Effect of Molecular Size on the Ability of Zwitterionic Polysaccharides to Stimulate Cellular Immunity

Wiltrud M. Kalka-Moll, Arthur O. Tzianabos, Ying Wang, Vincent J. Carey, Robert W. Finberg, Andrew B. Onderdonk and Dennis L. Kasper

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The large-molecular-sized zwitterionic capsular polysaccharide of the anaerobe *Bacteroides fragilis* NCTC 9343, designated polysaccharide (PS) A, stimulates T cell proliferation in vitro and induces T cell-dependent protection against abscess formation in vivo. In the present study, we utilized a modification of a recently developed ozonolytic method for depolymerizing polysaccharides to examine the influence of the molecular size of PS A on cell-mediated immunity. Ozonolysis successfully depolymerized PS A into structurally intact fragments. PS A with average molecular sizes of 129.0 (native), 77.8, 46.9, and 17.1 kDa stimulated CD4\(^+\)-cell proliferation in vitro to the same degree, whereas the 5.0-kDa fragment was much less stimulatory than the control 129.0-kDa PS A. Rats treated with 129.0-kDa, 46.9-kDa, and 17.1-kDa PS A molecules, but not those treated with the 5.0-kDa molecule, were protected against intraabdominal abscesses induced by challenge with viable *B. fragilis*. These results demonstrate that a zwitterionic polysaccharide as small as 22 repeating units (88 monosaccharides) elicits a T cell-dependent immune response. These findings clearly distinguish zwitterionic T cell-dependent polysaccharides from T cell-independent polysaccharides and give evidence of the existence of a novel mechanism for a polysaccharide-induced immune response.


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Materials and Methods

**Bacterial strain and isolation of capsular PS A**

*B. fragilis* NCTC 9343 was originally obtained from the National Collection of Type Cultures (London, U.K.). After rat-spleen passage, the bacteria were stored at \(-80^\circ\)C in peptone-yeast broth until use and then grown anaerobically as previously described (27, 28). PS A was extracted from the bacterial cells by hot phenol/water; digested extensively with RNase, DNase, and pronase/protease K; and purified by gel filtration in a 3% deoxycholate acid-containing buffer at pH 9.8. Further purification was achieved by gel filtration and anion-exchange chromatography as previously described (27, 28). The polysaccharide was subjected subsequently to isoelectric focusing to disaggregate this highly charged molecule. The final product was found to be essentially free of contaminating protein, nucleic acid, LPS, and lipids by the following assays: UV-scanning, protein measurements including the Lowry (29) and BCA (Pierce, Rockford, IL) methods, silver stain SDS-PAGE gels (30), and high resolution (500 MHz) proton nuclear magnetic resonance (NMR) spectroscopy (9–11, 27, 28).
Ozonolysis and purification of depolymerized PS A

PS A was suspended at a concentration of 5.93 mg/ml in buffer consisting of 900 μl of 0.01 M PBS with 0.05% NaN₃ (pH 7.2) and 100 μl of 0.2 M NaHCO₃ (final pH 7.6) (PBSA). Ozone was generated from compressed air through an ozone generator (More-Zon10, Taipei, Taiwan). A stream of ozone/air gas (21% O₃) was passed through the solution at room temperature at a flow rate of 3.17 ml/s. At time interval 0 and at various subsequent time intervals, 35 μl of the reaction solution was removed and diluted with 35 μl of PBSA, and the molecular size distribution of the products was determined by gel filtration chromatography on Superose 6, Superose 12, and Superdex 30 columns calibrated with dextran standards according to the fractionation ranges of the gel (Amer sham Pharmacia Biotech, Piscataway, NJ) at a flow rate of 0.5 ml/min (26, 31). The columns were eluted with PBSA, and the fractions were monitored by a differential refractometer. The ozone flow was terminated when products reached the desired molecular size. An aliquot of the depolymerized PS A was removed, and the reaction was then allowed to continue. The depolymerized saccharide was separated from native PS A by gel filtration chromatography on a Superdex 200 column (Amersham) equilibrated in 0.3 μM sodium phosphate buffer (pH 7.2) with 0.05% sodium azide. The fractions were again monitored by a differential refractometer. The polysaccharide of the desired size was desalted by gel filtration with G25 media (Amersham), lyophilized, and stored in 3 M NaCl at a concentration of 5 mg/ml at −20°C to avoid reaggregation.

