**Clostridium difficile** Recombinant Toxin A Repeating Units as a Carrier Protein for Conjugate Vaccines: Studies of Pneumococcal Type 14, *Escherichia coli* K1, and *Shigella flexneri* Type 2a Polysaccharides in Mice

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Unlike the native protein, a nontoxic peptide (repeating unit of the native toxin designated rARU) from *Clostridium difficile* toxin A (CDTA) afforded an antigen that could be bound covalently to the surface polysaccharides of pneumococcal type 14, *Shigella flexneri* type 2a, and *Escherichia coli* K1. The yields of these polysaccharide-protein conjugates were significantly increased by prior treatment of rARU with succinic anhydride. Conjugates, prepared with rARU or succinylated (rARUsucc), were administered to mice by a clinically relevant dosage and immunization scheme. All conjugates elicited high levels of serum immunoglobulin G both to the polysaccharides and to CDTA. Conjugate-induced anti-CDTA had neutralizing activity in vitro and protected mice challenged with CDTA, similar to the rARU alone. Conjugates prepared with succinylated rARU, therefore, have potential for serving both as effective carrier proteins for polysaccharides and for preventing enteric disease caused by *C. difficile*.

The immunogenicity of the surface polysaccharides of bacterial pathogens is improved when these antigens are bound covalently to a carrier protein (conjugate) (33, 35). Most carriers have been medically useful proteins, including inactivated tetanus, diphtheria, pertussis, and *Pseudomonas aeruginosa* toxins (1, 8, 9, 11, 12, 28, 33–35, 37). Thus, conjugate vaccines may confer immunity against pathogens whose protective antigens are the carrier proteins, including those that cause toxin-mediated diseases.

One variable affecting serum antibody (Ab) responses to the saccharide component is the carrier protein. For example, a genetically inactivated diphtheria toxin (CRM197) was a more effective carrier than the formalin-treated toxoid (1). In addition, treatment of two genetically inactivated medically important antigens, diphtheria toxin (CRM1) and *P. aeruginosa* exotoxin A (rEPA), with succinic anhydride improved the effectiveness of these two proteins as carriers for inducing polysaccharide Abs (28). Another variable is the total amount of protein injected in formulations containing several conjugates sharing the same carrier: interference with the maximal level of Ab to both the polysaccharide and the protein components was related to the dose of protein administered to young children (11). Carrier protein-mediated suppression may become a problem as the number of polysaccharide-protein conjugates considered for immunization increases (33, 35).

*Clostridium difficile* is a major cause of hospital-acquired diarrhea (15, 25, 27, 31). Antibiotic therapy often causes this normal inhabitant of the colon to overgrow and release two toxins, A (molecular weight [MW], 308,000) and B (MW, 270,000), that cause an enteric disease ranging from diarrhea to pseudomembranous colitis (3, 5, 24, 26, 27, 31). The presence of these toxins in intestinal fluids is diagnostic of this disease (24, 25). Of the two, toxin A is primarily responsible for the clinical symptoms. In animal models, serum neutralizing Abs to *C. difficile* toxin A (CDTA) confer immunity to this pathogen (10, 16, 23). There is clinical evidence that serum immunoglobulin G (IgG) neutralizing Abs to these toxins confer immunity to this disease (41, 42). CDTA has a series of contiguous repeating units at its COOH terminus comprising about one-third of the molecule (6, 13, 29). These repeating units are the region that recognizes the carbohydrate receptor of the host cells and that elicits serum Abs that neutralize the cytotoxic and lethal effects of toxin A (17, 24, 25, 30). A recombinant nontoxic peptide, containing these repeating units (rARU), has been created and shown to elicit neutralizing Abs that can protect laboratory animals against challenge with both toxin A and *C. difficile* (10, 16, 29, 30).

