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T Cells Activated by Zwitterionic Molecules Prevent Abscesses Induced by Pathogenic Bacteria*

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Immunologic paradigms classify bacterial polysaccharides as T cell-independent antigens. However, these models fail to explain how zwitterionic polysaccharides (Zps) confer protection against intraabdominal abscess formation in a T cell-dependent manner. Here, we demonstrate that Zps elicit a potent CD4+ T cell response *in vitro* that requires available major histocompatibility complex class II molecules on antigen-presenting cells. Specific chemical modifications to Zps show that: 1) the activity is specific for carbohydrate structure, and 2) the proliferative response depends upon free amino and carboxyl groups on the repeating units of these polysaccharides. Peptides synthesized to mimic the zwitterionic charge motif associated with Zps also exhibited these biologic properties. Lysine-aspartic acid (KD) peptides with more than 15 repeating units stimulated CD4+ T cells *in vitro* and conferred protection against abscesses induced by bacteria such as *Bacteroides fragilis* and *Staphylococcus aureus.* Evidence for the biologic importance of T cell activation by these zwitterionic polymers was provided when human CD4+ T cells stimulated with these molecules *in vitro* and adoptively transferred to rats *in vivo* conferred protection against intraabdominal abscesses induced by viable bacterial challenge. These studies demonstrate that bacterial polysaccharides with a distinct charge motif activate T cells and that this activity confers immunity to a distinct pathologic response to bacterial infection.

The study of T cell recognition of foreign antigens has been directed primarily toward an understanding of the host's immune system response to proteins or peptides. The recent demonstration that non-peptide-containing mycobacterial lipid and glycolipid antigens activate T cells in conjunction with CD1 molecules on the surface of antigen-presenting cells (APCs)[1] has broadened our understanding of antigens capable of T cell recognition (1–3). In those studies, the carbohydrate component of mycobacterial glycolipids was, in part, responsible for influencing T cell responses. This observation supported earlier work in which T cell recognition of glycoproteins was shown to be dependent on the content and distribution of sugar residues in these structures (4–8). However, purified carbohydrates and polysaccharides devoid of associated lipids and proteins did not elicit proliferative responses from T cells.

The Gram-negative anaerobe *Bacteroides fragilis* is the most common bacterial isolate from intraabdominal abscesses in humans (9, 10). In a rat model simulating human intraabdominal sepsis, *B. fragilis,* but not other anaerobic bacterial species, has the distinct ability to induce abscesses when implanted into the peritoneal cavities of animals (11–14). Anaerobic species other than *B. fragilis* require co-administration with a facultative organism to induce abscesses. Recently, we have shown that prophylactic or therapeutic subcutaneous administration of a capsular polysaccharide from this organism, PSA, aborts the formation of intraabdominal abscesses in rats challenged with *B. fragilis* or other intestinal bacteria capable of synergistically stimulating abscess formation (15). Studies aimed at understanding the cellular basis of protection against abscess formation demonstrated that splenic T cells obtained from PSA-treated animals confer protection when transferred to animals challenged with these abscess-inducing bacteria (15, 16). These studies suggested that PSA possessed novel immunomodulatory properties affecting T cell function.

PSA is a polysaccharide composed of oligosaccharide repeating units possessing constituent sugars with free amino and carboxyl groups (Fig. 1). The presence of oppositely charged groups on the same bacterial polysaccharide is distinctly uncommon because most polysaccharides contain either neutral or negatively charged groups. The presence of the free amino and carboxyl group on the PSA repeating unit is critical to its biologic function. Specific chemical neutralization of either of these charged substituents abrogates its ability to protect against abscess formation in animals (16).

The ability of *B. fragilis* PSA to elicit a protective host response that is dependent on T cells suggested an interaction between PSA and this cell type. In this study, we present defined chemical evidence that Zps are able to activate T cells. Proof that it is the charge motif that is critical for this activity was provided when chemical elimination of the charged group on the polysaccharide resulted in failure of these molecules to activate T cells. Furthermore, similarly charged microbial polysaccharides or peptides synthesized to mimic the charge motif of Zps activated T cells *in vitro,* and this interaction was functionally important in protection against abscess formation.

EXPERIMENTAL PROCEDURES

Polysaccharide Preparations—PSA was prepared from *B. fragilis* as described previously (17, 18). PSA used for proliferation experiments was subjected to isoelectric focusing in a Rotofor apparatus (Bio-Rad) to obtain the molecule in a disaggregated state (17). Following this proce-

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[1] The abbreviations used are: APC, antigen-presenting cell; PSA, polysaccharide A; Zps, zwitterionic polysaccharides; MHC, major histocompatibility complex; $NaIO_4$, sodium metaperiodate; $NaBH_4$, sodium borohydride; CP, capsular polysaccharide.

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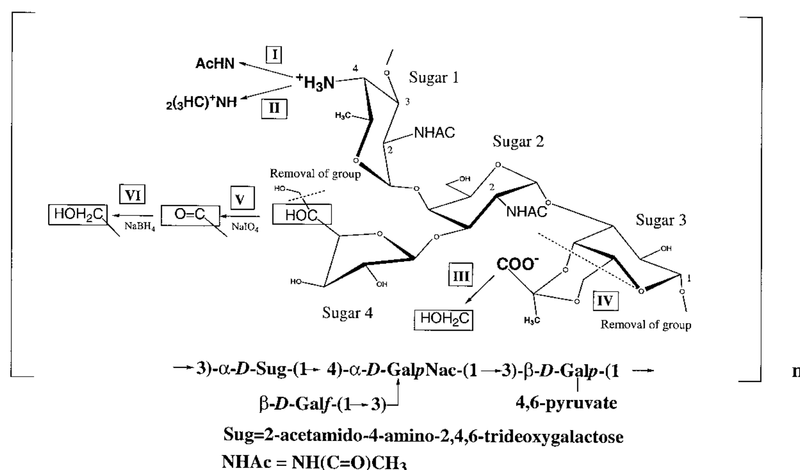

