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Structure-Function Relationships for Polysaccharide-Induced Intra-Abdominal Abscesses

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We have previously shown that in an animal model of sepsis positively and negatively charged groups on polysaccharide A of *Bacteroides fragilis* are essential for the induction of intra-abdominal abscess formation (A. O. Tzianabos, A. B. Onderdonk, B. Rosner, R. L. Cisneros, and D. L. Kasper, Science 262:416–419, 1993). By introducing chemical modifications into the structures of *B. fragilis* polysaccharide B as well as other abscess-inducing bacterial polysaccharides, we observed the following. (i) The presence of a nonacetylated free amino group on these polysaccharides appears to be required for abscess induction. (ii) No specific type of negatively charged group is essential to abscess induction by these polysaccharides. (iii) The density of free amino groups on these polysaccharides influences this pathobiologic host response.

Bacteroides fragilis is the anaerobic bacterial species most commonly isolated from clinical sites of infection, particularly abscesses (10). Attempts to characterize virulence factors unique to this organism have demonstrated that the capsular polysaccharide complex is a primary virulence factor in the pathogenesis of intra-abdominal abscess formation, promoting the induction of abscesses in an experimental rodent model of intra-abdominal sepsis (5, 8). The ability of this complex to induce the formation of abscesses is dependent on the presence of nonimmune $CD4^+$ $CD8^+$ T cells in the host (13).

The capsular polysaccharide complex comprises two distinct high-molecular-weight component polysaccharides, PS A and PS B. Each carbohydrate is composed of repeating oligosaccharide subunits possessing constituent sugars with free amino, carboxyl, and/or phosphonate groups (1, 9) (Fig. 1). Ionic interactions between the charged groups tightly link PS A and PS B to form a high-molecular-weight complex. Further studies using immunoelectron microscopy demonstrated that each of these polysaccharides is expressed on the surface of the bacterial cell (15).

Recently, we showed that PS A and PS B are potent abscess-inducing agents in the rat model of intra-abdominal sepsis and that both positively and negatively charged groups on polysaccharide structures are essential to this biologic function (14). Specific chemical modifications that neutralize or eliminate the free amino or carboxyl groups on PS A resulted in a loss of abscess-inducing potency of at least two orders of magnitude. These data strongly suggested that PS A requires both positively charged and negatively charged functional groups to promote abscess induction in this animal model. In that study, we also demonstrated that bacterial polysaccharides from other organisms that possess both positively and negatively charged groups as part of their repeating unit structures (C substance and the type 1 capsular polysaccharide from *Streptococcus pneumoniae*, as well as a chemically modified version of the Vi polysaccharide from *Salmonella typhi*) are also capable of inducing intra-abdominal abscesses (14). Those results demonstrated that a distinct structural motif is associated with the ability of polysaccharide molecules to induce intra-abdominal abscesses and led us to perform additional studies to extend those results. Presently, we report our studies with the more complex of the two *B. fragilis* polysaccharides, PS B, that were performed in order to more specifically define the structural attributes of these carbohydrates that influence the formation of intra-abdominal abscesses in experimental animals.

B. fragilis NCTC 9343 was originally obtained from the National Collection of Type Cultures (London, England), stored at −80°C in peptone-yeast broth until used, and grown anaerobically as previously described (9). The capsular polysaccharide complex from *B. fragilis* NCTC 9343 was isolated by hot phenol-water extraction and subsequent purification of PS A and PS B, also as described previously (9, 15). C substance (the group antigen from *S. pneumoniae*) (Fig. 2) and the *S. pneumoniae* type 1 capsular polysaccharide both possess positively charged free amino groups, and either phosphate or carboxyl functions as a negatively charged substituent (3, 6). C substance was a generous gift from Harold Jennings (National Research Council of Canada), and the *S. pneumoniae* type 1 capsule was obtained from the American Type Culture Collection (Rockville, Md.). Chemical modifications were introduced into the repeating unit structure of *B. fragilis* PS B (Fig. 1B) in order to further characterize the nature of the charged groups on polysaccharides that are required for the induction of abscesses. Modifications to polysaccharide structure were confirmed by nuclear magnetic resonance spectroscopy (14).

