Carbohydrate Research, 40 (1975) 89–97

IMMUNOCHEMICAL STUDIES ON L-RHAMNO-D-MANNANS OF *Sporothrix schenckii* AND RELATED FUNGI BY USE OF RABBIT AND HUMAN ANTISERA*

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(Received June 17th, 1974; accepted July 15th, 1974)

ABSTRACT

Antisera were prepared in rabbits against the human pathogenic yeast *Sporothrix schenckii* (strain 1099.12) grown at two different temperatures (25° and 37°). Precipitation and inhibition data showed that the former serum had a specificity directed against α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→3)-D-Man-(1→ determinants, whereas the latter had a broad specificity in which α-L-rhamnosyl or α-L-Rha*p*-(1→3)-D-Man- was the immunodominant structure. These results are consistent with data on the structures of the L-rhamno-D-mannans isolated from the organism grown at the two different temperatures. Human sera from patients with sporotrichosis were shown to have different specificities resembling the specificities developed in the rabbits. The rabbit antisera were also used to examine the cross-reactivity with L-rhamno-D-mannans from species of the genus *Ceratocystis*, which is reputed to include the ascigerous (perfect) state of *S. schenckii*. Polysaccharides from four species of *Ceratocystis* grown at 25° reacted with the antisera in a manner resembling that of the L-rhamno-D-mannan from *S. schenckii* grown at 37°. This is in accord with earlier data that showed that only *S. schenckii*, of the species studied, produces a polysaccharide with large amounts of α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→ side-chains when grown at 25°.

INTRODUCTION

Chemical and immunochemical studies on cell-wall polysaccharides have been widely used to classify microorganisms including yeasts and fungi[1,2]. By use of chemical methods we have studied the relationship between the human pathogen *Sporothrix schenckii* and members of the genus *Ceratocystis*. One or more species of

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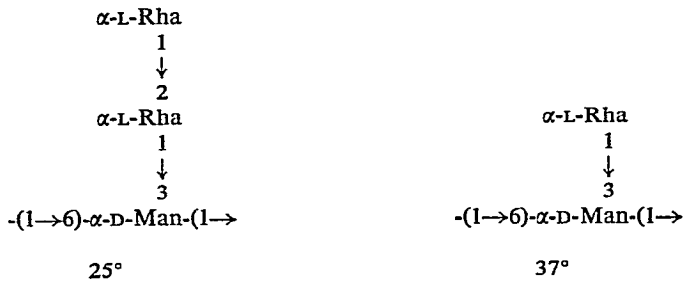
this genus may represent the sexual form of *S. schenckii*[3–5]. In a comparison of the fine structures, determined by methylation analysis[6] and proton and ^{13}C magnetic resonance spectroscopy[6,7] of the L-rhamno-D-mannans, we showed that *S. schenckii*, although closely resembling many species of *Ceratocystis*, produces a distinctive L-rhamno-D-mannan. The unique nature of *S. schenckii* was confirmed by studying the base composition of its DNA and the degree of hybridization of this DNA with the DNAs from various species of *Ceratocystis*[8]. On this basis, *S. schenckii* could be clearly distinguished from *C. stenoceras*—an ascomycete claimed[5,9] to be closely related to *S. schenckii*—and from most other species of *Ceratocystis* studied.

During these studies it was observed that the structure of the L-rhamno-D-mannan isolated from *S. schenckii* growing at 25° was different from that obtained from cultures grown at 37°. The main difference appeared to be the greater proportion of α-L-Rha-(1→2)-L-Rha-(1→ side-chains in the polysaccharides from cultures grown at the lower temperature[6]. The difference was greater in the polysaccharides from some strains than from others. The predominant structures formed at 25° or at 37° are represented in Scheme 1.



Scheme 1.

In the present study the specificity of antisera produced in rabbits against *S. schenckii* grown at the two temperatures was investigated. This criterion was used to explore further the relationship between *S. schenckii* and *Ceratocystis* species. Sera from patients with sporotrichosis were shown to differ in their specificities in a manner resembling the difference in the specificities of the two rabbit antisera.