FIG. 1. **Fine structure of *B. fragilis* PSA.** This polysaccharide is composed of approximately 200 tetrasaccharide repeating units and possesses free amino, *N*-acetyl, and carboxyl groups. Modifications to the PSA structure (*I–VI*) are described under "Experimental Procedures."

dure, the preparation was dialyzed against deionized water to remove ampholyte, lyophilized, and stored in 3 M NaCl to prevent aggregation. For some experiments, PSA was chemically modified to assess the role of structure on T cell activation. All modifications are shown in Fig. 1. Treatment with acetic anhydride was used to convert all free amino groups on Sugar 1 to *N*-acetyl groups (Modification I) as described previously (18). In addition, the saccharide was treated with 37% formaldehyde in the presence of sodium cyanoborohydride in order to convert the free amino group on PSA repeating unit to a tertiary dimethylamine such that it retained a positive charge (Sugar 1, Modication II). This conversion was confirmed by NMR spectroscopy, as evidenced by the resonance at 2.9 ppm (singlet) arising from the methyl protons of the dimethylamine. The negatively charged carboxyl groups associated with the pyruvate substituent were reduced by carbodiimide-mediated reduction (Sugar 3, Modification III) (18). The pyruvate ring was also removed by treatment with 5% acetic acid at 80 °C for 1 h (Sugar 3, Modification IV). The loss of the pyruvate group was confirmed by proton NMR in which a signal at 1.5 ppm due to the methyl proton of the pyruvate group disappeared after treatment. NMR analysis also revealed that other components of the PSA repeating unit were not affected by this treatment.

In order to demonstrate the specificity of T cell activation by PSA, this polymer was chemically oxidized with sodium metaperiodate ($NaIO_4$) as described previously (19). This chemical treatment selectively cleaves the C–C bond between vicinal hydroxyl groups found on carbohydrates. In the case of PSA, periodate oxidation (0.01 M $NaIO_4$ for 90 min at room temperature) specifically cleaves C-6 from the galactofuranose side-chain (Sugar 4, Modification V), leaving an aldehyde group (CHO) at C-5. Subsequent modification of the oxidized PSA involved reduction with sodium borohydride ($NaBH_4$) to reduce the aldehyde at C-5 to a hydroxymethyl group (Sugar 4, Modification VI) that converts the galactofuranose side-chain to arabinofuranose. Hydrolysis of this polysaccharide with 4 M trifluoroacetic acid for 2 h at 125 °C and subsequent analysis by gas chromatography-mass spectrometry of the resultant alditol acetate derivative demonstrated that >95% of the galactofuranose residues had been converted to arabinofuranose upon oxidation and reduction. PSA was treated with 30% hydrogen peroxide (2 h at room temperature), which oxidizes thiol-containing amino acids to sulfone derivatives but does not affect carbohydrate structure. All structural analyses of PSA and chemical modifications thereof were confirmed by NMR spectroscopy.

The *Streptococcus pneumoniae* type 1 and type 3 capsular polysaccharides (CPs) were obtained from the ATCC (Manassas, VA) and treated with 2 M NaOH for 1 h at 80 °C to remove the contaminating cell wall polysaccharide, C substance. Following purification by gel filtration chromatography, the *S. pneumoniae* polysaccharides were subjected to isoelectric focusing, dialyzed, lyophilized, and stored in 3 M NaCl to prevent aggregation. The free amino group of the type 1 CP was chemically modified in two separate experiments. This group was converted to an *N*-acetyl moiety by treatment with acetic anhydride or converted to a tertiary amine by treatment with formaldehyde in the presence of sodium cyanoborohydride as described above for PSA. These chemical modifications were confirmed by NMR spectroscopy as described above. β-Glucan (Alpha-Beta Technology, Worcestor, MA) was used as a negative polysaccharide control for some studies.

Preparation of Peptides—Peptides $(KD)_n$ were synthesized on a Rainin Symphony peptide synthesizer. Peptides were prepared with 4-alkoxybenzyl alcohol resins (PerSeptive Biosystems Inc., Framingham, MA) using Fmoc chemistry. Amino acids were activated with 2-(1H-benzotriazole-1-yl)-1,1,3,3 tetramethyluronium hexafluorophosphate for coupling. The peptides prepared were analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry and NMR spectroscopy. Mass spectra were acquired on a Voyager matrix-assisted laser desorption ionization-time of flight mass spectrometer. Proton NMR spectra were acquired on a Bruker AMX500 instrument with proton frequency of 500 MHz. Both analyses confirmed that the peptides were the expected structures. Preparations of poly-L-lysine with molecular masses ranging from 1000 to 4000 daltons were obtained commercially (Sigma). All peptides were subjected to isoelectric focusing as described above.

T Cell Proliferation Assay—T cell proliferation assays were performed on cells obtained from human leukopacs (discarded white cells from anonymous platelet donors) as described previously (20, 21). Mononuclear cells were separated by Ficoll-Hypaque sedimentation to eliminate red cells and polymorphonuclear leukocytes. The mononuclear layer, which consisted of T cells, B cells, and mononuclear cells was depleted of B cells and monocytes by passage over nylon wool column. A portion of these cells was saved prior to placement on nylon wool and used as autologous feeder cells following irradiation with 6.4 krad with a cesium source for 4.8 min. Nylon-passed cells, which were more than 98% CD3-positive (as determined by fluorescence-activated cell sorter analysis), were used as responder cells or further depleted with antibodies to CD4 (OKT4) or CD8 (OKT8) followed by negative selection with magnetic beads as described (20, 21). Responder cells (5×10^4 cells/well) were added to 2.5×10^5 irradiated feeder cells and cultured in U-bottomed 96-well plates (Corning-Costar Corp., Cambridge, MA) with RPMI 1640 and 5% fetal calf serum. At predetermined time points, cells were pulsed with 1 μCi of [^{3}H]thymidine/well 6 h prior to harvest in order to measure cell proliferation. Cells were washed extensively and harvested, and the amount of radioactive uptake was counted by liquid scintillation. Data were expressed as the average of triplicate wells ± the S.D. of cpm represented. For all proliferation experiments, data represent typical results from at least five different experiments. For antibody blocking experiments, T cells and APCs were mixed with LB3.1 (20 μg/ml), a class II specific monoclonal antibody (anti-DR) for 20 min at 37 °C. An isotype matched monoclonal antibody, 5E2B4, was added to cultures as an irrelevant control. Following incubation with antibody, *S. pneumoniae* type 1 CP was added (20 μg/ml) and allowed to incubate for 6 days, at which time proliferation was assessed as described above.

