<td>+</td>
<td>Columnar epithelium reactive</td>
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<td>Trachea</td>
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<td>Focal epithelial reactivity</td>
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<tr>
<td></td>
<td>Lung</td>
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<td>Endocrine</td>
<td>Thyroid</td>
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<td>Colloid extensively reactive</td>
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<td>Pituitary</td>
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<td>Focal staining of adrenohypophysis; inclusion cysts</td>
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<tr>
<td></td>
<td>Parathyroid</td>
<td>+</td>
<td>Focal staining; possibly thyroid colloid contamination</td>
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<tr>
<td>Other</td>
<td>Eye</td>
<td>+</td>
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<td></td>
<td>P'earst</td>
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<td></td>
<td>'acenta</td>
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<tr>
<td></td>
<td>Umbilical cord</td>
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<td>Skin</td>
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</table>

* Staining of mesothelium in the absence of parenchymal staining.

† Rare focal epithelial staining of uncertain significance.

‡ Smooth muscle staining in some specimens.
thelial linings were not sampled adequately in many of the tissue sections used for staining, and several potentially reactive tissues, such as parietal pleura and peritoneum, were not examined.

Tissues from several additional organs reacted with 2B4.14.1, including thyroid (colloid), pituitary gland (inclusion cysts, presumably of pharyngeal epithelial origin, and foci in the adenohypophysis), and kidney. Additionally, weak focal staining of myometrium and cervical smooth muscle was observed in some (but not all) uterine specimens. No staining of vascular endothelium was observed in any of the tissues studied.

The reactivity of 2B4.14.1 with tumors was not examined systematically. However, strong reactivity was observed with a gastric adenocarcinoma, and less intense, focal staining was seen with a colonic adenoma. No staining could be detected in sections of
breast carcinoma, melanoma, carcinoid tumor, or osteogenic sarcoma.

Renal Reactivity of 2B4.14.1

The staining pattern of 2B4.14.1 on sections of kidney was particularly interesting. In the cortex, intense staining of a small subpopulation of tubules and less intense staining of the parietal epithelium of Bowman's capsule was seen (Fig. 4A). In fortuitous sections, some of the stained tubules could be identified as proximal tubules by their contiguity with Bowman's capsule (Fig. 4B). However, most immunoreactive tubules had a low cuboidal epithelium characteristic of distal convoluted tubules. In many of these,
2B4.14.1 reactivity was patchy, with alternating staining and nonstaining epithelial cells (Fig. 4C).

Renal medullary staining generally was limited to a subpopulation of moderately sized tubules near the corticomedullary junction (Fig. 4D). The morphology and location of these tubules suggested that they belonged to the thick ascending limb of Henle's loop. Occasional intratubular casts also stained with 2B4.14.1.

**Recognition of THGP by 2B4.14.1**

Staining of distal convoluted tubules and the ascending limb of Henle's loop with antibodies against THGP, a major constituent of renal casts, has been reported by several investigators.\(^1\) In one study, a patchy distribution of tubular staining similar to that observed for 2B4.14.1 was documented by immunoelectron microscopy.\(^2\) The potential reactivity of 2B4.14.1 with THGP therefore was examined.

A western immunoblot of purified THGP with 2B4.14.1 is shown in Figure 5. Strong reactivity with a band of appropriate molecular weight (approximately 85,000 Daltons) was seen with the MAb. Successful blotting required reduction of the THGP sample before electrophoresis. A control blot stained with POP.I4.3, an immunoglobulin M kappa MAb that recognizes the human class II histocompatibility (HLA) antigen-associated invariant chain,\(^3\) did not react.

To ensure that the reactivity of 2B4.14.1 with THGP observed by immunoblotting was not an artifact induced by denaturation or reduction of the antigen, the ability of native purified THGP to inhibit tissue immunostaining with 2B4.14.1 was assessed. Preincubation of a 1:4000 dilution of 2B4.14.1 ascites with THGP at a concentration as low as 15 µg/ml totally abrogated staining of the cornea, kidney, and colon; results for representative sections of renal medulla are presented in Figure 6. Preincubation with THGP had no effect on the immunoreactivity of various control MAbs. Preincubation of 2B4.14.1 with purified THGP also blocked its ability to react with THGP in western blots (data not shown).

The distribution of THGP reportedly is limited to the kidney, as assessed by immunostaining and northern blotting with cDNA probes for the antigen.\(^4\) However, examination of corneal tissue for the possible presence of THGP has not been reported. We therefore stained sections of cornea with clone 10.32, a commercially available anti-THGP MAb. No reactivity was observed for endothelium or any other corneal layer. Parallel examination of sections of renal tissue revealed the expected staining of ascending limb of Henle's loop and distal convoluted tubules; this was somewhat more intense than that observed with 2B4.14.1 (data not shown). However, in agreement with published reports,\(^5\) no staining of Bowman's capsule or proximal tubules was seen.

