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Acanthamoebae Bind to Glycolipids of Rabbit Corneal Epithelium

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By use of a thin-layer chromatogram (TLC) overlay procedure, ^{35}S-labeled acanthamoebae were shown to bind to seven glycolipids of rabbit corneal epithelium. Corneal epithelial cells were grown in culture and were subjected to Folch extraction to isolate a chloroform-rich lower phase containing neutral glycosphingolipids (NGSL) and an aqueous upper phase containing gangliosides, i.e., sialic acid-containing glycolipids. Thin-layer chromatography of the upper phase revealed the presence of 10 ganglioside components. Acanthamoebae were shown to bind to four of these components, referred to as 2, 3, 6, and 7. On TLC plates, ganglioside components 2 and 3 migrated slightly ahead of the glycolipid standard GD1a, component 7 comigrated with standard GM_3, and component 6 migrated a little more slowly than GM_3. Likewise, of the 10 NGSL known to be present in the lower phase, acanthamoebae bound to components 1, 5, and 6. NGSL components 1, 5, and 6 migrated on TLC plates with relative mobilities similar to those of standards asialo GM_1, asialo GM_2, and ceramidetrihexoside, respectively. We propose that one or more of the *Acanthamoeba*-reactive glycolipids of corneal epithelium identified in this study may play a role in the pathogenesis of *Acanthamoeba* keratitis by mediating the adherence of the parasites to the cornea.

Acanthamoeba keratitis is a sight-threatening corneal infection (2, 9). It is caused by parasites of the genus *Acanthamoeba* and is characterized by intense pain and a slowly worsening clinical course. The number of reported cases of *Acanthamoeba* keratitis has increased sharply in recent years (26). This increase has been associated with an increase in the use of soft contact lenses (8, 12, 16, 24, 25). However, the disease also occurs, if less often, in noncontact lens wearers (23).

The mechanism by which acanthamoebae infect the human cornea has not been elucidated. It is generally believed that minor trauma to the corneal epithelium caused by contact lens wear or other noxious agents and exposure to contaminated solutions, including lens care products and tap water, are two important factors in the pathogenesis of the keratitis. Adherence of the pathogenic organisms to the tissues of the host is considered to be the initial step and an important virulence factor in the establishment of most infections. In studies of ocular as well as nonocular infections, cell surface glycosphingolipids of host tissues have been reported to act as receptors for a wide variety of pathogenic bacteria and viruses (13, 21). This study was designed to determine whether glycolipids of rabbit corneal epithelium contain binding sites for acanthamoebae.

An *Acanthamoeba* strain derived from an infected human cornea (MEEI 0184, probably *Acanthamoeba castellinii*), provided by Govinda S. Visvesvara, Centers for Disease Control, Atlanta, Ga., was used throughout this study. This strain has also been shown to cause a cytopathic effect in monkey kidney cell cultures (22). The parasites were axenically cultured in a Proteose Peptone-yeast extract-glucose (PYG) medium (5). For radiolabeling, acanthamoebae (2.0×10^6 parasites per ml, greater than 95% trophozoites) were incubated with PYG medium containing 60 μCi of [^{35}S]methionine (575 to 650 Ci/mmol; DuPont Co., Boston, Mass.) per ml for 48 h at 35°C.

Neutral glycosphingolipids (NGSL) and gangliosides were isolated from rabbit corneal epithelium grown in culture by using a modification of the extraction procedure of Folch et al. (10). This procedure yielded a lower phase containing NGSL, phospholipids, and cholesterol and an upper phase containing gangliosides. To eliminate phospholipids, the lower phase was saponified prior to use (20).