Animal Model of Intraabdominal Abscess Formation—The rat model of intraabdominal sepsis developed by Onderdonk was used (22). Male Lewis rats (180–200 g, Charles River Laboratories, Wilmington, MA) were used for all experiments. Animals were housed separately and received chow (Ralston Purina, St. Louis, MO) and water *ad libitum*. Animals were anesthetized with a single intraperitoneal injection of 0.15 ml of Nembutal (50 mg/ml, Abbott Laboratories, North Chicago, IL), and their abdomens were shaved and swabbed with a tincture of iodine. An anterior midline incision (1 cm) was made through the abdominal wall and peritoneum, and a gelatin capsule containing 0.5 ml of inoculum was inserted into the pelvis. The incisions were closed with interrupted 3.0 silk sutures, and the animals were returned to the cages. The inoculum contained a 1:1 mixture of *B. fragilis* NCTC 9343 (10^8 cfu/animal) or *S. aureus* PS 80 (10^7 cfu/animal, a kind gift from Dr.

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Jean Lee, Channing Laboratory, Harvard Medical School) and an adjuvant solution containing sterile rat cecal contents and 10% barium sulfate (w/v). Six days later, animals were necropsied in a blinded fashion and examined for the formation of one or more intraabdominal abscesses. Animal care was in accordance with the institutional guidelines set forth by Brigham and Women's Hospital and Harvard Medical School.

T Cell Transfer Studies—Cell transfer experiments were performed as described previously (15). Human CD4+ T cells were purified as described above and cultured *in vitro* with irradiated APCs in the presence of PSA or KD_{20} (20 μg/ml) for 5 days. T cells were harvested, examined for viability by trypan blue exclusion, and further enriched by passage over nylon wool (more than 95% pure CD4+ T cells as assessed by fluorescence-activated cell sorter analysis). Purified T cells were then counted and adjusted to an appropriate cell number (3×10^6/animal) prior to intracardiac transfer to animals (0.2 ml). Animals were challenged with *B. fragilis* 24 h later and assessed for abscess formation 6 days later.

Statistical Analyses—Comparison of abscess formation between groups of animals was made by χ^2 analysis, whereas comparison of groups in T cell proliferation assays was made by Student's *t* test (InStat, GraphPad Software, Inc., San Diego CA).

RESULTS

PSA Stimulates Human T Cell Activation—In proliferation assays with human T cells, PSA elicited a dose-dependent response (dose range, 10–0.1 μg/ml; Fig. 2*A*). This proliferative response peaked 6 days after culture with PSA. When tested at an optimal concentration of 1 ng/ml, the proliferative response to staphylococcal enterotoxin A also peaked at day 6 (Fig. 2). The depletion of T cells from these preparations abrogated the proliferative activity, whereas depletion of B cells did not (data not shown).

In previous studies, we have demonstrated the importance of the free amino group at C-4 of the 2-acetamido-4-amino-2,4,6-trideoxygalactose residue of PSA (Fig. 1, *Sugar 1*) and the carboxyl group associated with the pyruvate group on Sugar 3 in mediating *in vivo* biologic functions (14, 16, 23). In light of these data, we assessed the role of these chemical groups on T cell activation by PSA. A specific chemical modification converted the free amino groups on PSA to *N*-acetyl groups (Fig. 1, *Sugar 1, Modification I*). *N*-Acetylation of PSA abrogated T cell activation by PSA, a result indicating that free amino groups on PSA are critical for T cell activation (Fig. 2*B, PSA versus PSA: NAc*). Furthermore, conversion of this group to a tertiary amine that still retains its positive charge ($-HN^+(CH_3)_2$) by treatment with an aldehyde group under reducing conditions (as shown in Fig. 1, *Modification II*) significantly reduced T cell proliferation (medium control = 1123 ± 510 cpm; PSA = 11,215 ± 763 cpm; formaldehyde-treated PSA = 1256 ± 439 cpm). Chemical modification of the carboxyl group associated with the pyruvate substituent on PSA via carbodiimide-mediated reduction (Fig. 1, *Sugar 3, Modification III*) resulted in a 72% decrease in the proliferative response as compared with the unmodified PSA (tested at 20 μg/ml, 7937 ± 3264 cpm *versus* 27,886 ± 7890 cpm, respectively). This finding was supported by experiments in which the pyruvate ring was completely removed by acid hydrolysis (Fig. 1, *Sugar 3, Modification IV*), resulting in complete abrogation of the proliferative effect (medium = 750 ± 375 cpm; PSA (20 μg/ml) = 25,714 ± 1429; acid-treated PSA (20 μg/ml) = 552 ± 171 cpm).