NMR

Proton NMR experiments with native and depolymerized PS A were performed on a Bruker AMX500 spectrometer with a proton resonance frequency of 500.13 MHz. All ¹H spectra were recorded at 70°C in H₂O, and chemical shifts were referenced in relation to H₃PO₄ resonance at 4.00 ppm.

T cell proliferation assays

Mononuclear cells and CD4⁺ CD8⁻ cells from Leukopacks from different anonymous platelet donors were isolated by centrifugation of cells in Ficoll-Hypaque gradients and purified using nylon wool and immunomagnetic beads as previously described (32–34). The purity of the CD4⁺ cells was confirmed by FACS analysis (32–34). This analysis accommodated by clustered-measures analysis of variance (37). The development of one or more abscesses was considered a positive result.

Animal model of intraabdominal abscess formation

An animal model of intraabdominal abscess formation was used to evaluate the effect of molecular size on protection against abscess formation (35). In two independent experiments, groups of 10 outbred male Wistar rats (150–175 g; Charles River Laboratories, Wilmington, MA) were injected s.c. with a sterile solution of 100 μl of 0.15 M PBS containing 10.1, or 0.1 μg of PS A of 129.0, 46.9, 17.1, or 5.0 kDa; 100 μl of 0.15 M PBS were used as a negative control. Each group was treated with polysaccharide at ~24 h, 0 h (challenge), and 24 h. The challenge inoculum contained B. fragilis NCTC 9343, sterile cecal contents, and 10% barium sulfate. Another group of five animals was challenged with sterile cecal contents and 10% barium sulfate alone. Rats were anesthetized with a single i.p. injection of 0.15 ml of Nembutal (50 mg/ml; Abbott Laboratories, North Chicago, IL). An anterior midline incision (0.5 cm) was made through the abdominal wall and peritoneum, and a gelatin capsule with 0.5 ml of inoculum was inserted into the pelvis (36). Six days after challenge, the rats were sacrificed and examined for macroscopically visible i.p. abscesses by observers who were unaware of treatment status. Grossly visible abscesses were confirmed microscopically. The development of one or more abscesses was considered a positive result.

Statistical analysis

For PS A of each molecular size and negative controls, three independent T cell assays were performed, each in quadruplicate. The stimulation ratio for a saccharide was defined as the ratio of the cpm of the stimulated T cells in one well divided by the geometric mean of the cpm for the corresponding four wells with the negative controls. For each chain length, each assay yielded four correlated stimulation ratios. Log stimulation ratios were analyzed by clustered-measures analysis of variance (37). In vivo experiments were analyzed in a structured logistic regression model that permitted evaluation of separate dose-response relationships (dose in micrograms and moles) and direct interference on median abscess protective dose. Specifically, Pr(abscess/dose = χ₁, length = χ₂) = λ[1 + exp(β₁(χ₁ − γ₁))]⁻¹, where λ > 0 is the rate of abscess formation in positive controls; β₁ is the slope of the dose-response relation on the logistic scale for chain length χ₁; and γ₁ is the median protective dose (PD₅₀ = dose that would have protected 50% of the saline-treated rats from abscess formation) for the chain length χ₁ (38). This analysis accommodated the fact that not all positive control animals developed abscesses. Likelihood ratio tests were performed for hypotheses concerning commonality of dose-response slopes and PD₅₀s. Rats that died within 2 days after challenge were not included in the analysis because their deaths were due to anesthesia and surgery.