The rat model of intra-abdominal sepsis used in this study has been described previously (8, 14). The inoculum contained a 1:1 mixture of the test polysaccharide in phosphate-buffered saline and an adjuvant solution containing sterile rat cecal contents as well as 10% (wt/vol) barium sulfate. This mixture was surgically implanted into the abdominal cavities of the animals. Six days later, the animals were sacrificed and examined for intra-abdominal abscesses by an observer blinded to the experimental groups. A mathematical model was used to compare the biologic activities of modified and unmodified polysaccharides over a range of three doses (200, 20, and 2 μg) and to calculate the dose of each polysaccharide that induced abscesses in 50% of the animals (AD_{50}) (14). Abscesses

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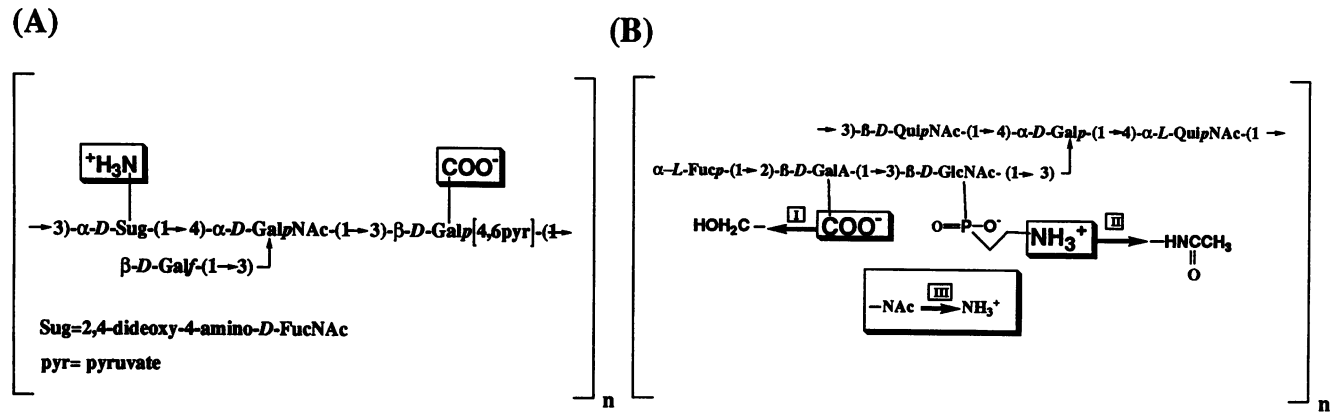

FIG. 1. Fine structures of the *B. fragilis* polysaccharide repeating units. (A) PS A is a tetrasaccharide repeating unit that has one positively charged amino group (conferred by a 2,4-dideoxy-4-amino-D-FucNAc residue) and one negatively charged carboxyl group (conferred by a pyruvate substituent). Charged groups are shown in boxes. (B) PS B is a hexasaccharide repeating unit with a negatively charged carboxyl group (conferred by a galacturonic acid residue) as well as a free amino group and phosphonate group (conferred by an unusual 2-aminoethylphosphonate substituent). This polymer was modified as follows: carbodiimide reduction of the negatively charged carboxyl group to a hydroxymethyl group (modification I); N acetylation of the free amino group (modification II); and de-N acetylation of the three amino sugars (β-D-QuiNAc, α-L-QuiNAc, and GlcNAc) converting *N*-acetyl groups to free amino groups.

induced by these polysaccharides were generally uniform in size, and those rats that possessed one or more fully formed abscesses were scored as positive. Animals that did not have any fully formed abscesses were scored as negative. Two control groups were included in all experiments: positive controls were challenged with intact *B. fragilis* mixed with adjuvant solution, while negative controls received adjuvant solution alone. In all cases, 100% of the positive control group and none of the negative control group developed abscesses (data not shown).