Specificity of T Cell Activation by PSA—We have carefully considered the possibility that the T cell proliferative response to PSA could reflect the presence of protein or peptide contamination. The following data specifically address this issue. 1) Purification of surface polysaccharides from *B. fragilis* involved procedures designed to degrade or denature proteins (extraction with hot phenol, repeated Pronase digestion, and boiling in 1M NaOH for 1 h) (24). 2) SDS-polyacrylamide gel electrophoresis, quantitative protein assays, and amino acid analysis

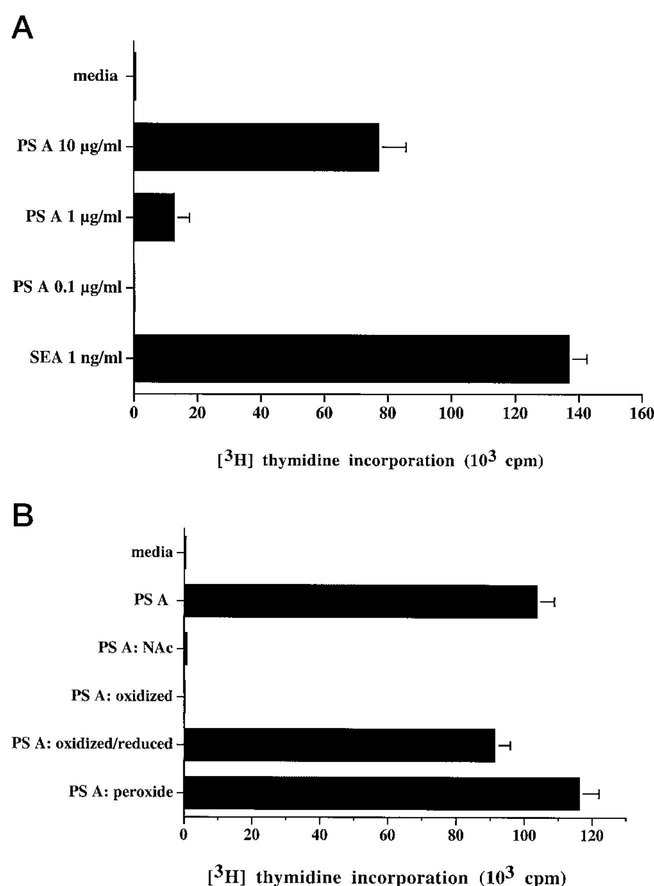

FIG. 2. **T cell activation by *B. fragilis* PSA and modified PSA derivatives.** *A*, 10-fold dilutions of PSA were co-cultured with human T cells (5×10^4 cells/200 μl) and irradiated APCs (2.5×10^5/200 μl) (17, 18) for 6 days. [^{3}H]Thymidine (1 μCi/well) was added during the last 6 h of culture. The response to PSA was dose-dependent and peaked 6 days after culture. The response to PSA typically varied with human T cell donors. In all assays, irradiated APCs cultured with PSA or staphylococcal enterotoxin A (*SEA*) alone did not proliferate in response to these antigens. The results shown are representative of at least five independent experiments. *B*, PSA was chemically *N*-acetylated by treatment with acetic anhydride as described in Fig. 1, *Modification I*. Conversion of the free amino groups of PSA to *N*-acetyl groups abrogated the proliferative response. Reduction of the negatively charged carboxyl group associated with the pyruvate ketal ring of the terminal galactose residue (Fig. 1, *Modification III*) reduced the proliferative response by 72%. PSA was subjected to selective oxidation by treatment with 0.01 M sodium metaperiodate (Fig. 1, *Modification V*). Oxidation by this method abrogated T cell activation by this polysaccharide (*PSA: oxidized* ($NaIO_4$)). However, upon reduction of the oxidized PSA with $NaBH_4$ (Fig. 1, *Modification VI*), the proliferative response to PSA was regenerated (*PSA: oxidized/reduced*). T cell proliferation assays revealed that the peroxide-treated PSA yielded activity equivalent to that of the untreated polysaccharide (*PSA versus PSA: peroxide*). Demonstration of comparable proliferative activity by the peroxide-oxidized PSA and regeneration of the proliferative activity of the periodate-oxidized and reduced PSA confirmed that the observed T cell response is attributable to the polysaccharide and not to a contaminating protein. All polysaccharides were tested at a concentration of 10 μg/ml. CD4+ T cells were used as the responder cell in these experiments.

reflected the absence of protein in polysaccharide samples. 3) Due to its charge motif, PSA ionically aggregates in aqueous solution, causing PSA to lose its ability to stimulate T cell proliferation. It is necessary to disaggregate this ionic complex via isoelectric focusing shortly before use for T cell activation to occur (data not shown). 4) Chemical treatment of PSA, which specifically alters carbohydrates but not proteins, abrogated proliferation by PSA. However, chemical regeneration of the affected carbohydrate groups restored T cell activation. For the last set of experiments, PSA was chemically oxidized by sodium

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metaperiodate ($NaIO_4$) treatment, which is selective for the cleavage of the C–C bond between vicinal hydroxyl groups on carbohydrates. In the case of PSA, periodate oxidation is exquisitely specific for removing the C-6 of the galactofuranose side chain (Fig. 1, *Sugar 4, Modification V*), creating an aldehyde group at C-5. When tested for T cell proliferation, periodate-oxidized PSA failed to elicit a response (Fig. 2*B, PSA versus PSA: oxidized*). After periodate oxidation, PSA was reduced with $NaBH_4$, converting the aldehyde group at C-5 to a hydroxymethyl group (Fig. 1, *Sugar 4, Modification VI*). This modification resulted in the conversion of the side-chain sugar to an arabinofuranose residue but left the original motif of the charged groups on the polysaccharide intact. The regeneration of the side-chain hydroxymethyl group on oxidized PSA restored the proliferative activity of this polysaccharide (Fig. 2*B, PSA versus PSA: oxidized/reduced*). NMR spectroscopy and gas chromatography-mass spectrometry confirmed that >95% of the repeating units were modified as described.

Generally, proteins are highly resistant to $NaIO_4$ oxidation; however, it is possible that this treatment could oxidize thiol groups present in cysteine residues associated with proteins or peptides to sulfoxide derivatives (25). If this were the case, reduction with $NaBH_4$ could reverse the oxidation procedure to regenerate this affected amino acid. Therefore, the results described above might be attributed to contamination by peptides containing cysteines. To eliminate this remaining possibility, PSA was treated with hydrogen peroxide, which oxidizes thiol groups on cysteine to sulfoxide derivatives (25) but does not affect carbohydrate structure. T cell proliferation assays with hydrogen peroxide-treated PSA revealed that the proliferative activity was equivalent to that of the untreated polysaccharide (Fig. 2*B, PSA versus PSA: peroxide*).

