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# Zwitterionic Polysaccharides Stimulate T Cells with No Preferential V $\beta$ Usage and Promote Anergy, Resulting in Protection against Experimental Abscess Formation<sup>1</sup>

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Zwitterionic polysaccharides (Zps) from pathogenic bacteria, such as *Bacteroides fragilis*, are virulence factors responsible for abscess formation associated with intra-abdominal sepsis. The underlying cellular mechanism for abscess formation requires T cell activation. Conversely, abscess formation can be prevented by prophylactic s.c. injection of purified Zps alone, a process also dependent on T cells. Hence, the modulatory role of T cells in abscess formation was investigated. We show that Zps interact directly with T cells with fast association/dissociation kinetics. V $\beta$  repertoire analysis using RT-PCR demonstrates that Zps have broad V $\beta$  usage. Zps-specific hybridomas responded to a variety of other Zps, but not to a nonzwitterionic polysaccharide, indicating cross-reactivity between different Zps. Furthermore, Zps-reactive T cell hybridomas could effectively transfer protection against abscess formation. Analysis of the proliferative capacity of T cells recovered from Zps-treated animals revealed that these T cells are anergic to subsequent stimulation by the different Zps or to alloantigens in an MLR. This anergic response was relieved by addition of IL-2. Taken together, the data show that this class of polysaccharides interacts directly with T cells in a nonbiased manner to elicit an IL-2-dependent anergic response that confers protection against abscess formation. *The Journal of Immunology*, 2004, 172: 1483–1490.

The Gram-negative anaerobe *Bacteroides fragilis* is the most common bacterial pathogen isolated from intra-abdominal abscesses in humans (1). The development of intra-abdominal abscesses is associated with sepsis and causes chronic disease that can be lethal in infected patients. Molecular and cellular mechanisms underlying abscess formation as well as host responses controlling the disease process remain poorly defined. The pathogenesis of *B. fragilis* in the majority of clinical cases is attributable to its capsular polysaccharide complex (2–4).

Two polysaccharides from the capsular polysaccharide complex of strain 9343 have been structurally elucidated. Each polymer, referred to as polysaccharides A and B (PS A and PS B),<sup>3</sup> are high molecular weight polysaccharides that are expressed on the surface of *B. fragilis* (5) and have repeating units that possess positively and negatively charged groups (6). Structural studies indicate that each of these polymers possesses a zwitterionic charge motif. The

zwitterionic features of these compounds are the basis for their distinct pathobiological and immunological properties. More recently, genetic analyses have revealed that this organism makes eight different surface-expressed polysaccharides implicated in phase variation (7).

Intraperitoneal injection of rodents with Zps and a sterile cecal contents adjuvant (SCCA) induces abscess formation at this site (6). The use of SCCA mimics the release of colonic contents from the intestine into the peritoneal cavity that is associated with intra-abdominal sepsis. The adjuvanticity of SCCA is responsible for the concomitant secretion of proinflammatory cytokines critical to abscess formation (8). These abscesses are histologically identical with those that develop in humans (9). Conversely, s.c. administration of Zps alone protects against abscess formation by abscess-inducing bacterial pathogens.

Using athymic or T cell-depleted animal models of infection, studies show that T lymphocytes are required for the initial induction of host responses leading to the formation of intra-abdominal abscesses, including those induced by Zps plus SCCA (10–12). Immunity to *B. fragilis*-induced abscess development in rats and mice can also be transferred to nonimmune recipients with T cells isolated from immune donors only (13, 14). Given the fact that bacterial polysaccharides are classically defined as T cell-independent Ag with regard to Ab production, the apparent involvement of T cells in the stimulatory/suppressive function of Zps is a surprising finding. Experimental models of sepsis have lately provided several clues to this issue. Transfer of CD4<sup>+</sup> T lymphocytes recovered from PS A-treated rats mediated protection against abscess formation, a process essentially dependent on IL-2 produced by T cells (15). PS A stimulates rat T lymphocyte proliferation in vitro, which is consistent with the T cell-mediated protective immunity seen in vivo (16). Involvement of the costimulatory receptor/ligand couple CD86/CD28 in CD4<sup>+</sup> T cell activation is required for PS A-triggered abscess development in rats (17).

