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Pseudomonas aeruginosa Infection of the Cornea and Asialo GM_1

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Extensive immunohistochemical and thin-layer chromatogram-immunostain analyses were carried out to establish whether asialo GM_1, a glycolipid which contains binding sites for *Pseudomonas aeruginosa*, is present in corneal epithelium. The data suggest that rabbit corneal epithelium does not contain detectable levels of asialo GM_1 even after corneas are scarified and incubated with trypsin, *P. aeruginosa*, or *P. aeruginosa* exoproducts to expose potential cryptic sites. Preliminary immunohistochemical analyses indicated that asialo GM_1 is also not found in human corneas.

There is much interest in cell surface glycoconjugates which serve as attachment sites for bacteria and play a role in the pathogenesis of infections (5). The study reported herein concerns a glycolipid which has been shown to have binding sites for *Pseudomonas aeruginosa*. In vitro studies conducted using a thin-layer chromatogram-bacterial overlay assay have demonstrated that *P. aeruginosa* binds to the GalNAcβ1-4Gal sequence present in asialo GM_1, asialo GM_2, and related glycolipids (6, 7) and more recently, the pilus adhesin of *P. aeruginosa* PAK and PAO has been shown to bind to asialo GM_1 (9) as well as to synthetic GalNAcβ1-4Gal disaccharides (15).

P. aeruginosa is a common cause of corneal infection, especially in wearers of extended-wear contact lenses (1). The mechanisms by which *Pseudomonas* organisms adhere to the cornea and cause infection have not been fully elucidated. Recently, on the bases of the observations that (i) scarified mouse corneas, when analyzed after preincubation with trypsin or *P. aeruginosa* organisms to expose cryptic sites, stained positively with antibodies to asialo GM_1 and (ii) preincubation of scarified corneas with anti-asialo GM_1 reduced the binding of the bacteria to the corneal surface, Hazlett et al. (3) suggested that asialo GM_1 is a corneal glycolipid receptor for *P. aeruginosa* binding. In the present communication, on the bases of immunohistochemical and high-performance thin-layer chromatography (HPTLC)-immunostain analyses, we report that rabbit corneal epithelium does not contain detectable levels of asialo GM_1.

In the studies reported here, rabbit corneal epithelia from animals in vivo, corneas in organ culture, and cells in tissue culture were analyzed for the presence of asialo GM_1. Since injury predisposes corneas to pseudomonal infection, scarified corneas and corneas with superficial epithelial wounds were analyzed.

Superficial epithelial wounds were produced by removing the epithelium from the central cornea (8-mm diameter) of anesthetized animals (New Zealand White rabbits) or rabbit eyes (Pel-Freez Biologicals, Rogers, Ark.) with a blade. Wounded corneas were allowed to heal partially in vivo or in organ culture (11) for 24 h and were then processed for preparing cryosections for immunohistochemical analyses. For HPTLC-immunostain analyses, epithelia were collected from the surface of healing corneas in organ culture by scraping with a blade, and harvested epithelia from 75 to 100 corneas were pooled, lyophilized, and processed for isolation of lipids. In one study, rabbit corneas were scarified with a 22-gauge needle (eight 4- to 5-mm linear incisions) as described by Hazlett et al. (3), and organ cultured in medium alone or medium containing trypsin (0.22 U/ml; Sigma Chemical Co., St. Louis, Mo.), *P. aeruginosa* (derived from a clinical bacterial corneal ulcer; 10^8 CFU/ml), or a stationary-phase culture supernatant of *P. aeruginosa* for 50 to 60 min prior to immunohistochemical analyses to expose any cryptic sites or to modify corneal glycolipids by glycosidases which may be produced by the organisms.

Both sparse and confluent primary cell cultures of rabbit corneal epithelium prepared as described previously (4, 11) were also analyzed for the presence of asialo GM_1. In some experiments, prior to harvesting the cells for HPTLC-immunostain analyses, we treated the cultures with *P. aeruginosa* (10^8 CFU/ml) or a stationary-phase culture supernatant of *P. aeruginosa* for 50 min.

For immunohistochemical analyses, frozen sections were stained with anti-asialo GM_1 (monoclonal antibody [MAb] TKH-7, obtained from S.-I. Hakomori and E. Nudelman, Biomembrane Institute, Seattle, Wash.) and antisulfatide (MAb BRD1; positive control, provided by F. B. Jungalwala, E. K. Shriver Center, Waltham, Mass.), using an avidin biotin peroxidase method (10). The specificity of MAb TKH-7 to asialo GM_1 was confirmed by HPTLC-immunostain analysis (Fig. 1). For HPTLC-immunostaining, lyophilized epithelia from cell cultures and corneas in organ culture were subjected to a series of extraction procedures to isolate either a total lipid fraction or a lower phase containing neutral glycolipids (2). Prior to use, lipid samples were saponified to eliminate phospholipids. To determine whether asialo GM_1 is present in the isolated lipid fractions, HPTLC-immunostaining as described by Yamamoto et al. (16) was performed. Briefly, duplicate aliquots of an asialo GM_1 standard or lipid samples derived from 1 to 4 mg of original cell protein were chromatographed on aluminum-backed HPTLC plates. After chromatography, one lane of each sample was stained with orcinol (8) to visualize glycolipids and the other was stained with MAb TKH-7 or BRD1.