T Cell Activation by the S. pneumoniae Type 1 CP—We next determined whether another bacterial polysaccharide with a charge motif similar to PSA could activate T cells *in vitro*. *S. pneumoniae* type 1 CP is among the few naturally occurring polysaccharides that have oppositely charged groups (26). The type 1 CP is a trisaccharide repeating unit that has the same sugar residue with a positively charged free amino group (2-acetamido-4-amino-2,4,6-trideoxygalactose residue) that occurs in PSA, and it has two galacturonic acid residues containing negatively charged carboxyl groups per repeating unit. In previous studies, we have demonstrated that, like PSA, the type 1 CP also protects animals against abscess formation (16). This protective activity is also dependent on the presence of the free amino group on its repeating unit structure. When tested for CD4+ T cell proliferation, this polysaccharide (after disaggregation by isoelectric focusing) yielded a response that peaked after 6 days of culture (Fig. 3*A*). T cell proliferation assays performed with *S. pneumoniae* type 3 capsule, which is a disaccharide repeating unit of glucose and glucuronic acid (27) and has only one negatively charged group per repeating unit, did not yield a response (Fig. 3*A*). As demonstrated with *B. fragilis* PSA, *N*-acetylation of the type 1 CP abrogated the proliferative response (Fig. 3*B*). Conversion of the free amino group of the type 1 CP to a tertiary dimethylamine that retained its positive charge resulted in a significant decrease in the proliferative activity. The activity of the treated polysaccharide was 3.5 times less active than the untreated control polysaccharide (Fig. 3*C*, $p < 0.001$). These results correlated with our findings with *B. fragilis* PSA in which the same chemical treatment also resulted in loss of activity.

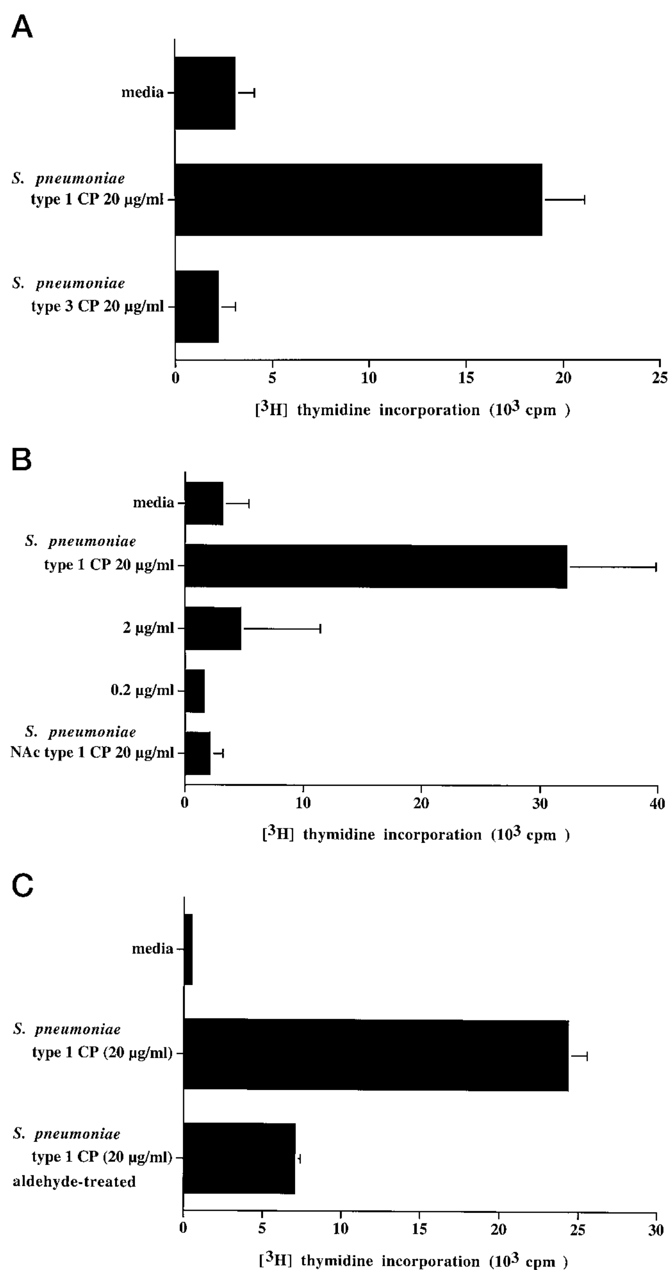

FIG. 3. **CD4+ T cell response to *S. pneumoniae* type 1 and type 3 CPs.** *A*, comparison of T cell proliferation by the type 1 CP compared with the type 3 CP. The type 3 CP consists of a repeating unit of glucose and glucuronic acid and did not elicit a T cell response in these assays. *B*, dose response and effect of *N*-acetylation of the *S. pneumoniae* type 1 CP. The type 1 CP elicited a potent T cell response that was typically 60–70% of the PSA response in this assay. *N*-Acetylation of type 1 capsular polysaccharide abrogated T cell proliferation. This modification was confirmed by NMR spectroscopy. *C*, effect of formaldehyde treatment on T cell activation by *S. pneumoniae* type 1 CP. Conversion of the free amino group on this polymer to a tertiary amine that retains its positive charge significantly reduced its ability to stimulate T cells ($p < 0.001$ compared with the untreated control, Student's t test).