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<sup>3</sup> Abbreviations used in this paper used in this paper: PS A, polysaccharide A; GBS, group B streptococcus; KD20, synthetic peptide made of alternative Lys and Asp residues; LN, lymph node; PS B, polysaccharide B; SCCA, sterile cecal content adjuvant; SEA, staphylococcal enterotoxin A; Sp1, Zps from *Streptococcus pneumoniae*; Zps, zwitterionic polysaccharide.

PS A-mediated T cell activation requires MHC class II molecules on APC (18). These data support the hypothesis of a direct interaction between PS A and T lymphocytes. However, the mechanism by which Zps interact with T cells remains undefined. To characterize this interaction, we investigated several parameters of Zps-reactive T cells using *in vitro* and *in vivo* models. Results obtained by determination of their V $\beta$  repertoire, their reactivity profile, and their specificity suggest a novel paradigm for Ag recognition in the T cell response to Zps Ag.

## Materials and Methods

### Ag preparation

PS A from *B. fragilis* and Zps from *S. pneumoniae* (Sp1) as well as the peptide KD20, a peptide composed of 20 alternating K (lysine) and D (aspartate) units, were prepared as described previously (5, 18). For proliferation experiments, the Zps as well as the KD20 Ag were disaggregated by isoelectric focusing in a Rotofor apparatus (Bio-Rad, Hercules, CA) as previously described (18). Subsequently, the preparation was dialyzed against deionized water to remove the ampholytes, lyophilized, and stored in 2 M NaCl to prevent reaggregation. The same purification procedure was applied to the nonzwitterionic capsular polysaccharide from group B streptococci (GBS). This polymer has one negatively charged group per repeating unit (19). Staphylococcal enterotoxin A (SEA) was purchased from Toxin Technology (Sarasota, FL).

### T cell proliferation assay

T cell proliferation assays using T cells recovered from spleens or mesenteric lymph node (LN) from naive or Zps-treated Lewis rats were performed in RPMI 1640 medium supplemented with 10% FCS. Spleens or LN were homogenized on a metal grid, and polymorphonuclear leukocytes and erythrocytes were removed by Ficoll-Hypaque gradient centrifugation. Subsequently, the T cells were enriched to 98% (as determined by FACS analysis with CD3-specific staining) by passage over a nylon wool column and were used as responder cells in proliferation assays. Autologous APC were derived either from homogenized whole spleen or thymus and were subjected to Ficoll-Hypaque centrifugation and irradiation (3000 rad). For the proliferation experiments, 50  $\mu$ l of T cell suspension ( $4 \times 10^6$  cells/ml) was added to 100  $\mu$ l of APC suspension ( $4 \times 10^6$  cells/ml) and cultured in round-bottom, 96-well plates (Corning Costar, Acton, MA) in the presence of 20  $\mu$ g/ml Zps. After 4, 6, or 8 days, cells were pulsed for 18 h with 1  $\mu$ Ci of [ $^3$ H]thymidine/well and harvested in an automatic cell harvester, and radioactive incorporation was counted by liquid scintillation. Data were expressed in cpm as the average of quadruplicate well counts  $\pm$  SD. For all proliferation experiments the data represent a typical experiment from at least three independent experiments.

### IL-2 intracellular staining

CD3 $^+$  T cells were enriched as described above, and a purity of >98% was confirmed by flow cytometry using anti-CD3 mAb G4.18-FITC (BD PharMingen, San Diego, CA). The Cytostain kit was used according to the manufacturer's protocol (BD PharMingen). Before analysis, cells were stimulated with ionomycin according to instructions. Mouse anti-rat mAb IL-2 coupled to R-PE was obtained from BioSource (Camarillo, CA). Gating was performed using isotype control Abs as the lower fluorescence gate.