MATERIALS AND METHODS

Polysaccharides and oligosaccharides. — The isolation and purification of the L-rhamno-D-mannans have been described previously[6]. The polysaccharides from *S. schenckii* (strains 1099.10 and 1099.12) and from *C. stenoceras* (strain 1099.11) are referred to as SS10-25, SS12-37, etc. indicating the organism (SS = *S. schenckii*), the strain (SS10 = *S. schenckii* 1099.10), and the temperature of the culture (25 or 37°), respectively. Briefly, they were isolated by potassium hydroxide extraction of the combined cells and ethanolic precipitate of the supernatants from cultures grown either at 25 or 37°, and purified by use of the copper-complex method[10]. L-Rhamno-

D-mannans from *C. ulmi*, *C. minor*, and *C. pilifera* and also 3-*O*-α-L-rhamnopyranosyl-D-mannose[11] were provided by Dr. P. A. J. Gorin; 2-*O*-α-D-mannopyranosyl-α,β-D-mannose and 6-*O*-α-D-mannopyranosyl-α,β-D-mannose by Dr. C. E. Ballou; and the trisaccharide *O*-α-L-rhamnopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→6)-D-galactose, isolated from *Rhamnus cathartica* L., by Dr. F. Pratviel-Sosa. The oligosaccharide *O*-α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→3)-L-mannose was isolated by partial acetolysis of polysaccharide SS12-25.

Analyses. — Sugars were determined as alditol acetates as described previously[12]. The reducing sugar residue of oligosaccharides was determined by reduction with sodium borohydride and identification of the alditol[13]. Methylation analysis and identification of *O*-methyl sugars were performed as described in earlier publications[6,12].

Immunochemical techniques. — Quantitative precipitin determinations were carried out as described by Lloyd and Bitoon[14]. Double diffusion in agar was performed in 1% agarose in phosphate-buffered 0.9% sodium chloride according to the method of Ouchterlony.

Rabbit sera. — Antisera were raised in female NZ white rabbits by injecting acetone-dried cells (1 mg) intravenously, three times a week for 1 month[15]. The animals were immunized with strain 1099.12 cells grown at either 25 or 37°. They were bled approximately 7, 10, and 14 days after the last injection and the sera from the three bleedings were combined.

Human sera. — Sera from patients with sporotrichosis were obtained from the diagnostic service, Department of Dermatology, Mycology Section, Columbia University.

Microorganisms. — The organisms and the origin of the various strains are described in Refs. 6 and 8.

EXPERIMENTS AND RESULTS

Isolation of a trisaccharide from S. schenckii. — The polysaccharide (1 g) from strain 1099.12 grown at 25° was partially acetolyzed for 5 h at 40° according to the procedure of Lee and Ballou[16]. The product was *O*-deacetylated with sodium methoxide in methanol and fractionated on a Biogel P-2 (<400 mesh) column. Five peaks were obtained. The major peak, which was eluted at the position of a trisaccharide, was purified by rechromatography on the same column and by preparative paper chromatography on Whatman 3MM paper in ethyl acetate–pyridine–water (5:3:2, v/v). The product (40.7 mg) contained D-mannose and L-rhamnose in the proportion 1.0:1.97 and had $[\alpha]_D^{24}$ −38° (*c* 0.3, water). After reduction with sodium borohydride, the product contained L-rhamnose and D-mannitol in the proportion 2.1:1.0 and had $[\alpha]_D^{24}$ −35° (*c* 0.3, water). Methylation analysis and identification of the products by g.l.c. of their alditol acetate derivatives showed the presence of 2,3,4-tri-*O*-methyl-L-rhamnose, 3,4-di-*O*-methyl-L-rhamnose, and 2,4,6-tri-*O*-methyl-D-mannose. It was concluded that the trisaccharide was *O*-α-L-rhamnopyranosyl-

(1→2)-*O*-α-L-rhamnopyranosyl-(1→3)-D-mannose. This structure corresponds to the longest side-chain present in the polysaccharide from strain 1099.12, as determined by analysis of the intact polysaccharide[6].