In the rat model, the AD_{50} of unmodified *B. fragilis* PS B was 4 μg (Table 1). Conversion of the carboxyl group on the galacturonic acid of PS B to a hydroxymethyl group via carbodiimide reduction (Fig. 1B, modification I) created a polysaccharide with one free amino group and one phosphonate group per repeating unit (a positive-to-negative-charge ratio of 1:1). This modification did not alter the ability of PS B to induce abscesses (AD_{50} = 3 μg; Table 1). N acetylation of the free amino group (NH_3^+) associated with the 2-aminoethylphosphonate substituent of PS B created a polysaccharide with two negatively charged groups (carboxyl and phosphonate) and no positive charges (Fig. 1B, modification II). This conversion of the free amino group to a secondary amine (NH_2R; R = acetyl group) via N acetylation significantly reduced abscess induction by this polymer (AD_{50} > 200 μg; $P < 0.005$ compared with the AD_{50} of unmodified PS B; Table 1); this observation is consistent with our previous finding (14) that N acetylation of PS A abrogated abscess induction.

Removal of acetyl groups from the three amino sugars present in the repeating unit introducing three free amino groups per repeating unit created a net positive charge on PS B, with a 4:2 ratio of positively to negatively charged groups (Fig. 1B, modification III). The de-N acetylation of PS B significantly reduced the abscess-inducing potential of this saccharide (AD_{50} > 200 μg; $P < 0.005$ compared with the AD_{50} of unmodified PS B; Table 1). This result indicated that increasing the density of the charged amino groups on PS B abrogated abscess-inducing potential and suggests that the presence of both positively and negatively charged groups on this polysaccharide is necessary but not sufficient to confer this biologic activity. It is apparent that the density of these charged groups per repeating unit may be another critical variable regulating the ability of these polysaccharides to induce abscesses. Perhaps the increased number of positively charged

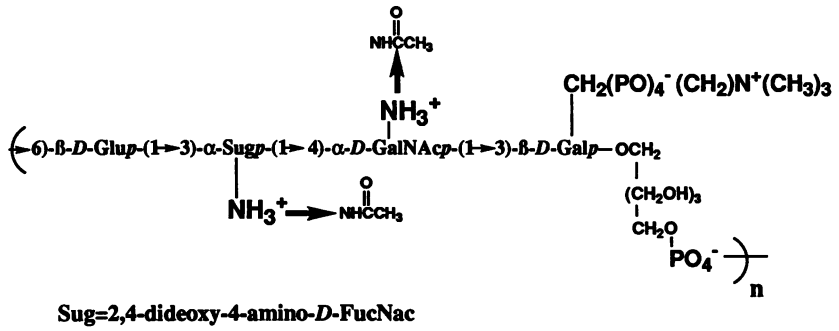

FIG. 2. Repeating unit structure of C substance. (A) C substance has a tetrasaccharide repeating unit with a total of three positive charges (conferred by a phosphatidylcholine substituent and two free amino groups) and two negative charges (conferred by phosphate groups). For some experiments, the two free amino groups of this polymer were N acetylated (large arrows), leaving this saccharide with one positive charge conferred by the phosphatidylcholine substituent.

TABLE 1. Abscess induction by unmodified and modified PS B from *B. fragilis*

Type of PS B	No. of rats with abscesses at indicated dose (μg)/no. tested			AD_{50} (μg)	*P* value[a]
	200	20	2		
Native	18/19	13/18	7/19	4	
Reduced (modification I)	17/18	15/20	9/19	3	NS
N acetylated (modification II)	4/20	5/20	1/20	>200	<0.005
De-N acetylated (modification III)	9/20	2/20	1/20	>200	<0.005

[a] Compared with AD_{50} of native PS B. NS, not significant.

amino groups on this polysaccharide prevents critical interactions with cell receptors (possibly present on T cells) that initiate the cascade of cellular events resulting in abscess formation.

We next assessed whether specific types of positively and negatively charged groups on polysaccharides are required for abscess induction. Investigation of the type of positive charge required on abscess-inducing polysaccharides was performed by testing N-acetylated versions of the *S. pneumoniae* type 1 capsule and C substance polysaccharide in animals. As we have previously shown (14), N acetylation of free amino groups on the *S. pneumoniae* type 1 capsule neutralizes its positive charge and markedly reduces the abscess-inducing capability of this polysaccharide (AD_{50} > 200 μg; $P = 0.018$ compared with the AD_{50} of the unmodified *S. pneumoniae* type 1 capsule). We now sought to determine whether there is a specific requirement for positively charged free amino groups on polysaccharides or whether any positively charged substituent can facilitate abscess induction. In this experiment, C substance was chemically modified via N acetylation and tested in animals. N acetylation of this polymer converts the two free amino groups of the repeating unit structure to *N*-acetyl groups but does not affect the positively charged nitrogen group on the phosphatidylcholine substituent (Fig. 2). The N-acetylated C substance exhibited reduced abscess-inducing ability (AD_{50} > 200 μg; $P < 0.05$ compared with the AD_{50} of unmodified C substance; Table 2). This finding was intriguing since N-acetylated C substance still possesses one positive charge (conferred by the nitrogen group on the phosphatidylcholine substituent) and two negative charges (phosphate groups; Fig. 2). This result indicated that free amino groups on these polysaccharides are necessary for abscess-inducing activity.