To identify which particular NGSL and gangliosides of corneal epithelium contain binding sites for acanthamoebae, a thin-layer chromatogram (TLC) overlay procedure (11, 15) was used. Briefly, duplicate aliquots of the lower phase, representing 1 mg of cell protein, and the upper phase, representing 4 mg of cell protein, were chromatographed on a TLC plate (HPTLC; Alufolien). Solvent systems consisting of chloroform–methanol–H_2O (65:25:4) and chloroform–methanol–0.25% $CaCl_2$ (50:40:10) were used for chromatography of the lower phase and upper phase, respectively. One lane of each sample was stained with an orcinol spray (14) to visualize glycolipids, and the other was processed for the *Acanthamoeba* binding assay. For the binding assay, the chromatograms were treated with 0.05 or 0.1% polyisobutylmethacrylate and then incubated with 2% bovine serum albumin in phosphate-buffered saline (PBS) and ^{35}S-labeled acanthamoebae (3.5×10^6 parasites per ml, 1 to 2 cpm per parasite) for 2 h each. After incubation with radiolabeled parasites, the plates were extensively washed with PBS containing 0.05% Triton X-100, air dried, sprayed with En^{3}Hance (Dupont, NEN Research Products, Boston, Mass.), and then subjected to fluorography. In one experiment, chromatograms of upper-phase samples were stained with resorcinol to visualize gangliosides.

Acanthamoebae were found to bind to three corneal epithelial NGSL (components 1, 5, and 6; Fig. 1) and four corneal epithelial gangliosides (components 2, 3, 6, and 7; Fig. 2). All four *Acanthamoeba*-reactive gangliosides reacted positively with resorcinol, a stain specific for sialic acid. To ensure reproducibility of the data, material from six and two different preparations of lower and upper phases, respectively, was analyzed. Reproducible results were ob-

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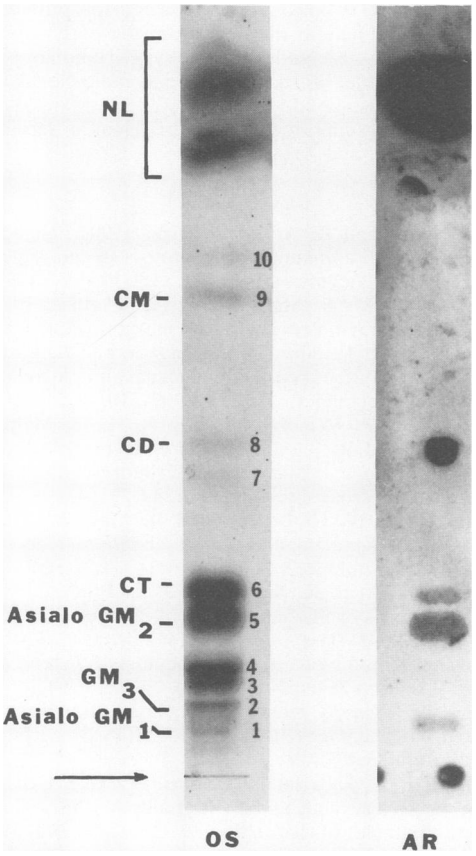

FIG. 1. Binding of acanthamoebae to neutral glycosphingolipids of rabbit corneal epithelium in culture. Migration positions of various standards are shown at the left. Abbreviations: OS, orcinol staining; AR, autoradiography after incubation with ^{35}S-labeled acanthamoebae; CM, ceramidemonohexoside; CD, ceramidedihexoside; CT, ceramidetrihexoside. The arrow indicates the origin.

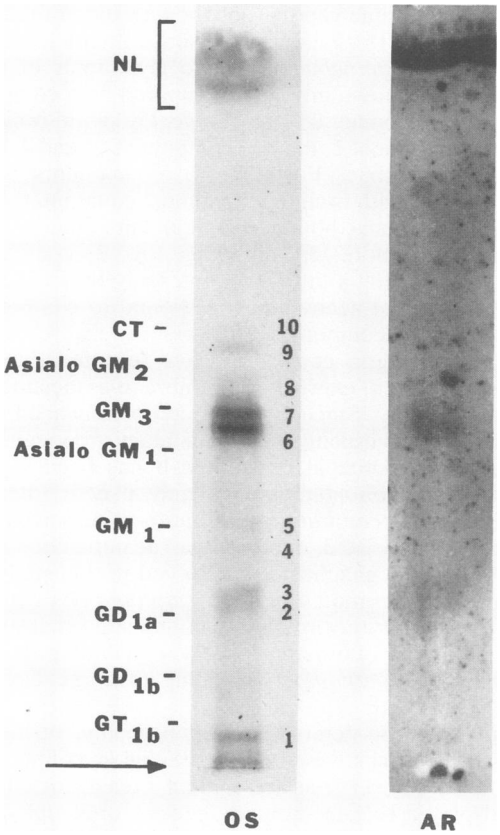

FIG. 2. Binding of acanthamoebae to gangliosides of rabbit corneal epithelium in culture. Migration positions of various standards are shown at the left. Abbreviations: OS, orcinol staining; AR, autoradiography after incubation with ^{35}S-labeled acanthamoebae; CT, ceramidetrihexoside. The arrow indicates the origin.