**Discussion**

We described the identification of an antigen expressed in human corneal endothelium by reactivity with 2B4.14.1, a MAb generated specifically against human corneal endothelial cells. To our knowledge, 2B4.14.1 represents the first MAb raised against corneal endothelium of any species. It also was found to react with kidney (Bowman's capsule and a subset of tubules), columnar epithelia from several sources, and thoracic and peritoneal mesothelia.

To date, information regarding antigenic molecules of the corneal endothelium is limited. Other investigators\(^6\) produced xenonantisera with endothelial reactivity by immunizing ducks with homogenized rabbit corneas. Cell surface expression of the antigens recognized by these sera reportedly was limited to metabolically active and/or proliferating cells, although the antigens appeared to be present in resting cells in a cryptic form. The only constitutively expressed cor-
neal antigens identified by these authors were said to exhibit wide tissue distribution. In other studies, immunoreactive serum proteins were described in bovine corneas, including the endothelial layer.28

The presence of HLA antigens on human corneal endothelial cells also was reported. Several studies detected HLA class I antigens on corneal endothelium.29,30 Although class II antigens appear to be absent from normal endothelium,29-31 they were detected on the endothelium of rejected corneal allografts31 and on cultured endothelial cells stimulated in vitro with interferon-γ.32

Antibodies against myosin, myoglobin, and a vascular endothelial antigen were reported to react with human corneal endothelium.33 Cultured corneal endothelial cells of various species also were found to produce fibronectin,34 collagen,35,36 several glycosaminoglycans,37 and a confluence-specific surface protein termed CSP-60.38 None of these antigens has a tissue distribution matching the reactivity pattern of 2B4.14.1.

We identified one of the renal antigens recognized by 2B4.14.1 as THGP. This antigenic specificity presumably explains the reactivity of 2B4.14.1 with distal convoluted tubules and the ascending limb of the loop of Henle, both documented sites of THGP expression.19-21 It is unlikely, however, that conventional THGP is the antigen recognized by 2B4.14.1 in other tissues, including Bowman’s capsule, proximal tubule, and corneal endothelium. Although purified THGP was able to block all tissue reactivities of 2B4.14.1, a MAb raised against THGP reacted exclusively with distal convoluted tubules and the ascending limb of Henle’s loop.

The 2B4.14.1-reactive epitope of THGP and other molecules recognized by the MAb has not been identified. One possible candidate for the epitope that we considered was the Sd<sup>α</sup> blood group substance, a pentasaccharide antigen shared by up to 96% of whites that is carried by THGP and expressed by colonic epithelium.39-42 However, erythrocytes from two Sd<sup>α</sup>-individuals did not bind 2B4.14.1 (as assessed by flow cytometry). Furthermore, no reactivity of Bowman’s capsule was detected in sections of human kidney stained by the ABC method with biotinylated *Dolichos biflorus* lectin, a sensitive probe for the Sd<sup>α</sup> oligosaccharide.39,40 Intense staining of tubules and casts in an appropriate distribution for THGP was present in these sections (data not shown). Other putative oligosaccharide ligands for 2B4.14.1 currently are being investigated.

It is possible that the 2B4.14.1-reactive tissue sites that do not bind conventional anti-THGP antibodies express an altered or incompletely processed form of THGP or a homologous protein sharing the same antigenic determinant. In this regard, it is interesting that western immunoblots of detergent-solubilized human renal tissue with 2B4.14.1 frequently exhibit a band at approximately 65,000 Daltons, close to the reported molecular weight of the peptide core of THGP.
THGP\textsuperscript{23,43} (data not shown). Further experiments investigating the nature of this additional 2B4.14.1 reactivity are in progress.

If the corneal antigen recognized by 2B4.14.1 is related to THGP, interesting questions regarding its functional role can be raised. Although the function of THGP is unclear, one of its postulated roles is the diminution of water permeability in the distal nephron.\textsuperscript{19,20} Because a primary function of corneal endothelium is to retard the influx of water into the cornea, the presence of an endothelial protein with this property is plausible. Both THGP and a similar or identical urinary protein, uromodulin, were reported to possess significant immunosuppressive function.\textsuperscript{39,44} A related corneal molecule therefore might be significant in the setting of corneal allografting.

**Key words:** cornea, endothelium, antigen, kidney, Tamm-Horsfall glycoprotein

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**References**