Precipitation studies. — In the study of the ability of two antisera (227 and 228), prepared against cells grown at 25 and 37°, respectively, to precipitate with L-rhamno-D-mannans prepared from the same organism (see Fig. 1), serum 227 was found to precipitate very strongly with the polysaccharide (SS12-25) from the immunizing

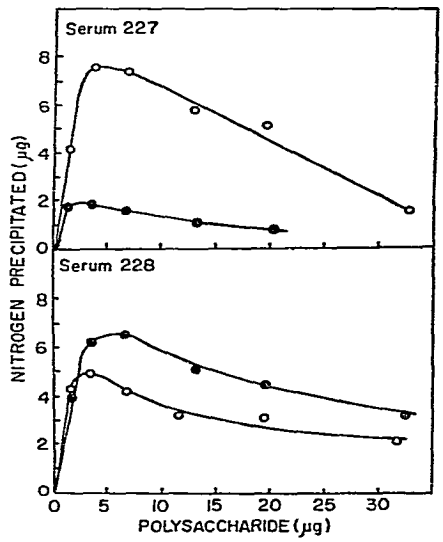


Fig. 1. Quantitative precipitation data showing reactivity of rabbit antisera with L-rhamno-D-mannans from *S. schenckii*: ○, SS12-25; and ●, SS12-37.

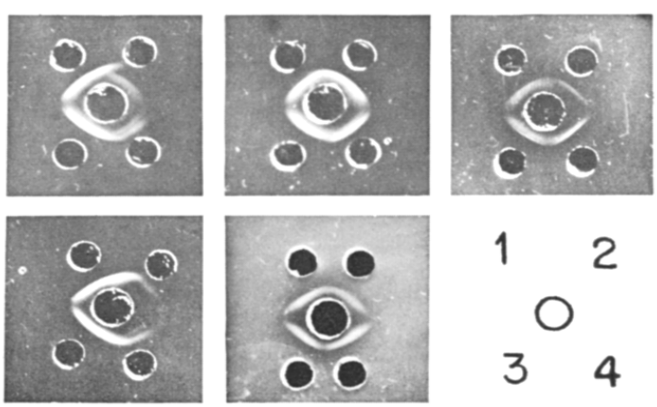


Fig. 2. Double-diffusion patterns of rabbit and human sera with L-rhamno-D-mannans. Center wells: Top left, 227; top center, 228; top right, Riv; lower left. Lop; and lower center, Guil. Outer wells: 1, SS12-25; 2, SS12-37; 3, SS10-25; and 4, SS10-37.

yeast but very poorly with the polysaccharide from cells grown at 37°. In double-diffusion in agar (see Fig. 2), the homologous reaction was again much stronger than the heterologous one, and the line spurred over the reaction line with the SS12-37 polysaccharide. Serum 228 reacted strongly with both SS12-25 and SS12-37 polysaccharides, although the homologous reaction with SS12-37 was slightly better than with SS12-25. In the Ouchterlony double-diffusion test, the two bands were of equal intensity and fused completely together. Antisera from two other rabbits gave very similar patterns of reactivity and will not be discussed separately.

In the study of the reactivity of sera 227 and 228 with the L-rhamno-D-mannans from another strain of *S. schenckii* (1099.10) (see Fig. 3), again the polysaccharide isolated from the cells grown at 25° (SS10-25) reacted much better with serum 227 than did the polysaccharide isolated from cells grown at 37° (SS10-37). With serum 228, the difference between the reactivities with SS10-37 and SS10-25 was more pronounced than with the corresponding polysaccharides isolated from 1099.12. Also shown in Fig. 3 are the precipitin curves of L-rhamno-D-mannans isolated from four species of *Ceratocystis* (*C. stenoceras*, *C. pilifera*, *C. minor*, and *C. ulmi*) with sera 227 and 228. The organisms were grown at 25°. All four polysaccharides reacted with the sera in a manner resembling that of SS12-37 and SS10-37, rather than that of SS12-25 and SS10-25.