tained with both preparations of upper phases. *Acanthamoeba* binding to NGSL components 1 and 5 was present in chromatograms of all six preparations, whereas binding to component 6 was observed in chromatograms of only four of the six preparations. With respect to standards, corneal epithelial NGSL 1, 5, and 6 migrated with relative mobilities similar to those of standards asialo GM_1, asialo GM_2, and ceramidetrihexoside, respectively (Fig. 1). With regard to gangliosides, components 2 and 3 were not well resolved and migrated slightly ahead of GD1a, component 7 comigrated with GM_3, and component 6 migrated a little slower than component 7 (Fig. 2). Acanthamoebae also bound to standard GM_3 but to a lesser degree than to component 7 (21a). Although NGSL 1 and 6 comigrated with asialo GM_1 and asialo GM_2, they were found not to react positively with monoclonal antibodies to asialo GM_1 and asialo GM_2 when tested by a solid-phase immunoassay using the procedure developed by Magnani et al. (15). In contrast, standards asialo GM_1 and asialo GM_2 were found to react positively with their respective monoclonal antibodies. Since nonspecific *Acanthamoeba* binding was observed in the area of the solvent front in the chromatogram where large amounts of neutral lipids such as cholesterol and fatty acids are expected to be present, in a separate experiment, we tested *Acanthamoeba* binding to various concentrations of cholesterol. In a TLC overlay assay, *Acanthamoeba* binding to cholesterol was observed at a concentration of 15 μg and higher. In the autoradiograph, the intensity of the band due to *Acanthamoeba* binding to 15 μg of cholesterol was weaker than those of the bands due to binding to any of the three *Acanthamoeba*-reactive corneal epithelial NGSL derived from 1 mg of cell protein. We have established previously (21) that in the lower phase derived from 1.0 mg of cell protein, there is less than 1 μg of any particular NGSL. *Acanthamoeba* binding to cholesterol is not likely to be of relevance in the pathogenesis of *Acanthamoeba* keratitis because it was observed at a relatively high concentration of cholesterol. Furthermore, because of the nonpolar nature of the sterol, most of the molecule, with the exception of its hydroxyl group, is likely to be buried within the cell membrane and not available for binding interactions. To further ensure that *Acanthamoeba* binding to corneal epithelial glycolipids observed in this study was not simply due to binding to neutral lipids, thin-layer chromatograms were stained with iodine vapors and the staining patterns were compared with those of the *Acanthamoeba* binding patterns. No correlation was observed between the ability of corneal epithelial glycolipids to bind to the amoeba and stain with iodine vapors. While component 5 (Fig. 1), which reacted intensely with iodine vapors (not shown), also stained intensely with acanthamoebae, components 6 and 7 (Fig. 2), which did not react with iodine vapors, also stained with acanthamoebae and component 9 (Fig. 2), which stained

intensely with iodine vapors (not shown), did not react with acanthamoebae. The following evidence points to the specificity of *Acanthamoeba* binding to glycolipids of corneal epithelium. As shown in Fig. 1, compared with components 3 and 4, component 1 stained weakly with orcinol; in contrast, component 1, but not components 3 and 4, stained with acanthamoebae. Furthermore, components 5 and 6 (Fig. 1) stained with orcinol with almost equal intensity, but more acanthamoebae bound to component 5 than to component 6. Furthermore, as shown in Fig. 2, component 1 stained with orcinol a little more intensely than did components 2 and 3, but acanthamoebae bound to components 2 and 3 and not to component 1.

Recently Pellegrin et al. (22) have reported that *Acanthamoeba* species express a membrane-associated neuraminidase activity. Since microbial neuraminidase has been implicated as a virulence factor in many nonocular viral, bacterial, and protozoal infections, it has been suggested that the *Acanthamoeba* enzyme may play a role in the pathogenesis of *Acanthamoeba* keratitis (22). Since gangliosides contain sialic acid, the binding of acanthamoebae to the corneal epithelial gangliosides observed in this study could well have been promoted by the *Acanthamoeba* neuraminidase. However, a recent study has indicated that mannose but not a number of other saccharides, including sialic acid, could inhibit *Acanthamoeba* binding to corneal epithelial cells (17).