FIGURE 1. PS A of B. fragilis NCTC 9343 has a tetrasaccharide repeating unit with a balanced positively charged amino group and negatively charged carboxyl group. Oxonolysis cleaves the β-α-galactopyranosyl of the backbone of PS A to give the corresponding acid (arrow). The structure of PS A is as follows:
FIGURE 2. The kinetics of ozone treatment of native PS A of *B. fragilis* NCTC 9343 followed a logarithmic curve. At each of the three time points, 8, 11, and 23 min, one-third of the sample was removed for in vitro and in vivo assays. The average molecular size of the polysaccharide was halved after 9 min and reduced to one-fifth of the original size after 18 min. *ru* = repeating units.

Results

**Generation of PS A molecules with different chain lengths**

Native PS A with an average molecular size of 129.0 kDa (upper limit, 245.5 kDa; lower limit, 74.3 kDa), which corresponds to approximately 155 repeating units, was oxidized with ozone to produce PS A polymers of decreased molecular size. After treatment for 6, 8, 11, 16, 21, and 23 min, the resulting average polysaccharide sizes were determined by gel filtration chromatography (Fig. 2). The kinetics of the decrease in polysaccharide size following ozone treatment appeared to fit a logarithmic function. The molecular size distribution of the ozonolysis products were typical of polysaccharide preparations, eluting in one symmetrical peak (data not shown). Samples corresponding to the generation of PS A with the average molecular sizes of 129.0, 77.8 (upper limit, 204.2 kDa; lower limit, 20.5 kDa), 46.9 (upper limit, 155.0 kDa; lower limit, 11.8 kDa), and 17.1 kDa (upper limit, 77.2 kDa; lower limit, 4.2 kDa) were used for the biological assays. Efforts to generate PS A of ≤10 kDa yielded (at 54 min) an oligosaccharide with an average molecular size of 5 kDa (upper limit, 21.5 kDa; lower limit, 1.6 kDa) that corresponded to 6 repeating units. Proton NMR analysis of native and depolymerized PS A chain lengths showed the saccharides to have intact repeating units (Fig. 3).

**Effect of PS A chain length on induction of T cell proliferation in vitro**

PS A with average polymer sizes of 129.0, 77.8, 46.9, and 17.1 kDa elicited T cell proliferation significantly greater than that in the negative control. The stimulation ratios of the quadruplicate experiments in three independent assays showed that PS A as small as 17.1 kDa was as potent in inducing T cell proliferation as native PS A (average 6 repeating units) had the same structure as the polymer (10).

**Effect of polysaccharide chain length on prevention of intraabdominal abscess formation**

PS A of 129.0 (from Fig. 4A), 46.9, 17.1, and 5.0 kDa was tested for the ability to protect rats against *B. fragilis* induced abscess formation. In these experiments, 87.5% of saline-treated rats developed intraabdominal abscesses (Fig. 5). None of the control rats (challenged with barium sulfate and sterile cecal contents only) developed abscesses (data not shown). After *B. fragilis* challenge, 21%, 26.3%, and 31.6% of rats treated with 10 μg of 129.0-kDa PS A, 46.9-kDa, and 17.1-kDa PS A, respectively, developed abscesses. Of rats treated with 10 μg of 5.0-kDa PS A, 68% developed abscesses. Protection was dose dependent.

The dose of the respective PS A preparations that theoretically would have protected 50% of saline-treated rats from abscess formation (PD_{50}) was calculated with a structured logistic regression