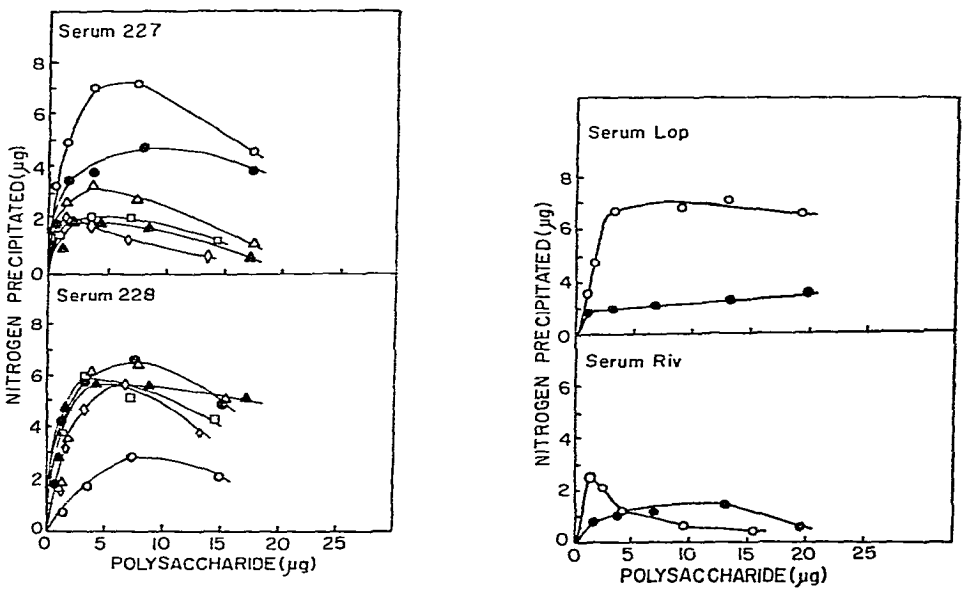


Fig. 3. Quantitative precipitation curves showing reactivity of rabbit antisera with L-rhamno-D-mannans from a different strain of *S. schenckii* and from species of *Ceratocystis*: ○, SS10-25; ●, SS10-37; △, *C. stenoceras*; □, *C. pilifera*; ▲, *C. ulmi*; and ◇, *C. minor*.

Fig. 4. Quantitative precipitation curves of reactivity of human sera with L-rhamno-D-mannans from *S. schenckii*: ○, SS12-25; and ●, SS12-37.

Figure 4 shows the ability of sera from two patients with infections of *Sporothrix schenckii* to precipitate with SS12-25 and SS12-37 polysaccharides. Serum Lop resembled rabbit serum 227 in that it reacted much more strongly with SS12-25 than with SS12-37. Serum Riv also reacted more strongly with the former antigen, although the difference with the two antigens was not as pronounced. The results of double diffusion in gels were in agreement with these reactivity patterns (see Fig. 2). Figure 2 also shows the patterns obtained with one human serum that was not available in amounts sufficient for quantitative precipitin studies. This serum (Guil) reacted equally well with SS12-25 and SS12-37, with complete fusion in a manner resembling that of antiserum 228. In all the gel-diffusion plates, the strong reaction with the polysaccharides isolated from cells grown at 25°, which spurred over the other

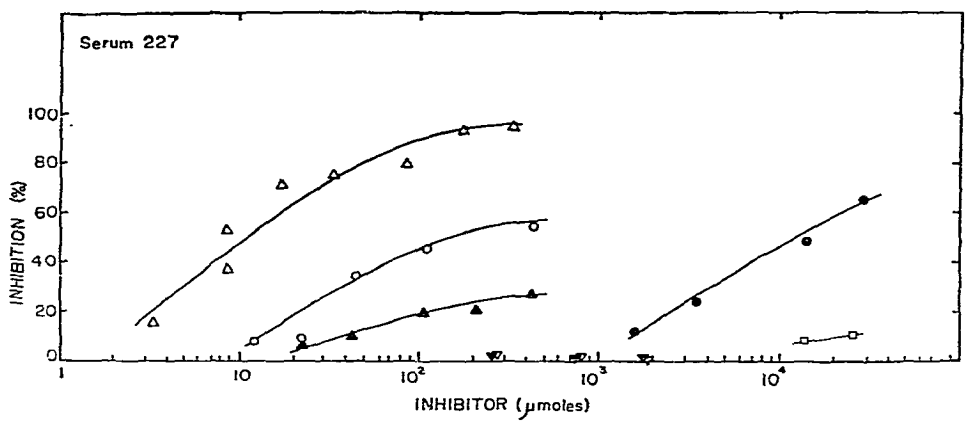


Fig. 5. Inhibition of serum 227 by oligosaccharides and monosaccharides: △, α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→3)-D-Man; ○, α-L-Rha*p*-(1→3)-D-Man; ▲, α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→6)-D-Gal; ●, L-rhamnose; □, D mannose; ▼, α D-Man*p*-(1→2)-D-Man; and ▽, α-D-Man*p*-(1→6)-D-Man.