It remains to be determined which, if any, of the seven *Acanthamoeba*-reactive corneal epithelial glycolipids identified in this study serve as attachment sites for acanthamoebae and play a role in the pathogenesis of *Acanthamoeba* keratitis. Interestingly, in a recent study, we have shown that *Acanthamoeba*-reactive NGSL components 1, 5, and 6 also contain binding sites for *Pseudomonas aeruginosa* (21). However, it remains to be determined whether the parasites and the bacteria bind to the same sites on the receptor. Clearly, the mechanism of *Acanthamoeba* adherence to the cornea may vary among animal species and parasitic strains. To this end, Niederkorn et al. (18) have recently reported that the *A. castellanii* strain (ATCC 30868) that they used failed to produce a cytopathic effect and did not bind to corneal epithelia of mice, rats, cotton rats, horses, guinea pigs, cows, chickens, dogs, and rabbits but adhered to and infected human, pig, and Chinese hamster corneas. In contrast, our strain binds to corneal epithelium of intact rabbit corneas (21a). In this study, a rabbit model was chosen because we previously analyzed lectin binding patterns of corneal epithelia of eight species and have established that the lectin binding patterns of the corneal epithelia of rabbits and dogs are most similar to that of human corneal epithelium (19).

In a recent study, it was shown that mannose residues, found in glycoproteins but not in glycolipids, could inhibit binding of acanthamoebae to corneal epithelium in culture (17). Likewise, other studies (1, 7) have implicated the presence of a mannose-specific lectin on the surface of acanthamoebae. These studies and other reports (3, 4, 6, 7) which implicated the presence of mannose residues on the corneal epithelium lead us to suggest that in addition to cell surface glycolipids, plasma membrane glycoproteins of corneal epithelium may serve as attachment sites for acanthamoebae and play a role in the pathogenesis of *Acanthamoeba* keratitis.