In immunohistochemical analyses, anti-asialo GM_1 failed to react with all corneas analyzed. Scarified corneas failed to react with the antibody even after they were exposed to trypsin, *P. aeruginosa*, or *P. aeruginosa* culture supernatant (Table 1; Fig. 2). In HPTLC-immunostain analyses also, anti-asialo GM_1 failed to react with the lower-phase (Fig. 3; Table 1) or total lipid (not shown) samples of all epithelia analyzed. A positive reaction with the antibody could not be detected even when

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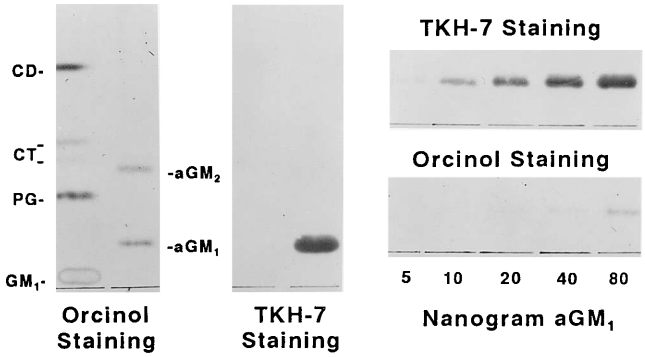

FIG. 1. HPTLC-immunostaining showing specificity of MAb TKH-7 and sensitivity of the assay. CD, ceramidedihexoside; CT, ceramidetrihexoside; PG, paragloboside; aGM_1, asialo GM_1; aGM_2, asialo GM_2.

lipid samples of corneal epithelium were overloaded on HPTLC plates (Fig. 3) or cultures were treated with *P. aeruginosa* or *P. aeruginosa* culture supernatant for 50 min prior to analysis. Samples derived from as much as 4.0 mg of the original cell protein did not produce even a weak positive reaction with anti-asialo GM_1. In contrast, samples derived from 1 mg of original cell protein were sufficient to detect a positive reaction with antiparagloboside (13) and antisulfatide (12a). The HPTLC-immunostain assay used was sensitive. An intense positive reaction was produced with 100 ng of asialo GM_1, and even as little as 5 ng of standard asialo GM_1 could be detected (Fig. 1). At this sensitivity level of the assay, even low levels of antigen, if present in corneal epithelia, would have been detected. As shown in Fig. 3, in lipid samples of corneal epithelia, an orcinol-positive component with a relative mobility similar to that of standard asialo GM_1 was visualized. Since this component did not react with MAb TKH-7, it is likely to be a glycolipid(s) distinct from asialo GM_1. In some experiments, prior to HPTLC-immunostaining, standard asialo GM_1 was added to the lipid samples of corneal epithelium. Positive immunostaining of such mixtures with MAb TKH-7 ensured

TABLE 1. Various rabbit corneal epithelia tested for reactivity with anti-asialo GM_1 (MAb TKH-7) by immunohistochemical and HPTLC-immunostain analyses[a]

Cornea	Assay method[b]	Treatment
Superficially injured	IH[c]	In vivo healing for 24 h
Scarified	IH	Organ culture in media alone
Scarified	IH	Incubation with trypsin
Scarified	IH	Incubation with *P. aeruginosa*
Scarified	IH	Incubation with *P. aeruginosa* supernatant
Normal	IH	None
Normal	HPTLC-IS	None
Superficially injured	HPTLC-IS	Organ culture in media alone
Epithelial cell cultures[d]	HPTLC-IS	None
Epithelial cell cultures	HPTLC-IS	Incubation with *P. aeruginosa*
Epithelial cell cultures	HPTLC-IS	Incubation with *P. aeruginosa* supernatant

[a] Reactivity with MAb TKH-7 was not detected in any case.
[b] IH, immunohistochemical analysis; IS, immunostain.
[c] Frozen sections of superficially injured human corneas were also tested for reactivity with MAb TKH-7 before and after incubation with trypsin, *P. aeruginosa*, and *P. aeruginosa* culture supernatant ($n = 2$ for each treatment). Reactivity with antibody was not detected in any case.
[d] Both confluent ($n = 6$) and sparse ($n = 3$) cultures were analyzed.