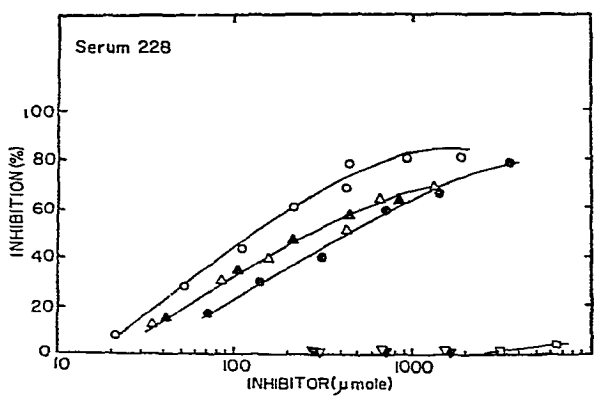


Fig. 6. Inhibition of serum 228 by oligosaccharides and monosaccharides. Symbols are the same as in Fig. 5.

line, was much easier to observe with the SS12 than with the SS10 antigens. With some sera, two bands were produced with SS12-37 and SS10-37.

Inhibition studies. — L-Rhamnose and D-mannose and certain oligosaccharides containing these two sugars were able to inhibit the reaction of two rabbit antisera with their homologous antigens (see Figs. 5 and 6). Serum 227 was best inhibited by the trisaccharide α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→3)-D-Man and less well by α-L-Rha*p*-(1→3)-D-Man and α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→6)-D-Gal. L-Rhamnose was a poor inhibitor of this antiserum, and D-mannose and the two D-mannose-containing oligosaccharides were inactive. In contrast, serum 228 was inhibited very well by L-rhamnose and by all three L-rhamnose-containing oligosaccharides. The disaccharide α-L-Rha*p*-(1→3)-D-Man was slightly better than the other two oligosaccharides. Again, the D-mannose-containing oligosaccharides were inactive.

DISCUSSION

The antisera prepared against *S. schenckii* grown either at 25° or at 37° have specificities directed against L-rhamnose-containing determinants, as would be expected from the absence of terminal D-mannose groups in the polysaccharides[6]. A detailed examination of their specificities shows, however, that serum 228 prepared against cells grown at 37° was relatively less specific as compared with serum 227. Thus, the former was inhibited quite well by L-rhamnose, and only slightly better by the L-rhamnose-containing oligosaccharides. In contrast, serum 227 was inhibited best by α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→3)-D-Man. Other L-rhamnose-containing oligosaccharides were poorer inhibitors, and L-rhamnose inhibited only about 1/1000 as well as the trisaccharide. The specificity of this antiserum was further demonstrated by the observation that α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→6)-D-Gal was a poorer inhibitor than was α-L-Rha*p*-(1→3)-D-Man, even though it contained an α-L-rhamnosyl-α-L-rhamnosyl sequence. The differences in the specificities of the two sera were also evident from the precipitation studies. Serum 227 precipitated much better with SS12-25 and SS10-25 than with the corresponding polysaccharides isolated from organisms grown at 37°. Serum 228, on the other hand, precipitated quite well with SS12-25, as well as with SS12-37. In agreement with these observations, SS12-25 and SS10-25 have been shown[6] to have larger proportions of α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→ side-chains than do SS12-37 and SS10-37. Studies of other fungal mannans have demonstrated the importance of the side-chains in the immunochemical determinants of these antigens also[17–20].