Based upon the high incidence of enteric disease caused by *C. difficile* in hospitalized patients, there is a need for an effective vaccine for this pathogen. Probably because of their high MWs, we were unable to synthesize conjugates of *C. difficile* toxins A and B (unpublished results). We studied rARU as a carrier protein for conjugates of the capsular polysaccharide of *Escherichia coli* K1 (possessing a capsular polysaccharide identical to that of group B meningococcus), pneumococcus type 14, and the O-specific polysaccharide of *Shigella flexneri* type 2a (2, 8, 9, 12, 28, 37). Pneumococcus type 14 was chosen because it has been a major type isolated from patients over a long time span and from different diseases caused by pneumococci (2, 32). *S. flexneri* type 2a was chosen because it is the most common *Shigella* type from patients in developing countries (8, 9, 35). *E. coli* K1 was chosen because there is yet no vaccine for systemic infections caused by these two pathogens (12). All three conjugates elicited high levels of Abs to their respective polysaccharides and to CDTA.
TABLE 1. Composition of C. difficile recombinant enterotoxin A (rARU) conjugates of pneumococcal type 14 (Pn14) and E. coli K1 (group B meningococcal) capsular polysaccharides and S. flexneri type 2a O-specific polysaccharides (SF-rARU) derivatized with adipic acid dihydrazide and bound to rARU or rARUsucc by water-soluble carbodiimide condensation as described elsewhere with the exception that the pH of the reactants was maintained with 0.1 M MES, pH 6.0 (8, 9, 37). E. coli K1 polysaccharide was both derivatized with adipic acid dihydrazide and bound to rARU or rARUsucc by treatment with EDC (12). The composition of the adipic acid dihydrazide-derivatized polysaccharides and of the conjugates is shown in Table 1. Note that low yields of conjugates, using rARU as the carrier, were obtained with the pneumococcal type 14 and S. flexneri type 2a polysaccharides. We were unable to synthesize a conjugate of the K1 polysaccharide with rARU.

Vaccination of mice. Female 5-week-old general-purpose Swiss Albino mice at the National Institutes of Health or outbred hsd/ICR mice (Harlan-Sprague-Dawley, Inc., Indianapolis, Ind.) at Techlab were injected subcutaneously (s.c.) with 0.1 ml containing 2.5 μg of polysaccharide in the conjugate every 2 weeks. Mice (n = 10) were exsanguinated 2 weeks after the first injection and 1 week after the second and third injections.

SERологIC methods. IgG and IgM antibodies to S. flexneri type 2a lipopolysaccharide (LPS) and to E. coli K1 polysaccharides were measured by enzyme-linked immunosorbent assay (ELISA) as described previously (8, 12). IgG anti-pneumococcal type 14 polysaccharide was assayed by ELISA, and total polysaccharide Ab was assayed by radioimmunoassay (RIA) as described previously (18, 36, 37).

Abs to CDTA were measured by ELISA, with CDTA as the coating antigen, and by in vitro neutralization of cytotoxicity (24). Human intestinal HT-29 cells (ATCC HTB 38) were maintained in 96-well plates with McCoy’s 5A medium supplemented with 10% fetal calf serum in a 5% CO2 atmosphere (HT-29 cells were chosen because of their high sensitivity to CDTA probably because of the high density of the carbohydrate receptor on their surface). Serial twofold dilutions of sera were incubated with 0.4 μg of CDTA per ml for 30 min at room temperature. CDTA-serum mixtures were added to the wells at a final concentration of 20 ng of the toxin per well (about 200 times the minimal cytotoxic dose for HT-29 cells) in a final volume of 0.2 ml. The neutralization titer is expressed as the reciprocal of the highest dilution that completely neutralized cytotoxicity.

Goat Abs to toxin A. Affinity-purified caprine Abs elicited by formalin-treated CDTA were prepared by immunoaffinity chromatography with toxin A bound to AgiIgel-10 (Bio-Rad Laboratories, Hercules, Calif.) as described previously (22, 23).

Protection against lethal challenge of mice with CDTA. hsd/ICR mice were injected s.c. with 6.94 μg of rARU. The dose of 2.5 μg of polysaccharide for the conjugates contained 6.94 μg of rARU for SF-rARU and 3.9 μg of rARU for SF-rARUsucc (see above). Controls were mice injected with 0.1 ml of phosphate-buffered saline (PBS) by the same scheme. The mice were challenged by

MATERIALS AND METHODS

CDTA and recombinant enterotoxin (rARU). CDTA was purified as described elsewhere (24). rARU contains the entire C-terminal repeat region (861 amino acids plus 4 amino acids upstream) and has an MW of 104,844. Approximately two-thirds of toxin A, including the enzymatic domain that is necessary for cytotoxicity, has been removed (13, 29). Intraperitoneal (i.p.) injection of 0.5 mg of SF-rARUsucc while retaining its antigenicity as measured by double immunosorbent assay (DIASA) conjugates of pneumococcal type 14 (Pn14) and E. coli K1 (group B meningococcal) capsular polysaccharides and S. flexneri type 2a O-specific polysaccharides (SF-rARU) was passed through a 2.5- by 50-cm Sephadex G-50 column in 0.2 M NaCl, and filtered, and stored at 4°C.