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REFERENCES

1. **Allen, P. G., and E. A. Dawidowicz.** 1990. Phagocytosis in *Acanthamoeba*: a mannose receptor is responsible for the binding and phagocytosis of yeast. J. Cell. Physiol. **145:**508–513.
2. **Auran, J. D., M. B. Star, and F. A. Jakobiec.** 1987. *Acanthamoeba* keratitis: a review of the literature. Cornea **6:**2–26.
3. **Bonvicini, F., P. Versura, R. Caramazza, and R. Laschi.** 1982. Detection of surface corneal concanavalin A receptors by colloidal gold technique. Cornea **1:**309–310.
4. **Bonvicini, F., P. Versura, F. Caruso, M. C. Maltarello, R. Caramazza, and R. Laschi.** 1983. Lectin receptors on human corneal epithelium: visualization by fluorescence microscopy and electron microscopy. Cornea **2:**237–241.
5. **Bornes, J. T., W. G. Bornes, and D. Meyers.** 1970. Axenic cultivation of large populations of *Acanthamoeba castellanii* (JBM). J. Parasitol. **56:**904–906.
6. **Brandon, D. M., S. K. Nayak, and P. S. Binder.** 1988. Lectin binding patterns of the human cornea. Cornea **7:**257–266.
7. **Brown, R. C., H. Bass, and J. P. Coombs.** 1975. Carbohydrate binding proteins involved in phagocytosis by *Acanthamoeba*. Nature (London) **254:**434–435.
8. **Centers for Disease Control.** 1986. *Acanthamoeba* keratitis associated with contact lenses—United States. Morbid. Mortal. Weekly Rep. **35:**405–408.
9. **Cohen, E. J., C. J. Parlato, J. J. Arentsen, G. I. Genvert, R. C. Eagle, M. R. Wieland, and P. R. Laibson.** 1987. Medical and surgical treatment of *Acanthamoeba* keratitis. Am. J. Ophthalmol. **103:**615–625.
10. **Folch J., M. Lees, and S. G. H. Stanley.** 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. **226:**497–509.
11. **Hansson, G. C., K. A. Karlsson, G. Larson, N. Stromberg, and J. Thurin.** 1985. Carbohydrate-specific adhesion of bacteria to thin-layer chromatograms: a rationalized approach to the study of host cell glycolipid receptors. Anal. Biochem. **146:**158–163.
12. **Johns, K. J., C. M. Parrish, M. R. Seal, G. W. Jerkins, W. R. Berrie, D. W. Litchford, W. R. Sullivan, J. E. Boone, J. H. Elliott, and D. M. O'Day.** 1989. *Acanthamoeba* keratitis in Tennessee: a growing problem in patients wearing contact lenses. J. Tenn. Med. Assoc. **15:**584–588.
13. **Karlsson, K.** 1989. Animal glycosphingolipids as membrane attachment sites for bacteria. Annu. Rev. Biochem. **58:**309–350.
14. **Laine, R. A., K. Stellner, and S. Hakomori.** 1974. Isolation and characterization of membrane glycosphingolipids, p. 205–244. *In* E. D. Korn (ed.), Methods in membrane biology, 2nd ed. Plenum Publishing Corp., New York.
15. **Magnani, J. L., S. L. Spitalnik, and V. Ginsburg.** 1987. Antibodies against cell surface carbohydrates: determination of antigen structure. Methods Enzymol. **138:**195–207.
16. **Moore, M. B., J. P. McCulley, C. Newton, L. M. Cobo, G. N. Foulks, D. M. O'Day, K. J. Johns, W. T. Driebe, L. A. Wilson, R. J. Epstein, and D. J. Doughman.** 1987. *Acanthamoeba* keratitis: a growing problem in soft and hard contact lens wearers. Ophthalmology **94:**1654–1661.
17. **Morton, L. D., G. L. McLaughlin, and H. E. Whiteley.** 1991. Effects of temperature, amebic strain, and carbohydrates on *Acanthamoeba* adherence to corneal epithelium in vitro. Infect. Immun. **59:**3819–3822.
18. **Niederkorn, J. Y., J. E. Ubelaker, J. P. McCulley, G. L. Stewart, D. R. Meyer, J. A. Mellon, R. E. Silvany, Y. He, M. Pidherney, J. H. Martin, and H. Alizadeh.** 1992. Susceptibility of corneas from various animal species to *in vitro* binding and invasion by *Acanthamoeba castellani*. Invest. Ophthalmol. Visual Sci. **33:** 104–112.
19. **Panjwani, N., and J. Baum.** 1986. A histochemical comparison

of human corneal glycoconjugates with eight other species. Invest. Ophthalmol. Visual Sci. Suppl. **27**:64.

20. **Panjwani, N., G. Michalopoulos, J. Song, T. S. Zaidi, G. Yogeeswaran, and J. Baum.** 1990. Neutral glycolipids of migrating and nonmigrating rabbit corneal epithelium in organ and cell culture. Invest. Ophthalmol. Visual Sci. **31**:689–695.
21. **Panjwani, N., T. S. Zaidi, J. E. Gigstad, F. B. Jungalwala, M. Barza, and J. Baum.** 1990. Binding of *Pseudomonas aeruginosa* to neutral glycosphingolipids of rabbit corneal epithelium. Infect. Immun. **58**:114–118.

21a. **Panjwani, N., et al.** Unpublished data.

22. **Pellegrin, J. J., E. Ortega-Barria, M. Barza, J. Baum, and M. E. A. Pereira.** 1991. Neuraminidase activity in *Acanthamoeba* species trophozoites and cysts. Invest. Ophthalmol. Visual Sci. **32**:3021–3026.
23. **Sharma, S., M. Srinivasan, and C. George.** 1990. *Acanthamoeba* keratitis in non-contact lens wearers. Arch. Ophthalmol. **108**: 676–678.
24. **Stehr-Green, J. K., T. M. Bailey, F. H. Brandt, J. H. Carr, W. W. Bond, and G. S. Visvesvara.** 1987. *Acanthamoeba* keratitis in soft contact lens wearers. A case control study. JAMA **258**:57–60.
25. **Stehr-Green, J. K., T. M. Bailey, and G. S. Visvesvara.** 1989. The epidemiology of *Acanthamoeba* keratitis in the United States. Am. J. Ophthalmol. **107**:331–336.
26. **Wilhelmus, K. R.** 1991. The increasing importance of *Acanthamoeba*. Rev. Infect. Dis. **13**(Suppl. 5):S367–S368.