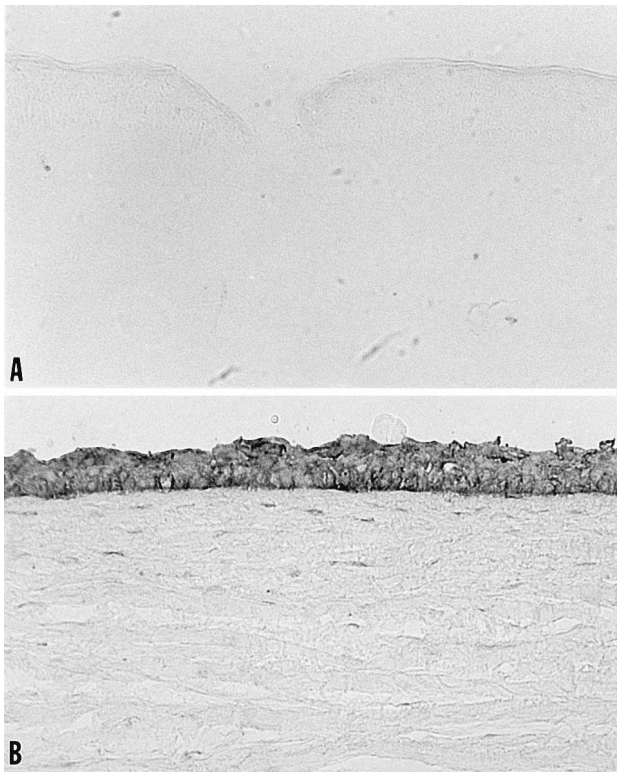

FIG. 2. Scarified rabbit cornea stained with anti-asialo GM_1 (MAb TKH-7) after organ culture for 60 min in medium containing trypsin (A) and a normal cornea stained with antisulfatide (B; MAb BRD1, positive control). Asialo GM_1 was not detected in scarified corneas cultured in medium alone, medium containing trypsin, medium containing *P. aeruginosa*, or medium containing *P. aeruginosa* culture supernatant (see Table 1 for details).

that the antibody binding sites were not masked by the presence of lipids in the same region in the chromatograms as asialo GM_1.

Data presented in this report, using rabbit corneas, differ from data reported by Hazlett et al. (3) in which a positive immunohistochemical staining of scarified mouse corneas was detected with anti-asialo GM_1. We have no explanation for the differences between the results of the two studies. Immunohistochemical analyses are usually more convincing when supported by biochemical analyses such as those carried out in the present study. Furthermore, in the study reported by Hazlett et al. (3), even the stromal matrix reacted positively with the antibody, an observation difficult to reconcile with the fact that glycolipids are not usually found in the extracellular matrix. The studies utilized different MAbs, and assays were performed on different animal species. In an attempt to determine whether the differences between the two studies could be species related, we also performed immunohistochemical analyses of human and mouse corneas. Frozen sections of normal and wounded human corneas did not react with anti-asialo GM_1 (Table 1, footnote *c*), and a nonspecific reaction was detected in mouse corneas. Since corneas are rich in immunoglobulins, it would be expected that the secondary antibody, anti-mouse immunoglobulin M, would react with the mouse corneas even in the absence of a primary antibody. In the studies by Hazlett et al. (3), however, nonspecific staining of mouse corneas with anti-mouse immunoglobulins was not detected.

In brief, the present study indicates that rabbit corneal epithelium does not contain detectable levels of asialo GM_1.

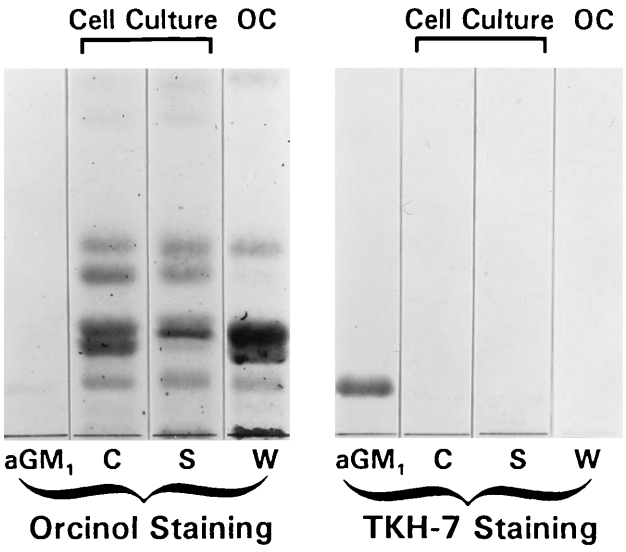

FIG. 3. Immunostaining of glycolipids in the lower-phase samples of epithelia from wounded rabbit corneas (W) in organ culture (OC) and confluent (C) and sparse (S) cell cultures. Samples derived from 4 mg of the original cell protein were loaded in each lane. Asialo GM_1 (aGM_1) standard stained intensely with the antibody at a 100-ng concentration. Asialo GM_1 was not detected in any lipid samples of corneal epithelia nor even when corneal epithelial cell cultures were incubated with *P. aeruginosa* or *P. aeruginosa* culture supernatant for 50 min prior to isolation of lipid fractions for analysis (see Table 1 for details).

Specific molecules of corneal epithelium which serve as attachment sites for *P. aeruginosa* remain to be identified. We have previously shown that several components in the lipid samples prepared from rabbit corneal epithelial cell cultures contain *P. aeruginosa* binding sites (12). The identity of these components must await further studies.

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