Affigel-10 (Bio-Rad Laboratories, Hercules, Calif.) as described previously (22, 23).

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TABLE 2. Serum pneumococcal type 14 polysaccharide Abs (Pn14) elicited in mice by conjugates composed of C. difficile recombinant toxin A repeating units (rARU) alone or succinylated (rARUsucc) bound to pneumococcal type 14 polysaccharide.*

| Conjugate | IgG for injection: | IgM for injection: |
|-----------|---------------------|---------------------|
| First     | Second              | Third               |
| Pn14-rARU | 0.90 (0.77–1.26)    | 4.38 (1.97–11.6)    | 6.41 (4.56–7.37) |
| Pn14-rARUsucc | 0.71 (0.42–1.65) | 6.10 (3.55–7.40) | 9.76 (7.10–12.4) |

* Six-week-old mice were injected s.c. with 2.5 μg of pneumococcal type 14 polysaccharide as a conjugate at 2-week intervals. Mice (n = 10) were exsanguinated 2 weeks after the first injection and 7 days after the second and third injections, and their sera were assayed for IgG and IgM anti-pneumococcal type 14 polysaccharide by ELISA. A hyperimmune serum, arbitrarily assigned a value of 100 EU, was the reference (37). Statistical significance of IgG results: second- and third-injection values versus first-injection values for both conjugates, P < 0.005; third-injection values versus second-injection values for both conjugates, not significant; third injection of Pn14-rARUsucc versus that of Pn14-rARU, no significance.
TABLE 4. Serum LPS Abs elicited in mice by *S. flexneri* 2a O-specific polysaccharide (SF) bound to *C. difficile* recombinant toxin A repeating units (rARU) alone or succinylated (rARUsucc)a

| Immune | IgG for injection: | IgM for injection: |
|--------|--------------------|--------------------|
|        | Second             | Third              | Second             | Third              |
| SF-rARU | 0.75 (0.40–1.43)   | 1.61 (1.13–3.38)   | 6.92 (4.85–12.2)   | 7.18 (2.74–18.2)   |
| SF-rARUsucc | 0.37 (0.03–1.63) | 2.48 (1.35–5.14)   | 1.54 (0.18–54.5)   | 4.06 (1.74–8.77)   |

a Five-week-old mice were injected s.c. with 2.5 μg of *S. flexneri* type 2a O-specific polysaccharide alone or as a conjugate at 2-week intervals. Mice (*n* = 10) were exsanguinated before and 7 days after the third injection, and their sera were assayed for IgG anti-LPS by ELISA. A hyperimmune serum pool, arbitrarily assigned a value of 100 EU, served as a reference (29). Polysaccharide alone did not elicit Abs (data not shown). Statistical significance: third-injection value versus second-injection value for IgG anti-SF-rARUsucc, *P* = 0.04.

RESULTS

Pneumococcal type 14 Abs. Both conjugates elicited statistically significant rises of IgG Abs after the first and the second injections (*P* < 0.005) (Table 2). The third injection of both conjugates elicited rises in IgG (4.38 to 6.41 ELISA units [EU] for Pn14-rARU and 6.10 to 9.76 EU for Pn14-rARUsucc) and IgM (4.82 to 7.57 EU for Pn14-rARU and 6.16 to 8.54 EU for Pn14-rARUsucc), but these were not statistically significant. Pneumococcal type 14 polysaccharide alone elicited only trace levels of Abs in mice (37); PBS did not elicited Abs (data not shown).

The correlation coefficients of Ab levels induced by both conjugates after each injection, as measured by RIA or ELISA, were statistically significant (Table 3).

*S. flexneri* type 2a IgG LPS Abs. Both SF-rARU and SF-rARUsucc elicited LPS Abs after the second injection compared to prevaccination levels (*P* = 0.001) (Table 4). Rejection for the third time elicited a rise of IgG anti-LPS for both conjugates, but the rise was statistically significant only for SF-rARUsucc (2.48 versus 0.37 EU, *P* = 0.04). The *S. flexneri* type 2a IgG anti-LPS levels induced by the two conjugates were not statistically different.