T Cell Activation by Synthetic Zwitterionic Peptides—In order to demonstrate the role of the zwitterionic charge motif in T cell activation, a dipeptide repeating unit was synthesized to mimic the repeating unit structure of PSA. For this purpose, different repeating unit sizes of lysine and aspartic acid were synthesized and tested for their ability to stimulate CD4+ T cells. KD peptides consisting of 15, 20, or 25 repeating units each stimulated T cell activation *in vitro* (Fig. 4). However, peptides consisting of less than 15 repeating units (1, 5, and 10 repeats) did not stimulate T cell activation. Testing of a peptide with positively charged groups only, poly-L-lysine, did not stimulate T cell proliferation (data not shown). These data clearly indicate that zwitterionic repeating unit polymers other than

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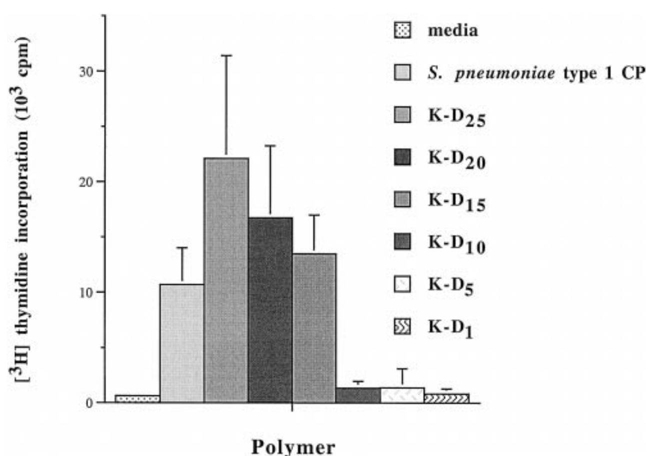

FIG. 4. **Effect of repeating unit size on CD4+ T cell proliferation.** KD peptides (20 μg/ml) of varying size were assessed for their ability to stimulate T cell activation 6 days postincubation. Culture of polymers consisting of 15, 20, or 25 repeats with T cells and APCs resulted in T cell proliferation. Incubation with peptides with 1, 5 or 10 repeats did not stimulate T cell activation. The *S. pneumoniae* type 1 CP (20 μg/ml) was included as a positive control.

polysaccharides stimulate T cell activation and that this activity depends on the repeating unit size of the polymer.

Protection against Abscess Formation by Zwitterionic Peptides—We next investigated whether these peptides could protect animals against abscess formation *in vivo*. Animals were administered 50 or 5 μg of the 25-repeating-unit KD peptide and challenged with *B. fragilis*. The results are shown in Table I, Experiment A. Treatment with the higher dose of $(KD)_{25}$ yielded significant protection in animals compared with the saline-treated control group (17% compared with 78%, respectively; $p < 0.0005$). However, treatment with the lower dose of the peptide failed to protect. *S. pneumoniae* type 1 CP yielded significant protection of animals at the 50-μg dose but not at the 5-μg dose. Administration of poly-L-lysine at the higher dose did not protect against abscess formation. Finally, treatment of animals with $(KD)_{25}$ protected animals against intra-abdominal abscess formation by *S. aureus* (Table I, Experiment B). Animals treated with saline and challenged with *S. aureus* had a 80% abscess rate, whereas treatment with 50 μg of $(KD)_{25}$ reduced abscess formation to 20% ($p < 0.02$).

The effect of the peptide repeating unit size on protection was examined. Animals were treated according to the regimen described above with a 50 μg/dose of each repeating unit size (Table II). Treatment with the 15-, 20-, or 25-repeating-unit peptide resulted in a significant level of protection. However, treatment with peptide repeating units less than 15 repeats did not yield significant protection compared with animals treated with saline. In fact, for peptides of less than 15 repeats, the level of protection diminished as the repeating unit size decreased.

Characterization of T Cell Response to PSA—Characterization of the T cell response to PSA demonstrated that CD4+ T cells were preferentially activated. In tritiated thymidine uptake assays performed on T cell populations, the CD4+ T cell response was comparable to the CD3+ response and 2.4 times greater than the CD8+ T cell response (Fig. 5*A*). Incubation of CD4+ T cells with PSA in the absence of irradiated feeder cells failed to elicit proliferation, thereby establishing the requirement for APCs in this system (Fig. 5*B*). In initial experiments, depletion of MHC class II-bearing cells (by negative selection with the class II specific antibody LB3.1) abrogated the proliferative activity of PSA (data not shown). In further experiments, preincubation of irradiated APCs with LB 3.1 inhibited T cell activation by PSA (Fig. 5*C*), whereas incubation of an isotype matched antibody (5E2B4) with APCs did not have this effect. The loss of activity following antibody blockade of MHC class II molecules suggested that available class II molecules are required for PSA-mediated T cell activation.

TABLE I
Protection against abscess formation by the peptide KD

Treatment	Dose	Abscess formation	P value[a]
	μg	*No. with abscess/total (%)*	
Experiment A: Protection against *B. fragilis*			
Saline		14/18 (78)	
$(KD)_{25}$	50	3/18 (17)	<0.0005
$(KD)_{25}$	5	10/17 (59)	>0.05
S. pneumoniae type 1 CP	50	4/20 (20)	<0.0001
S. pneumoniae type 1 CP	5	7/16 (44)	>0.05
Poly-L-lysine	50	8/10 (80)	>0.05
Experiment B: Protection against *S. aureus*			
Saline		8/10 (80)	
$(KD)_{25}$	50	2/10 (10)	<0.02

[a] Compared with saline-treated control. Comparison of abscess formation between groups of animals was made by χ^2 analysis (InStat, GraphPad Software, Inc.).

TABLE II
Protection against abscess formation by different repeating unit sizes of KD

Treatment	Repeating unit size	Abscess formation	P value[a]
		No. with abscess/total (%)	
Saline		7/7 (100)	
KD	25	2/9 (22)	0.003
KD	20	1/10 (10)	0.0004
KD	15	5/10 (50)	0.04
KD	10	6/10 (60)	>0.05
KD	5	8/10 (80)	>0.05
KD	1	6/7 (86)	>0.05
S. pneumoniae type 1 CP		3/10 (30)	0.001

[a] Compared with saline-treated control. Comparison of abscess formation between groups of animals was made by χ^2 analysis (InStat, GraphPad Software, Inc.).