### Zps-T cell association/dissociation assay

T cell binding assays were performed using radiolabeled PS A or GBS prepared as previously described (18). For binding assays, T cells from Lewis rats were cultured in 96-well plates ( $1 \times 10^6$  cells/well) with PS A or GBS (50  $\mu$ g/ml) for increasing periods of time and were harvested in an automatic cell harvester. Radioactivity was measured by liquid scintillation. For dissociation experiments, PS A was added to  $1 \times 10^6$  T cells for 30 min, at which time increasing doses of unlabeled PS A or GBS were added, and the amount of bound labeled polysaccharide assessed 30 min later as described above. Data were expressed in cpm as the average of quadruplicate well counts  $\pm$  SD. For all proliferation experiments, the data represent a typical experiment from at least three independent experiments.

### Inhibition of T cell proliferation by TCR-specific F(ab') $_2$

TCR blocking assays were performed similarly to the T cell proliferation assays, except that naive rat spleen T cells were preincubated for 30 min with 1  $\mu$ g/ml F(ab') $_2$  from mAb R73 (20) directed against the rat TCR

$\alpha\beta$ -chains before adding PS A at 20  $\mu$ g/ml and irradiated rat APC. mAb R73 as well as the isotype-matched nonbinding control mAb MOPC-21 were purchased from BD PharMingen and treated as described by Mariani et al. (21) to obtain F(ab') $_2$  from these Abs. T cells were harvested after 6 days of culture to measure overnight [ $^3$ H]thymidine incorporation.

### Characterization of TCR V $\beta$ usage by semiquantitative and analytical RT-PCR

To characterize the V $\beta$  repertoire of Zps-reactive cells, T cells were stimulated as described above for 6 days *in vitro* with SEA, Sp1, or Con A or were left unstimulated, and total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA). cDNA was obtained using the Superscript Pre-amplification System (Invitrogen, Carlsbad, CA). PCR was performed in a 96-well spectrofluorometric thermal cycler (PRISM 7700; PE Applied Biosystems, Foster City, CA) using the molecular beacon technology for real-time quantification of PCR products (22) and UDG SuperMix (Invitrogen) to prevent amplification of carryovers. Molecular beacons hybridizing to the constant region of the TCR  $\beta$ -chain and the rat  $\beta$ -actin (FAM as a fluorophore, DABCYL as quencher, and ROXX as internal fluorescence standard) were designed and provided by I. Nazarenko (Invitrogen). The forward primer mapped within the variable region and the reverse primer hybridized within the constant region (see Table I). PCR conditions were: T $_{denaturation}$ , 95°C for 15 s; T $_{annealing}$ , 62°C for 30 s; T $_{extension}$ , 72°C for 30 s; 40 cycles. PCR standardization was performed with oligonucleotides for  $\beta$ -actin serially diluted in 10-fold steps. All proliferation experiments were performed at least three times, and the PCR reaction was systematically conducted in duplicate. All V $\beta$  region amplicons produced a fragment of ~600 bp, allowing for comparative fluorescence intensity.

For the characterization of V $\beta$  repertoire of T cell hybridomas, analytical RT-PCR was conducted using the same set of forward and reverse (lacking the molecular beacon) primers. PCR conditions were as follows: T $_{denaturation}$ , 94°C for 30 s; T $_{annealing}$ , 59°C for 45 s; T $_{extension}$ , 72°C, 1 min; 35 cycles.

### Generation of T cell hybridomas

T cell hybridomas were generated by cell fusion of *in vitro*-activated T cells and the mouse thymoma cell line BW5147.G.1.4 (23). To obtain Zps-activated T cells,  $\sim 2 \times 10^8$  naive rat spleen or LN T cells were isolated and incubated with PS A or Sp1 in the presence of twice as many irradiated APC for 6 days in round-bottom, 96-well plates ( $\sim 1 \times 10^6$  cells/well). Subsequently, the fusion was performed following the procedure described by Uede et al. (24), and freshly formed T cell hybridomas were plated in flat-bottom 96-well plates. The first cell clusters were visible after 10 days in culture. To test the reactivity and specificity of the T cell hybridomas, cell clusters were expanded and replicated in 24-well plates. T cell hybridoma supernatants recovered after 48 h of incubation in the presence of 10  $\mu$ g/ml Ag and APC were analyzed by ELISA specific for IL-2. 200 PS A- and Sp1-reactive T cell hybridomas were screened three times for IL-2 secretion, and only 10 of each that tested positive in three independent wells were used for TCR V $\beta$ -chain analysis. Two single-cell clones, 4E8 and 3B5, were obtained after repeated limiting dilution cloning and screening for stimulation by Sp1 and PS A, respectively.