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4 Although the native PS A used in experiments shown in Fig. 4, A and B, is from the same lot, the quantitative level of stimulatory activity of the control native polysaccharide differed in the two sets of experiments. Isoelectric focusing is used to disaggregate the polysaccharides. The results shown in Fig. 4B were obtained with PS A used shortly after isoelectric focusing. We believe the T cell-stimulatory activity of this material was greater than that seen in Fig. 4A because testing quickly followed this disaggregation step and the polysaccharides were used promptly in T cell proliferation assays. We have found that these zwitterionic polysaccharides aggregate very quickly in storage, resulting in reduced T cell-proliferative activity (data not shown). Because of the length of the time required for preparation of different chain length sizes of PS A used in Fig. 4A, these polymers had less in vitro activity.
The PD$_{50}$ was 0.31 mg for rats treated with 129.0-kDa PS A, 1.67 mg for rats treated with the 46.9-kDa molecule, and 0.68 mg for rats treated with 17.1-kDa PS A; step-down likelihood ratio testing in the structured logistic model yielded a PD$_{50}$ common to PS A of these three sizes of 0.73 mg (95% confidence interval (CI) 0.29, 1.88). Mathematical extrapolation indicated that a significantly higher dose (187.1 mg) of 5.0-kDa PS A would have protected 50% of rats against abscess formation ($p < 0.001$). Fig. 6 shows that the required dose of 5.0-kDa PS A is well beyond the upper limit of doses used in this experiment.

FIGURE 5. Protection of rats against $B$. fragilis-induced intraabdominal abscess formation by PS A of various molecular sizes. The rat model of intraabdominal abscess formation was used in two independent experiments, with 10 rats per group in each experiment. Numerators indicate numbers of animals developing abscesses, while denominators indicate total numbers of animals surviving surgery.

FIGURE 6. Statistical analysis of studies determining PD$_{50}$ values. Rats were treated with 10 µg ( ), 1 µg ( ), and 0.1 µg ( ) of PS A of the molecular sizes noted on the x-axis; control rats were treated with saline ( ). Vertical displacements of symbols indicate proportion of rats developing intraabdominal abscesses. The lines depict structured logistic regression fit assuming a common logistic slope for all lengths. PS A of 17.1, 46.9, and 129.0 kDa (average molecular sizes) shared a PD$_{50}$ value (0.73 mg), while 5.0-kDa PS A (average molecular size) had a distinct and extremely high PD$_{50}$ (187.1 µg, $p < 0.001$).

Discussion

Ozonolysis of PS A successfully generated saccharides as small as 5.0 kDa, an average molecular size corresponding to 6 repeating units. PS A of all molecular sizes retained the native structure. Three biologically critical structural features of PS A needed to be preserved on depolymerization: 1) the galactofuranose side chain; 2) the acid-labile 4,6-pyruvate structure; and 3) the very reactive free amino group (39). Traditional methods would have destroyed these biologically active groups. Enzymes specific for the glycosidic linkages in the backbone of PS A have not yet been described. Therefore, ozonolysis, which was shown to depolymerize polysaccharides by cleaving specifically $\beta$-D-aldosidic linkages, represented a promising alternative. In preliminary studies in which we used the previously described method, including per-acetylation and deacetylation, to achieve the most selective oxidation, we obtained smaller structurally intact polymers but found it impossible to selectively deacetylate the molecule to regain the positive charge in only a single position. Without the protection of possibly reacting groups, two $\beta$-D-aldosidic linkages were at risk of oxidation: one between the galactofuranose side chain and the galactopyranose of the backbone, and the other between the galactopyranose bearing the pyruvate group and the fucose of the backbone. As confirmed by NMR spectroscopy, the $\beta$-D-aldosidic
linkage of the galactofuranose side chain was less reactive to ozonolysis, a result suggesting that ozonolysis, even without protection of the hydroxyl groups, specifically cleaved B-α-aldo-sidic linkages of backbone sugars under stereoelectronic control (Fig. 2) (26).

In this study, characterization of the T cell response to PS A of different chain lengths demonstrated that the biological activity of the saccharide resides in a polymer as small as 17.1 kDa. Our statistical analysis took into account the fact that the three independent T cell assays for PS A of each molecular size were performed with CD4+ cells from different donors. Despite the individual variability of the response, PS A molecules with average molecular sizes between 129.0 and 171.1 kDa stimulated CD4+ cells to a similar degree (p = 0.76). The 5.0-kDa PS A was significantly less capable of stimulating T cell proliferation than its native polymer (p = 0.035). These in vitro results correlated with results in vivo: the 129.0-, 46.9-, and 17.1-kDa PS A molecules were equally able to protect rats against abscess formation (PD50 = 0.73 μg), whereas 5.0-kDa PS A was not protective (PD50 = 187.1 μg, p < 0.001).