Biologic Role for T Cell Activation by Zps and Zwitterionic Peptides—To assess the biologic function of T cells stimulated by these polymers, *in vitro*-stimulated human CD4+ T cells were transferred to rats and challenged with an abscess-inducing bacterial inoculum in the animal model described above. T cells were cultured *in vitro* with PSA (20 μg/ml) or an irrelevant polysaccharide (β-glucan) for 5 days and transferred to rats via the intracardiac route. The following day, animals were challenged with *B. fragilis,* and they were examined 6 days later for the presence of intraabdominal abscesses. Results are shown in Table III, Experiment A. Animals receiving CD4+ T cells stimulated *in vitro* with PSA showed a significant reduction in abscess formation compared with animals receiving T cells cultured in medium only (9% *versus* 76% abscess rate, respectively; $p < 0.0001$). T cells cultured with a glucan polymer (β-glucan) did not confer protection (79% abscess rate). Similar studies with KD_{20} were performed (Table III, Experiment B). The number of animals given T cells cultured with 20 μg/ml of this peptide was significantly lower than those given T cells stimulated with medium (10% compared with 90%; $p < 0.002$). T cells stimulated with poly-L-lysine yielded an 80% abscess rate in recipient animals.

DISCUSSION

Previous studies have indicated that polysaccharide antigens elicit T cell-mediated immune responses. For example, different T cell subsets can regulate the magnitude of antibody responses to specific pneumococcal and meningococcal polysac-

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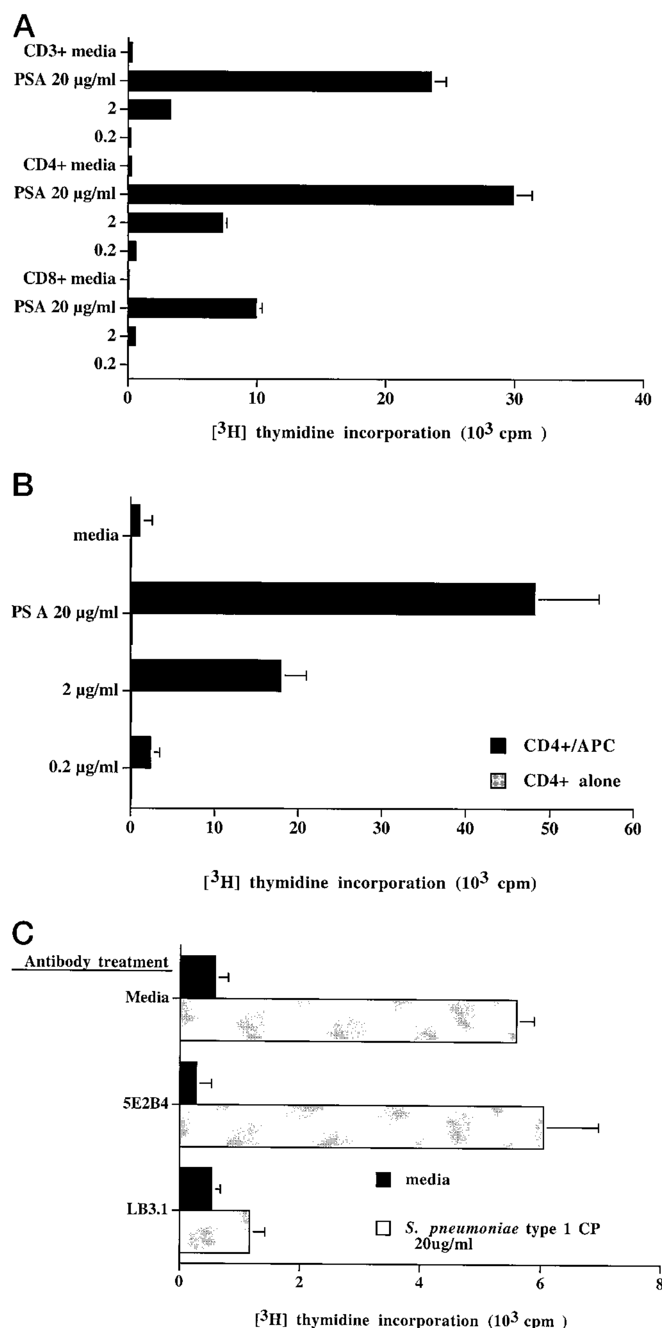

FIG. 5. **Characterization of T cell response to PSA.** *A,* phenotypic T cell response to PSA. Culture of PSA with unfractionated T cells (CD3+) or CD4+ cells yielded similar proliferative responses. However, the response of CD8+ cells to PSA in this assay was 2.4-fold less than that of CD4+ cells at the highest dose tested (20 μg/ml). *B,* requirement for APCs. Ten-fold dilutions of PSA were cultured with purified CD4+ cells alone or CD4+ cells mixed with irradiated APCs. Culture of PSA with CD4+ cells in the absence of irradiated APCs failed to elicit any proliferative response. Culture of PSA with both CD4+ cells and APCs yielded a dose-dependent response in these assays. *C,* blocking of T cell proliferation by MHC class II-specific antibody. Incubation with LB3.1 inhibited proliferation of T cells in response to *S. pneumoniae* type 1 CP. Incubation with 5E2B4 did not inhibit this response, yielding a proliferation level comparable to the medium control.

charides (28, 29), and cell-mediated immunity plays a distinct role in the host response to the *Pseudomonas aeruginosa* capsular polysaccharide (30). Furthermore, models of nonpeptide antigen recognition by T cells advanced by a series of *in vitro* studies with mycobacterial antigens has augmented the evidence that T cells are important in the immunosurveillance of

TABLE III
Protection against abscess formation by human CD4+ T cells stimulated in vitro with PSA

In vitro T cell stimulus (20 μg/ml)	Abscess formation in recipient animals	*P* value[a]
	No. with abscess/total (%)	
Experiment A		
Medium	16/21 (76)	
PSA	2/23 (9)	<0.0001
β-Glucan (20 μg/ml)	7/9 (79)	
Experiment B		
Medium	9/10 (90)	
KD_{20} (20 μg/ml)	1/10 (10)	<0.002
Poly-L-lysine	8/10 (80)	

[a] Compared with animals given T cells incubated with medium alone. Comparison of abscess formation between groups of animals was made by χ^2 analysis (InStat, GraphPad Software, Inc.).

foreign antigens other than proteins (1–3, 31, 32). More recently, a putative carbohydrate antigen derived from a pollen allergen has been shown to stimulate CD8+ T cells *in vitro* (33). The structural characterization of this antigen and the relevance to *in vivo* host responses is unknown.