### T cell hybridoma transfer studies

T cell transfer studies were performed in a similar manner as described previously (4). Briefly, T cell hybridoma 4E8 was freshly cultured to ensure >95% viability as determined by trypan blue exclusion. Cell density was adjusted to  $5 \times 10^7$  cells/ml, and 100  $\mu$ l was added to 100  $\mu$ l of irradiated fresh spleen APC. The Sp1 Ag was added at a final concentration of 20  $\mu$ g/ml, and stimulation was allowed for 48 h. Subsequently, the activated T cell hybridomas were harvested, washed three times in medium, and adjusted to a cell density of  $5 \times 10^6$  cells/ml. Two hundred microliters of T cell hybridoma suspension or dilutions thereof were administered via intracardiac injection. Cell viability was >95% upon transfer of cells. Twenty-four hours later, the animals were challenged with *B. fragilis* and SCCA and were assessed for abscess formation as described below.

### Animal model of intra-abdominal abscess formation

The rat model for abscess formation was previously reported by Onderdonk et al. (25). Male Lewis or Wistar 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 pyrogen-free sterilized water *ad libitum*. Animals were anesthetized with a single i.p. injection of 150  $\mu$ l of Nembutal (50 mg/ml; Abbott Laboratories, North Chicago, IL), and their abdomens were shaved and



swabbed with iodine tincture. An anterior midline incision (1 cm) was made through the abdominal wall and peritoneum, and an inoculum of *B. fragilis* and adjuvant (0.5 ml total) was inserted into the pelvis. The inoculum consisted of a 1/1 mixture of *B. fragilis* NCTC 9343 ( $10^8$  CFU/animal) and an adjuvant solution containing sterile rat cecal contents. The incisions were closed with silk sutures, and animals were returned to cages. Six days later animals were necropsied in a blinded fashion and examined for the formation of one or more intra-abdominal abscesses. Animal care was performed in accordance with the institutional guidelines set forth by Brigham and Woman's Hospital and Harvard Medical School.

#### Ex vivo T cell proliferation experiments and one-way MLR

The ex vivo T cell proliferation experiments were performed in essentially the same manner as the T cell proliferation assays described above with the following modifications: T cells were recovered every other day from rats treated with five doses of  $10 \mu\text{g}$  of PS A in  $100 \mu\text{l}$  of PBS administered s.c. The control animals received  $100 \mu\text{l}$  of PBS only. Two rats per group (PS A- or PBS-treated animals) were treated to recover enough responder T cells from the spleen to perform proliferation experiments. Responder cells ( $2 \times 10^5$  cells/well) were mixed with twice the number of APCs. APCs used in this experiment included splenic cells, irradiated splenic cells, or irradiated thymocytes. For the one-way MLR, responder T cells from PS A- or PBS-treated animals were added to  $100 \mu\text{l}$  ( $4 \times 10^6$  cells/ml) of irradiated feeder cells that were either of the autologous (Lewis spleen cells) or the allogeneic (Wistar spleen cells) type. [ $^3\text{H}$ ]thymidine incorporation was measured after 6 days. The proliferation experiments were performed in a blinded fashion and were unblinded after cell harvesting and counting.