IgG *E. coli* K1 Abs. K1-rARUsucc elicited a significant rise of K1 Abs after all three injections: first injection, 1.35 EU; second, 12.4 versus 1.35 EU, *P* = 0.0001; and third, 104 versus 12.4 EU, *P* = 0.002.

CDTA Abs. All five conjugates elicited high levels of anti-CDTA (194 to 613 EU/ml) (Table 5). Since the 2.5-μg immunizing dose of the conjugate was based upon its polysaccharide component, the amount of rARU injected was different for each conjugate. To illustrate, on a protein weight basis, Pn14-rARU, with 1.29 μg of rARU, elicited 194 μg of CDTA Ab/ml (150.3 μg of Ab/μg of rARU injected). In contrast, Pn14-rARUsucc, which contained 7.3 μg of rARU per dose, elicited 371 μg of CDTA Ab/ml (50.8 μg of Ab/μg of rARUsucc injected). Pn14-rARU induced less anti-CDTA per μg of rARU than did Pn14-rARUsucc, but the higher total amount of anti-CDTA elicited by the latter was due to its higher content of rARU. The difference between the level of anti-CDTA elicited by Pn14-rARU (194 μg of CDTA Ab/ml) and that elicited by Pn14-rARUsucc (371 μg of CDTA Ab/ml) was significant (*P* = 0.01).

SF-rARU, containing 3.9 μg of rARU, elicited 437 μg of CDTA Ab/ml (112.0 μg of Ab/μg of rARU injected) compared to 518 μg of CDTA Ab/ml for SF-rARUsucc (34.9 μg of Ab/μg of rARUsucc injected). Although the specific immunogenic activity for the rARUsucc was lower than that of the rARU in the *S. flexneri* type 2a conjugates, there was no statistical difference between the levels of CDTA Ab elicited by the two conjugates (437 μg of Ab/ml for SF-rARUsucc versus 242 μg of Ab/ml for SF-rARU).

K1-rARUsucc, which elicited 390 EU of CDTA Ab/ml, had i.p. injection of 150 ng of CDTA (about three times the minimum lethal i.p. dose) 7 days after the third injection of rARU conjugates. A blood sample for Ab assay was obtained from all mice 4 h before challenge.

Statistical analysis. Comparison of geometric means was performed by the unpaired *t* test. For values too low to be detected, a value of one-half the detectable level was assigned. Comparison of the RIA versus ELISA for pneumococcal type 14 antibodies used the Pearson correlation coefficient. Log-transformed Ab data were analyzed using SAS software.

TABLE 5. Serum Abs (micrograms per milliliter) to CDTA elicited in mice by recombinant enterotoxin A (rARU) or polysaccharides bound to rARU alone or succinylated (rARUsucc)a

| Conjugate | rARU injected (μg) | Geometric mean (25th to 75th percentile) by ELISA for injection: |
|-----------|--------------------|---------------------------------------------------------------|
|           | First              | Second             | Third              |
| rARU      | 6.94               | ND                 | ND                 | 717 (621–863)   |
| Pn14-rARU | 1.29               | 3.70 (2.55–5.08)   | 80.1 (69.8–131)   | 194 (113–236)   |
| Pn14-rARUsucc | 7.30 | 7.94 (5.21–11.3) | 183 (146–175) | 371 (274–463) |
| SF-rARU   | 3.90               | ND                 | 433 (258–609)     | 613 (485–778)   |
| SF-rARUsucc | 6.94 | ND                 | 191 (118–291)     | 518 (366–615)   |
| SF-rARU   | 3.90               | ND                 | ND                 | 437 (372–547)   |
| SF-rARUsucc | 6.94 | ND                 | ND                 | 242 (172–443)   |
| K1-rARUsucc | 8.08 | 10.7 (6.75–17.2) | 84.9 (72.5–131)   | 390 (279–470)   |

a Six-week-old mice were injected s.c. with 2.5 μg of polysaccharide as a conjugate at 2-week intervals. Groups of mice (*n* = 10) were exsanguinated before the first injection and 7 days after the second and third injections, and their sera were assayed for anti-CDTA by ELISA. A hyperimmune serum, arbitrarily assigned a value of 100 EU, served as a reference. ND, not done. Statistical significance: 183 versus 7.94, *P* = 0.0001; 371 versus 183, *P* = 0.0005; 80.1 versus 3.70, *P* = 0.0001; 194 versus 80.1, *P* = 0.007; 7.94 versus 3.70, *P* = 0.01; 183 versus 80.1, *P* = 0.004; 371 versus 194, *P* = 0.01.