Results from the present study show that highly purified microbial polysaccharide antigens that possess free amino and negatively charged groups stimulate human CD4+ T cell activation. We performed a series of chemical modifications of PSA to demonstrate that the observed T cell proliferation is specific for carbohydrate structure. Loss of this activity following periodate oxidation is likely due to the generation of aldehydes following periodate oxidation that interact with free amino groups on PSA to form intermediate Schiff bases. The occupation of free amino groups with intra- and/or intermolecular aldehydes in Schiff base formation rather than in the interaction with T cells and/or APCs may have resulted in the lack of proliferation by the oxidized form of PSA. Rhodes and coworkers (34) have shown that Schiff base formation between T cells and APCs are critical in providing signals for T cell activation. In addition, the generation of aldehydes on PSA could allow it to interact inappropriately with serum proteins. These groups can also isomerize or form acetals under certain conditions. Demonstration of comparable proliferative activity by the peroxide-oxidized product and recovery of proliferative activity via $NaBH_4$ reduction of periodate-oxidized PSA confirmed that the observed T cell response is attributable to the carbohydrate and not to a contaminating protein.

Chemical modifications to PSA and the *S. pneumoniae* type 1 CP demonstrated that positively charged free amino groups and negatively charged groups on these polymers are required for T cell activation. Although these saccharides possess different repeating unit structures, the presence of these charged groups is sufficient for T cell activation. Conversion of the free amino groups on each of these repeating unit structures to tertiary amines that retained a positive charge resulted in loss of activity. This result demonstrated that a primary amine is necessary for activity and correlates with our previous studies with C substance, the group polysaccharide from *S. pneumoniae*. This saccharide has positively charged free amino groups (NH_3^+) and a trimethylamine group ($-N^+(CH_3)_3$) on each repeating unit and exhibits the same biologic activity as PSA and the *S. pneumoniae* type 1 CP in the animal model of intraabdominal abscess formation. Conversion of the free amino groups to neutrally charged *N*-acetyl moieties following chemical *N*-acetylation did not affect the positively charged trimethylamine group on this repeating unit and resulted in the loss of *in vivo* biologic function (35).

The importance of the zwitterionic charge motif associated with Zps was underscored by our finding that synthetically

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derived repeating unit peptides designed to possess this motif also exhibit these biologic properties. Using these molecules, we have shown that repeating unit size is critical to their biologic function. These data clearly demonstrate that the *in vitro* T cell activation results correlate with their ability to protect animals *in vivo* against abscess formation and that KD peptides less than 15 repeating units long do not possess these biologic activities.

Zps preferentially stimulate CD4+ T cells and require accessory cells to support T cell stimulation by PSA. Further study showed that blockade of MHC class II molecules by a specific antibody on APCs abrogated T cell activation. In addition, depletion of class II-bearing APCs abrogated proliferation by PSA in this system. The role of the T cell receptor in this process is currently undefined, and it is unclear whether Zps behave as superantigens or conventional antigens. Further studies are under way to determine the mechanism by which these molecules activate T cells.

Previous studies have indicated that molecules possessing positively and negatively charged groups can regulate the activity of host immune cells. Amphoteric molecules such as copolymer I and mylein basic protein have been described as modulating T cell function in experimental models of autoimmune disease (36, 37). Copolymer I is a synthetic random protein consisting of alanine, glutamic, lysine, and tyrosine that specifically inhibits the T cell response elicited by mylein basic protein and suppresses experimental allergic encephalomyelitis. Copolymer I is believed to interact directly with HLA-DR MHC class II molecules (38). Recent studies have shown that zwitterionic glycosphingolipids exhibit immunomodulatory biologic activities on peripheral blood mononuclear cells, as well as on T- and B-lymphocyte populations (39–42). Lochnit *et al.* (41) have shown that the ability of these molecules to activate immune cell function is dependent upon component substituent groups (such as phosphocholine and phosphoethanolamine) that possess both positive and negative charges. Like Zps, the removal of these groups via chemical modification resulted in the loss of biologic activity.

We have recently shown that PSA possesses mitogenic activity for mouse B cells and rat T cells (43). The reason for this differential stimulatory effect may be due to the affinity of the polysaccharide for different receptors on lymphocytes from these two species or the inability of T or B cells from these species to respond. In the present study, we demonstrate that human CD4+ T cells respond preferentially to PSA and that this activity is specific for the carbohydrate. Human B cells did not respond to PSA. The finding that human and rat T cells are activated by PSA is intriguing and supports our use of the rat model to test the biologic activity of this molecule.

Importantly, the role for T cells activated by PSA and KD_{20} was demonstrated by their ability to prevent abscess formation *in vivo*. Human T cells stimulated *in vitro* with these polymers protected against abscess formation. These data provide direct evidence of the specificity of T cell activation by Zps and KD_{20} with prevention of this disease process. Furthermore these data suggest that: 1) T cells stimulated by these molecules *in vitro* suppress abscess formation in animals, and 2) the protective efficacy may be attributable to the production of a T cell-derived cytokine(s) that exerts activity in a xenogeneic fashion. Recently, we have shown that interleukin-2 produced by splenic T cells of animals treated with Zps mediates protection against abscess formation (23). The ability of Zps to activate T cells that confer this protective activity correlates with these findings. We are currently investigating the mechanisms by which interleukin-2 confers this protective effect.

In summary, these data expand the repertoire of antigens known to stimulate T cells and ascribe a biologic function for nonpeptide activation of these lymphocytes. These studies support the concept that polysaccharide-T cell interactions are critical to the modulation of host immune responses to certain bacterial infections.

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T Cells Activated by Zwitterionic Molecules Prevent Abscesses Induced by Pathogenic Bacteria

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