In the context of what is known about the influence of the size of T cell-independent polysaccharides on immunogenicity, our results strengthen the conclusion of earlier in vivo experiments classifying PS A as a T cell-dependent Ag and clearly distinguish this molecule from polysaccharides with only negatively charged or no charged residues. Like that of other T cell-independent polysaccharides, the immunological activity of PS A correlates with its molecular size. However, in contrast to these other polysaccharides, PS A retains its full antigenicity at least down to a size as small as 17.1 kDa. T cell-independent saccharides investigated, in particular, dextrans and capsular polysaccharides from group B streptococci, Streptococcus pneumoniae, Neisseria meningitidis, and Haemophilus influenzae, are thought to bind membrane-bound Ig receptor on B cells to induce a humoral response. Affinity of Ab binding was shown to be positively correlated with increasing chain length. Up to hundreds of repeating units in the case of group B streptococcal and S. pneumoniae type 14 capsular polysaccharides are necessary to elicit optimal binding activity, thus supporting the hypothesis that anticapsular Abs recognize a conformational epitope fully expressed only in high m.w. forms of the T cell-independent saccharide molecules (21, 24, 40). Although six to seven sugar residues of a linear or(1→6)-linked glucose have been proven sufficient to fill the Ag-binding site of Ig s (16–18), such small-sized saccharides do not induce humoral response in primates. Nonzwitterionic polysaccharides smaller than 80,000–90,000 Da do not induce Ab response (25), and even T cell-independent polysaccharides with a molecular size between 80,000 Da and 200,000 Da are only variably immunogenic in humans (40). Our findings with PS A clearly distinguish this molecule from those T cell-independent polysaccharides.

The mechanism through which T cells are stimulated by a zwitterionic polysaccharide is not known, but the results suggest two possible pathways. Since APCs and a minimal PS A size of 7–22 repeating units are necessary to stimulate CD4+ cell proliferation, we hypothesize that the Ag-presenting molecules on APCs and the signaling molecules on CD4+ cells are linked by zwitterionic polysaccharides. In the first of the two possible scenarios, if PS A were presented (for example) in the binding groove of a MHC class II molecule (with or without being processed), it would need to have, depending on its primary, secondary, tertiary, and quaternary organization, a minimal molecular size between 1602 Da and about 2996 Da to fit into the cleft (41–48). Conceivably, hydrogen bonds between charged atoms along the PS A and residues of the α-helical regions and between PS A and the β-sheet of HLA-DR (for instance) are a component of the binding interaction that is essential to T cell activation. The conformation of PS A might allow most of its charged residues to get into contact with the T cell receptor. The ability of PS A to interact with T cells from different donors (different haplotypes) suggests promiscuous binding motif(s) due to the existence of different epitopes in the PS A molecules that bind different MHC determinants. In the second scenario, a zwitterionic polysaccharide might act as a T cell mitogen. For instance, for PS A to interact with both MHC and T cell receptor molecules outside their normal Ag-specific sites (in a manner similar to that by which superantigens induce T cell activation), a minimal size would be required. Most of the known superantigens have a molecular mass between 15,000 and 40,000 Da (49–56). Superantigens bind as intact proteins, and no large conformational change occurs upon complex formation (50). However, in comparison to the binding of peptides in the MHC class II cleft, hydrogen bonds seem to play a minor role in the binding of a superantigen to its ligand.

These results demonstrate that a small zwitterionic polysaccharide, successfully generated by ozonolysis, comprising 22 repeating units fully elicits cell-mediated immunological response. These findings clearly distinguish this molecule from T cell-independent polysaccharides and give evidence of the existence of a novel mechanism for a polysaccharide-induced immune response.

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