#### Statistical analysis

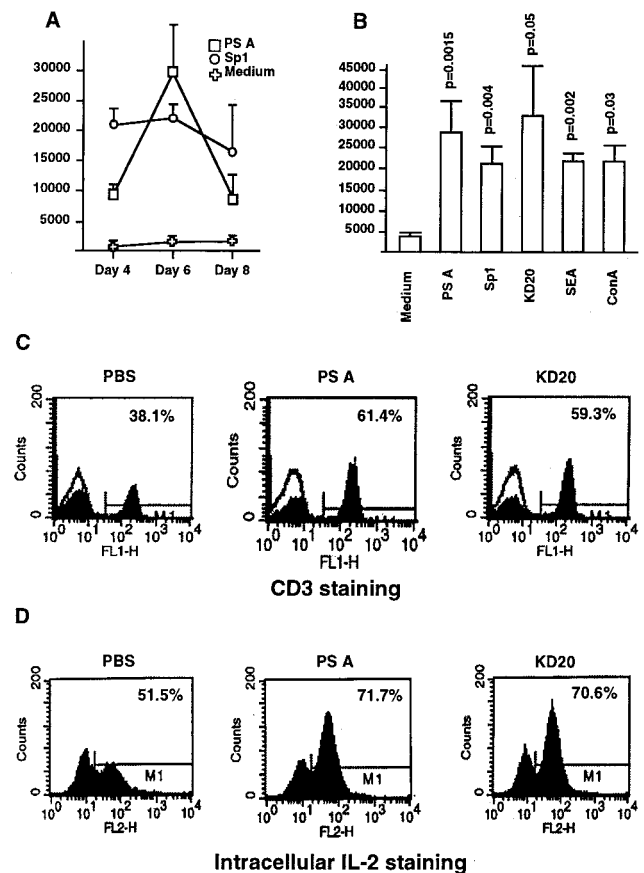
Comparison in T cell proliferation assays between groups of mice (see Figs. 1, A and B; 2, C and D; 5, B and C; and 6) was evaluated by paired Student's *t* test using Instat software (Mac version 2.01; GraphPad, San Diego, CA). SDs and *p* values were calculated throughout using the same application.

## Results

#### In vitro and in vivo stimulation of rat T cells by Zps and KD20

Previous studies have shown that Zps and synthetic peptides with repeating zwitterionic structure stimulate T cells from humans (18). In the present study we investigated the T cell response in rats to correlate these data with in vivo studies using the rat model of intra-abdominal abscess formation. No proliferative response was noted on day 1 or 2, with a very weak response seen on day 3 (data not shown) and a good response between days 4 and 9 (Fig. 1A). The in vitro proliferation experiments with T cells harvested from Lewis rats showed that both PS A from *B. fragilis* and Sp1 from *S. pneumoniae* elicited a response that peaked on day 6. The proliferative peak observed on day 6 lies between the fast proliferation kinetics of a superantigen (SEA) or a polyclonal mitogen (Con A), which peaks on day 3, and that of a nominal protein Ag (tetanus toxoid), which peaks on day 9 or 10 (data not shown). On day 6 the proliferative capacity of PS A and Sp1 compares with those measured for KD20, SEA, and Con A (Fig. 1B).

