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

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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⁺ T 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. The Journal of Immunology, 2000, 164: 719–724.

The Gram-negative anaerobe Bacteroides fragilis is the bacterium most commonly isolated from intraabdominal abscesses in humans (1). In the 1970s, this organism’s capsular polysaccharide complex was purified and identified as its major virulence factor for abscess induction (2–4). In subsequent studies, both abscess formation and protection against abscess development were shown to be T cell dependent in rodent models of abdominal sepsis (5–8). The capsular polysaccharide complex of B. fragilis comprises two zwitterionic polysaccharides, which are termed polysaccharide (PS) A and PS B. PS A, the more active of the two molecules, induces T cell proliferation in vitro, an effect that depends on direct contact of APCs with T cells (9). T cells activated by PS A in vitro confer protection against abscess formation in vivo (our unpublished data). The unusual immunologic properties of this polysaccharide depend on the positively charged amino and negatively charged carboxyl groups on its repeating-unit structure (Fig. 1) (8, 10–15). Polysaccharides have been believed to be relatively thymus-independent Ags (T1-2 Ags) that activate B cells by extensively cross-linking their membrane-bound Ig receptor to induce Ab production. Although six to seven sugar residues have been identified as sufficient to fill the Ag-binding site of Igs (16–18), the affinity of Ab binding of some polysaccharides has been positively correlated with increased chain length, which allows full expression of putative conformational epitopes (19–24). Besides antigenicity, in humans the immunogenicity of polysaccharides is known to be a chain length-dependent phenomenon. Polysaccharides with a molecular mass below 80,000–90,000 Da do not elicit an Ab response (25).

Since PS A is unusual among polysaccharides in that it induces a T cell-dependent response, it is important to understand the structural features of PS A that are required for the stimulation of T cells. We have addressed whether molecular size influences T cell dependency. We looked at in vitro and in vivo parameters of the T cell response, i.e., T cell proliferation in vitro and protection of rats against abscesses induced by viable B. fragilis. To depolymerize PS A, we used a modification of a recently developed ozonolytic technique (26). The results show that a relatively short saccharide chain length is required to induce the T cell-dependent biologic properties.

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°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).

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3 Abbreviations used in this paper: PS, polysaccharide; NMR, nuclear magnetic resonance; PDₙ₀, dose that would have protected 50% of the saline-treated rats from abscess formation; CI, confidence interval.
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, Tapei, Taiwan). A stream of ozone/air gas (21% O₂) was passed through the solution at room temperature at a flow rate of 3.17 ml/min. 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 (Amersham 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 then separated from native PS A by gel filtration chromatography on a Superdex 200 column (Amersham) equilibrated in 0.3 mM 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 2H₂O, and chemical shifts were referenced in relation to H₂O resonance at 4.26 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, as well as the Ag concentration, were shown to be optimal in preliminary experiments. Earlier studies had revealed maximal T cell proliferation at day 8 with continuous stimulation by native PS A. Therefore, after 8 days, cells were pulsed with [3H]thymidine (1 Ci/well) for an additional 6 h and harvested with a semiautomated cell harvester. Incorporation of [3H]thymidine by CD4⁺ CD8⁻ T cells was quantitated in a scintillation counter, and the results were expressed as cpm. Three independent T cell proliferation assays, each with CD4⁺ cells from different donors, were performed for PS A of each molecular size.

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 triplicate. 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 = χ, length = λ) = Φ[1 + exp{β + (χ − γ)}]^−1, where λ > 0 is the rate of abscess formation in positive controls, β is the slope of the dose-response relationship on the logistic scale for chain length λ, and γ is the median protective dose (PD₅₀ = dose that would have protected 50% of the saline-treated rats from abscess formation) for the chain length (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 did not die 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. Ozonolysis 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:

\[
\begin{align*}
\text{4,6-pyruvate} & \\
\leftarrow-3\alpha-\text{D-(2,4-dideoxy-4-amino-D-FucNac)-(1→4)}\alpha-\text{D-Galp-Nac-(1→3)}\beta-\text{D-Galp-(1→3)}
\end{align*}
\]
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

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 PS A of larger molecular size (Fig. 4). Because of this result, a smaller chain-length size of PS A was generated and tested in T cell proliferation assays. To accomplish this, native PS A from the same lot was prepared by isoelectric focusing and then depolymerized by ozonolysis into 5.0-kDa average molecular sized molecules. The native PS A (Fig. 4B) was shown to have the identical structure and purity as the native PS A produced previously (Fig. 3) from this lot. The 5.0-kDa polysaccharide was structurally intact (Fig. 3) but was significantly less stimulatory to CD4+ T cells than native PS A (p = 0.035) (Fig. 4B) (9). The ability to induce T cell proliferation was dose dependent (data not shown).

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 (PD50) was calculated with a structured logistic regression

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.
model. The PD$_{50}$ was 0.31 µg for rats treated with 129.0-kDa PS A, 1.67 µg for rats treated with the 46.9-kDa molecule, and 0.68 µg 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 µg (95% confidence interval (CI) 0.29, 1.88). Mathematical extrapolation indicated that a significantly higher dose (187.1 µg) 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.

On a molar basis, the PD$_{50}$ values for the 129.0-, 46.9-, and 17.1-kDa PS A preparations were nearly identical (10$^{-12.16}$, 10$^{-13.33}$, 10$^{-13.38}$, respectively). Step-down likelihood ratio testing for common PD$_{50}$s among the different chain lengths revealed a common PD$_{50}$ for 17.1-kDa and 46.9-kDa PS A (10$^{-13.36}$ mol) with separate values for 5.0-kDa PS A (PD$_{50}$ = 10$^{-16.65}$ mol, 95% CI = 10$^{-15.92}$ mol, 10$^{-17.68}$ mol) and 129.0-kDa PS A (PD$_{50}$ = 10$^{-12.18}$ mol, 95% CI = 10$^{-11.65}$ mol, 10$^{-12.67}$ mol).

**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 β-D-aldosidic linkages, represented a promising alternative. In preliminary studies in which we used the previously described method, including peracetylation 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 β-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 β-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 \( \beta\)-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 (PD\(_{50}\) = 0.73 \( \mu \)g), whereas 5.0-kDa PS A was not protective (PD\(_{50}\) = 187.1 \( \mu \)g, \( p < 0.001 \)).

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, dextrins 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 \( \alpha-(1\rightarrow6)\)-linked glucose have been proven sufficient to fill the Ag-binding site of Igs (16–18), such small-sized saccharides do not induce humoral response in primates. Nonwzitterionic 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 zwiterionic polysaccharide is not known, but the results suggest two possible pathways. Since APCs and a minimal PS A size of \( \sim 7–22 \) 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 zwiterionic 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 \( \alpha\)-helical regions and between PS A and the \( \beta\)-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 zwiterionic 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 zwiterionic 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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References