Our studies with *B. fragilis* PS B, as well as other abscess-inducing polysaccharides, have shown that no structural requirement for a certain type of negatively charged group on these polymers exists. Rather, polysaccharides with different negatively charged groups were shown to be potent inducers of abscesses. More specifically, polysaccharides whose negatively charged groups were of the carboxyl type only (*B. fragilis* PS A and the *S. pneumoniae* type 1 capsule), of the phosphate or phosphonate type only (*S. pneumoniae* C substance and *B. fragilis* PS B subjected to modification I), or of both the carboxyl and phosphonate types (*B. fragilis* PS B) were able to induce abscess formation (14) (Tables 1 and 2).

TABLE 2. Abscess induction by native and modified C substance

S. pneumoniae polysaccharide type	No. of rats with abscesses at indicated dose (μg)/no. tested			AD_{50} (μg)	*P* value[a]
	200	20	2		
C substance[b]	17/18	12/18	6/19	5	
C substance (N acetylated)	5/10	1/10	1/10	>200	<0.05

[a] Compared with AD_{50} of unmodified C substance.

[b] Experimental data with unmodified C substance were previously reported (14) and are shown here for comparison. These groups were tested in the same experiments.

These data underscore the relative effects of the charged substituent groups on abscess-inducing polysaccharides. First, the importance of the free amino group on abscess-inducing polysaccharides does not appear to be attributable to its charge. However, it is possible that the highly reactive nature of this substituent is important in forming bonds with other chemical structures such as carbonyl groups (C=O) present on eukaryotic cell surfaces. The reaction of free amino and carbonyl groups (both present on effector cells of the immune system) has been demonstrated previously to form reversible covalent bonds (known as Schiff bases) that are necessary for antigen-specific T-cell activation (11, 12, 17). Perhaps a similar interaction that is necessary to stimulate the immunologic events that lead to abscess induction occurs between the free amino group of polysaccharides and carbonyl groups on effector cells. We are currently investigating this possibility.

It is unclear what role the negative charges play in this system, since either carboxyl groups or phosphate/phosphonate groups can suffice as the source of this charge. It is possible that the negative charges on abscess-inducing polysaccharides somehow stabilize the conformation of the polymer in a manner that allows for interaction of the polysaccharide with the host immune system via the free amino group of the repeating-unit structure.

We have demonstrated previously that bacterial polysaccharides with no charged groups or with one negatively charged group (a carboxyl group) per repeating unit do not induce abscesses (14). This work demonstrated that the capsular polysaccharide from *S. pneumoniae* type 14 (7), which has no charged groups, failed to induce abscesses at the highest concentration tested (200 μg). Capsular polysaccharides of group B *Neisseria meningitidis* (2) or of group B streptococcus types Ia and III (4, 16) have one negative charge per repeating unit and were also poor inducers of abscesses.

It is evident from these studies that oppositely charged groups on polysaccharides mediate abscess induction by these polymers. However, more specific structural requirements than the simple presence of oppositely charged groups exist for polysaccharides that exhibit abscess-inducing capability. We have documented a requirement for a free amino group as the type of positively charged group on the polysaccharides tested and have shown that the density of these groups influences the capacity of these macromolecules to induce abscesses. Thus, this work demonstrates that abscess induction in the peritoneal cavities of rodents is mediated by a distinct structural motif exhibited by bacterial polysaccharides and delineates a novel host-parasite interaction that mediates this pathobiologic response to bacterial infection.