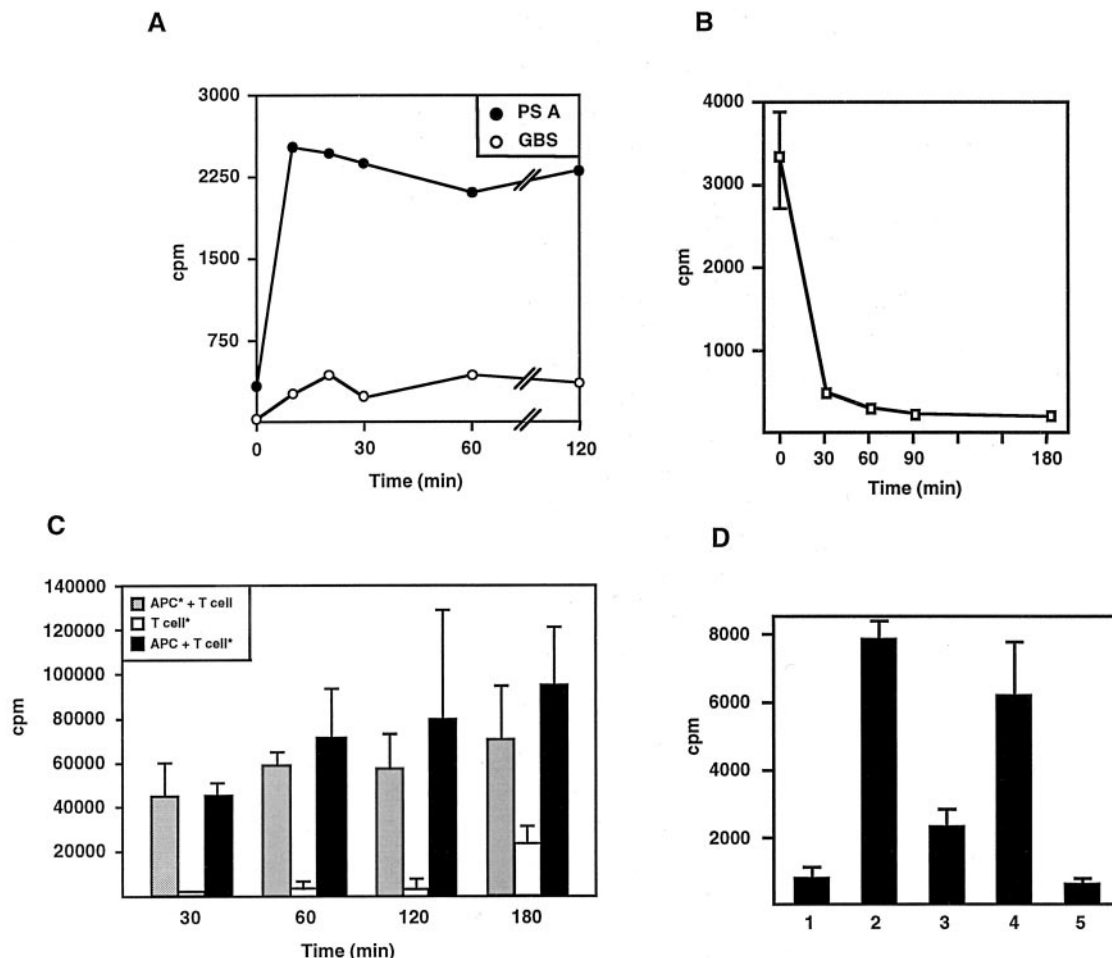
To analyze the proliferative response of T cells within a physiological context, rats were injected s.c. with PS A, KD20, or PBS as a negative control. Ten days later, T cell proliferation in the spleen was measured by FACS analysis using surface CD3 and intracellular IL-2 staining, respectively. Despite the high background of IL-2 staining in PBS-treated animals, treatment with PS A resulted in a consistent and reproducible increase by 20% of splenic T cell staining for CD3 (Fig. 1C) and IL-2 (Fig. 1D) markers. The treatment of rats with KD20 resulted in a similar increase in CD3 and IL-2 staining (Fig. 1D), clearly showing that both compounds can activate T cells in vivo. Because T cells and IL-2 are key players in the immunological cascade leading to abscess formation (26), this indicates a strong correlation between in vitro and in vivo effects of Zps.



**FIGURE 1.** Proliferation of spleen T cells with Zps, superantigen, and mitogen in the presence of irradiated APC measured by incorporation of [ $^3\text{H}$ ]thymidine (cpm). *A*, In vitro proliferation kinetics on days 4, 6, and 8 with polysaccharides from *B. fragilis* (PS A), *Streptococcus pneumoniae* (Sp1), and medium as a negative control. *B*, In vitro proliferation values on day 6 with zwitterionic polysaccharides PS A and Sp1, zwitterionic peptide KD20, superantigen from SEA (1 ng/ml), and lectin mitogen Con A (5  $\mu\text{g}/\text{ml}$ ). *C*, Ex vivo FACS analysis with CD3 and IL-2 (*D*) markers of T cells from rats that had been stimulated in vivo with PBS (control), PS A, and KD20, respectively. An increased number of CD3-positive and IL-2-secreting activated T cells were observed in the PS A and KD20 groups. The white profile is the isotype Ab control; the solid profile is the positive staining, indicated as percentage.