Specificity differences are also reflected in the Ouchterlony gel-diffusion patterns. With polysaccharide SS12-25, serum 227 produced a strong line that spurred over the weak line produced with SS12-37. Serum 228 produced lines of about equal intensity with both polysaccharides. The difference in the specificities of the two sera against SS10-25 and SS10-37 was less pronounced, presumably reflecting the fact that the difference in the proportion of dirhamnosyl to monorhamnosyl side chains is also less pronounced in the SS10 than in the SS12 polysaccharides[6].

Sera from patients with sporotrichosis contain varying amounts of antibodies to the L-rhamno-D-mannan of *S. schenckii*[14]. The data presented in Figs. 2 and 4 show that these sera too vary in their specificities. Serum Lop reacted with SS12-25 and SS12-37 in a manner very similar to that of rabbit serum 227 and probably, therefore, has a strong specificity directed towards structures containing the α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→ group. Serum Guil, at least as judged by gel-diffusion patterns, reacted equally well with SS12-25 and SS12-37, and thus resembles rabbit serum 228. The other two human sera showed an intermediate pattern of reactivity. It is not clear whether these differences in specificity arose from: (a) differences in the immune response among different individuals to the same antigen, or (b) infections with different strains of *S. schenckii*—different strains of *S. schenckii* are known to produce L-rhamno-D-mannans which differ in their fine structures[6,7], or (c) different growth conditions of the organisms at different sites of infection. Some infections of *S. schenckii* are confined to fairly superficial, subcutaneous sites, whereas others become systemic. Such differences in environment might lead to the formation of different antigens, which may induce an immune response that differs in a way similar to that observed in rabbits immunized with cells grown at 25° or at 37°.

The reactivity of the rabbit sera with L-rhamno-D-mannans from four *Ceratocystis* species confirms and extends the conclusions, arrived at earlier by use of chemical methods, concerning the possible relationship between members of this genus and *S. schenckii*. The cross-reactivity with other species has been demonstrated by Ishizaki and Wheat who have reported briefly[21] on the cross-reactions of 46 of 57 species of *Ceratocystis*. The polysaccharides from *C. stenoceras*, *C. minor*, *C. pilifera*, and *C. ulmi* were shown in the present study to react strongly with serum 228 and poorly with serum 227. In this respect, they resembled SS12-37 rather than SS12-25. These data reinforce the findings of the methylation analysis[6] and n.m.r. studies[6,7] that the L-rhamno-D-mannans of species other than *S. schenckii* have single residues of L-rhamnose as their principal side-chains when they are isolated from cultures grown at 25°. The n.m.r. spectra of *S. schenckii* L-rhamno-D-mannans isolated at 25° are characterized by signals at τ 4.41 in their proton spectra and at δ_c 96.6 in their carbon-13 spectra. The latter signal has been assigned[7] to the C-1 of the 2-*O*-substituted L-rhamnose residues in the side-chains. Among the species of *Ceratocystis* studied was *C. stenoceras*, which has been suggested by Mariat and associates[3,5,9] as being the perfect form of *S. schenckii*. Certainly, on the basis of the chemical and immunochemical studies of their L-rhamno-D-mannans, this species of *Ceratocystis* is no more closely related to *S. schenckii* than are a number of other species. *C. minor*, by virtue of the close correspondence of its DNA guanine and cytosine content to that of *S. schenckii*, and the high degree of hybridization of their DNA samples[8], appeared to us to be a much better candidate to be the perfect form of *S. schenckii*. On the basis of the reactivity of its L-rhamno-D-mannan with the rabbit antisera, however, *C. minor* is no more related to *S. schenckii* than are *C. stenoceras* and the other two species studied. The lack of correlation between the structures of the somatic antigens of *S. schenckii* and *C. minor* and their close genetic relationship, as judged by the

homology in their DNA samples[8], raises questions as to the advisability of using the polysaccharide phenotype to assess the relationship between microorganisms.

ACKNOWLEDGMENTS

This investigation was supported by a U.S. Public Health Service grant (AI-08478) from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, and by the Brown–Hazen Fund of The Research Corporation. K.O.L. was a Research Career Development Awardee of the Public Health Service. L.R.T. was a Fellow of the Brazilian National Research Council on leave of absence from the Federal University of Rio de Janeiro, Brazil.

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