b hsd/ICR mice. The remainder were NIH Swiss Albino mice.
comparable specific immunogenic activity of its rARU component (48 \mu g of Ab/ml of rARUsucc).

Neutralizing Abs to CDTA. Individual sera obtained 7 days after the third injection of the conjugates were assayed individually for their neutralization of approximately 200 times the cytotoxic dose of CDTA on human intestinal HT-29 cells (Table 6). All sera from mice immunized with the conjugates had a neutralizing titer of $64$ (data not shown). The geometric mean and range of neutralizing titers for each immunogen are shown in Table 6. Conjugate-induced Ab levels approached or surpassed the neutralizing activity of an affinity-purified goat Ab, containing 0.5 mg/ml, that was raised against formalin-inactivated CDTA.

In vivo protection of mice against CDTA. hsd/ICR mice were injected with SF-rARU, SF-rARUsucc, or rARU (see above). One week after the third injection, the mice were challenged i.p. with a lethal dose (150 ng) of CDTA. Almost all mice vaccinated with either conjugate or the rARU were protected (Table 7). Based upon the amount injected, rARU and SF-rARU elicited similar levels of anti-CDTA. As expected, SF-rARUsucc elicited lower levels of anti-CDTA than did the other two immunogens, but the recipients were comparably protected.

**DISCUSSION**

Although we were able to bind polysaccharides to rARU, only low yields of conjugates were obtained. Precipitation followed concentration of the rARU to 3 to 5 mg/ml. Accordingly, low concentrations of reactants had to be used for the conjugation step, reducing its efficiency and resulting in low yields of conjugates. We obtained high yields of conjugates only with the succinylated rARU. Succinic anhydride (dihydro-2,5-furandione) reacts rapidly with the $\varepsilon$-amino groups of lysines and with the N-amino acid termini of proteins in aqueous solutions at pH 7 to 8 by replacing the amino group with a carboxyl group (28). Carbodiimide-mediated condensation, designed to bind the hydrazide on the polysaccharide to the carboxyls of proteins, is likely accompanied by side reactions that include forming amide bonds between $\varepsilon$-amino groups of rARU.

| Immunogen      | Reciprocal neutralization titer |
|----------------|---------------------------------|
|                | GM     | Range |
| Pn14-rARU      | 194    | 104   |
| Pn14-rARUsucc  | 371    | 111   |
| SF-rARU        | 613    | 194   |
| SF-rARUsucc    | 518    | 111   |
| K1-rARUsucc    | 390    | 181   |
| Goat antitoxin (0.5 mg/ml) | 128 |
| PBS            | 0      |

Neutralizing titers were the highest serum dilution that completely inhibited the cytotoxicity of CDTA (20 ng/well) on HT-29 cells (Materials and Methods). The titers represent the geometric means (GM) ± standard deviations from general-purpose Swiss Albino mice ($n = 10$) obtained 7 days after the third injection. Anti-CDTA was also measured by ELISA, and the individual values are expressed as micrograms of Ab per milliliter of serum.

TABLE 6. Serum neutralizing activity against in vitro cytotoxicity of CDTA for HT-29 cells

| Immunogen      | Reciprocal neutralization titer |
|----------------|---------------------------------|
|                | GM     | Range |
| rARU           | 6.94   | 19/20 |
| SF-rARU        | 3.90   | 17/20 |
| SF-rARUsucc    | 6.94   | 19/20 |
| PBS            | 0      | 2/15  |

Mice (hsd/CSR) were injected i.p. with 150 ng of CDTA 7 days after the third injection of rARU or conjugate. Mean Ab level (values in parentheses are 25th to 75th percentiles) of sera used for pool. Mice ($n = 10$) from each group were bled 4 h before challenge with CDTA.

Highest dilutions of sera (range) that completely neutralized the cytotoxicity of CDTA (20 ng/well) on HT-29 cells.