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REFERENCES

1. **Baumann, H., A. O. Tzianabos, J.-R. Brisson, D. L. Kasper, and H. J. Jennings.** 1992. Structural elucidation of two capsular polysaccharides from one strain of *Bacteroides fragilis* using resolution NMR spectroscopy. Biochemistry **31:**4081–4089.
2. **Bhattacharjee, A. K., H. J. Jennings, C. P. Kenny, A. Martin, and I. C. P. Smith.** 1975. Structural determination of the sialic acid polysaccharide antigens of *Neisseria meningitidis* serogroup B and serogroup C with carbon 13 nuclear magnetic resonance. J. Biol. Chem. **250:**1926–1932.
3. **Jennings, H. J., C. Lugowski, and N. M. Young.** 1980. Structure of the complex polysaccharide C-substance from *Streptococcus pneumoniae* type 1. Biochemistry **19:**4712–4719.
4. **Jennings, H. J., K.-G. Rosell, and D. L. Kasper.** 1983. Structure of native polysaccharide antigens of types Ia and Ib group B *Streptococcus*. Biochemistry **22:**1258–1264.
5. **Kasper, D. L., A. B. Onderdonk, J. Crabb, and J. G. Bartlett.** 1979. Protective efficacy of immunization with capsular antigen against experimental infection with *Bacteroides fragilis*. J. Infect. Dis. **140:**724–731.
6. **Lindberg, B., B. Lindqvist, J. Lonngren, and D. A. Powell.** 1980. Structural studies of the capsular polysaccharide from *Streptococcus pneumoniae* type 1. Carbohydr. Res. **78:**111–117.
7. **Lindberg, B., J. Lonngrenn, and D. A. Powell.** 1977. Structural studies on the specific type 14 pneumococcal polysaccharide. Carbohydr. Res. **58:**177–186.
8. **Onderdonk, A. B., D. L. Kasper, R. L. Cisneros, and J. G. Bartlett.** 1977. The capsular polysaccharide of *Bacteroides fragilis* as a virulence factor: comparison of the pathogenic potential of encapsulated and unencapsulated strains. J. Infect. Dis. **136:**82–89.
9. **Pantosti, A., A. O. Tzianabos, A. B. Onderdonk, and D. L. Kasper.** 1991. Immunochemical characterization of two surface polysaccharides of *Bacteroides fragilis*. Infect. Immun. **59:**2075–2082.
10. **Polk, B. F., and D. L. Kasper.** 1977. *Bacteroides fragilis* subspecies in clinical isolates. Ann. Intern. Med. **86:**569–571.
11. **Rhodes, J.** 1989. Evidence for an intercellular covalent reaction essential in antigen-specific T cell activation. J. Immunol. **143:** 1482–1489.
12. **Rhodes, J.** 1990. Erythrocyte rosettes provide an analogue for Schiff base formation in specific T cell activation. J. Immunol. **145:**463–469.
13. **Shapiro, M. E., D. L. Kasper, D. F. Zaleznik, S. Spriggs, A. B. Onderdonk, and R. W. Finberg.** 1986. Cellular control of abscess formation: role of T cells in the regulation of abscesses formed in response to *Bacteroides fragilis*. J. Immunol. **137:**341–346.
14. **Tzianabos, A. O., A. B. Onderdonk, B. Rosner, R. L. Cisneros, and D. L. Kasper.** 1993. Structural features of polysaccharides that induce intra-abdominal abscesses. Science **262:**416–419.
15. **Tzianabos, A. O., A. Pantosti, J. R. Brisson, H. J. Jennings, and D. L. Kasper.** 1992. The capsular polysaccharide of *Bacteroides fragilis* comprises two ionically linked polysaccharides. J. Biol. Chem. **267:**18230–18235.
16. **Wessels, M. R., V. Pozsgay, D. L. Kasper, and H. J. Jennings.** 1987. Structure and immunochemistry of an oligosaccharide repeating unit of the capsular polysaccharide of type III group B *Streptococcus*. J. Biol. Chem. **262:**8262–8267.
17. **Zheng, B., S. J. Brett, J. P. Tite, M. R. Lifely, T. A. Brodie, and J. Rhodes.** 1992. Galactose oxidation in the design of immunogenic vaccines. Science **256:**1560–1563.