**FIG. 1.** Repeating units of polysaccharides used for conjugates of C. difficile rARU.
lysines with adjacent carboxyls of the protein (intramolecular cross-linking) or with adjacent protein molecules (intermolecular cross-linking). Prior conversion of these amino residues by succinylation reduces the intra- and intermolecular amide formation during the conjugation step and provides additional carboxyl groups capable of binding the hydrazide-derivatized polysaccharide. Treatment with succinic anhydride has been shown to inactive diphtheria and tetanus toxins and stabilize the resultant toxoids against aggregation (38). The by-product of succinic anhydride hydrolysis in water is the metabolite succinic acid.

The rARUsucc protein served as an effective carrier for conjugates of three medically useful and structurally different polysaccharides. Figure 1 shows that the pneumococcal type 14 polysaccharide is a neutral high-MW branched copolymer (20), *S. flexneri* type 2a O-specific polysaccharide is a comparatively lower-MW neutral branched copolymer (7, 19), and each subunit of *E. coli* K1, a linear high-MW homopolymer, is negatively charged (4). Thus, the use of rARU as a carrier is likely to be applicable to all polysaccharides.

All three conjugates elicited high levels of serum CDTA Abs with in vitro neutralizing activity and in vivo protection. On a weight basis, rARU as a component of the three conjugates was as immunogenic as rARU alone. As expected, the specific immunogenic activity of rARU was greater than that of succinic acid derivatives of rARU. However, the higher protein content of the succinylated derivatives compensated for this difference. The minimum protective level of CDTA Abs is, as yet, not known.

There were no significant differences between the levels of polysaccharide Abs elicited by the pneumococcal type 14 and *S. flexneri* type 2a O-specific polysaccharide conjugates with rARU and the levels elicited by rARUsucc. The levels of Abs to each of the three polysaccharides elicited by the conjugates prepared with rARUsucc were similar to those reported previously (12, 23, 37).

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serum IgG antibody is sufficient to confer protection against infectious diseases by inactivating the inoculum. J. Infect. Dis. 171:1387–1398.

35. Robbins, J. B., R. Schneerson, P. Anderson, and D. H. Smith. 1996. Prevention of systemic infections, especially meningitis, caused by Haemophilus influenzae type b: impact on public health and implications for other polysaccharide-based vaccines. JAMA 276:1181–1185.

36. Schiffman, G., R. M. Douglas, M. J. Bonner, M. Robbins, and R. Austrian. 1980. Radioimmunoassay for immunologic phenomena in pneumococcal disease and for the antibody response to pneumococcal vaccine. I. Method for the radioimmunoassay of anticapsular antibodies and comparison with other techniques. J. Immunol. Methods 33:130–144.

37. Schneerson, R., L. Levi, J. B. Robbins, D. A. Blyla, G. Schiffman, and T. Lagergård. 1992. Synthesis of a conjugate vaccine composed of pneumococcus type 14 capsular polysaccharide bound to pertussis toxin. Infect. Immun. 60:3528–3532.

38. Schwendeman, S. P., H. R. Costantino, R. K. Gupta, G. R. Siber, A. M. Klibanov, and R. Langer. 1995. Stabilization of tetanus and diphtheria toxoids against moisture-induced aggregation. Proc. Natl. Acad. Sci. USA 92:11234–11238.

39. Scott, T. A., and E. H. Melvin. 1953. Determination of dextran with anthrone. Anal. Chem. 25:1656–1661.

40. Shields, R., and W. W. Burnett. 1960. Determination of protein-bound carbohydrate in serum by a modified anthrone method. Anal. Chem. 32:885–886.

41. Warny, M., J.-P. Vaerman, V. Avesani, and M. Delmée. 1994. Human antibody response to Clostridium difficile toxin A in relation to clinical course of infection. Infect. Immun. 62:384–389.

42. Warny, M., J. P. Vaerman, M. Delmée, and C. Lefebvre. 1995. Gamma globulin administration in relapsing Clostridium difficile-induced pseudomembranous colitis with a defective antibody response to toxin A. Acta Clin. Belg. 50:36–39.

43. Yao, K., and T. Ubuka. 1987. Determination of sialic acids by acidic ninhydrin reaction. Acta Med. Okayama 41:237–241.