#### Evidence for Zps-T cell interaction

In addition to T cell activation by Zps, more specific evidence suggested that this proliferation might be mediated by the TCR. FACS analysis of Zps-reactive T cell hybridomas (see below) showed that they all exhibited surface expression of CD3 and TCR  $\alpha\beta$ -chains. In contrast, unstable clones, which had become nonreactive, had lost both CD3 and TCR  $\alpha\beta$ -chains or had barely detectable expression levels (our unpublished observations). Further, we have previously shown that Zps-dependent T cell proliferation requires MHC class II, APC, and CD28-CD86 interaction (17). In polysaccharide-T cell binding experiments, rapid association kinetics of PS A to T cells compared with the negatively charged polysaccharide GBS were observed at a dose of  $50 \mu\text{g}/\text{ml}$  (Fig. 2A). The dose used was a saturating dose in these studies. The use of lower doses of PS A showed similar binding kinetics (data not shown). These data indicate that a charge-specific interaction between Zps and cell surface receptors may take place as a prelude to T cell proliferation. In a dissociation kinetics assay the amount of bound PS A returned to baseline after 90 min, suggesting that a low affinity equilibrium exists between Zps ligands and cognate



**FIGURE 2.** Evidence for Zps-T cell interaction. Association kinetics (A) and dissociation kinetics (B) of  $^3\text{H}$ -labeled Zps binding to T cells are shown. In agreement with A, Zps bind directly to T cells and dissociate rapidly (30 min). C, ZPS pulsing experiment. T cells ( $\square$  and  $\blacksquare$ ) or APC ( $\text{▨}$ ) were pulsed with Sp1 for 30, 60, 120, or 180 min. After the pulsing period, the cells were washed three times and added to naive APC ( $\blacksquare$ ), T cells ( $\text{▨}$ ), or medium only ( $\square$ ) and were allowed to proliferate for 6 days before measuring [ $^3\text{H}$ ]thymidine incorporation. \*, Ag-pulsed cells (T cells or APC). D, T cell proliferation-blocking experiment with  $\text{F}(\text{ab}')_2$  directed against the rat TCR  $\alpha\beta$ -chains. Column 1, medium; column 2, Zps; column 3, Zps and anti-TCR  $\text{F}(\text{ab}')_2$ ; column 4, Zps and isotype control  $\text{F}(\text{ab}')_2$ ; column 5, anti-TCR  $\text{F}(\text{ab}')_2$ .

receptor(s) on T cells (Fig. 2B). Moreover, T cells are able to proliferate after a short preincubation period (30 min) with Sp1 after washing and incubation with APC without novel addition of Ag (Fig. 2C,  $\blacksquare$ ). Similarly, pulsing of APC with Sp1 before mixing with T cells leads to T cell activation (Fig. 2C,  $\text{▨}$ ). Finally, further evidence shows that T cell proliferation by Zps is inhibited by  $\text{F}(\text{ab}')_2$  specific for the TCR  $\alpha\beta$ -chains (Fig. 2D). Together, these findings indicate the direct involvement of the TCR in the Zps-mediated T cell proliferation process and prompted us to further investigate the nature of the T cell-Zps interaction.

#### Characterization of the $\text{V}\beta$ T cell repertoire responding to Zps

To discern the possible role of TCR in specific recognition, we determined the  $\text{V}\beta$  repertoire used by Zps. To this end, semiquantitative RT-PCR using  $\text{V}\beta$ -specific primers was performed on T cell total RNA that had been previously stimulated in vitro with Con A, SEA, or Sp1, respectively. Con A and SEA were selected as comparative stimuli because they represent the two extremes of  $\text{V}\beta$  usage: Con A is a polyclonal mitogen, whereas SEA is a superantigen that selectively stimulates T cells expressing particular TCR  $\text{V}\beta$  genes ( $\text{V}\beta 11$  and  $\text{V}\beta 17$  in the rat). For RT-PCR, the reverse primer was derived from a conserved region in the constant domain of the TCR  $\beta$ -chain and was used in combination with

either forward primer specific for 22  $\text{V}\beta$ -coding regions (Table I and Fig. 3A). Con A-stimulated cells exhibited a polyclonal response (Fig. 3B). In contrast, stimulation with SEA revealed selective usage of  $\text{V}\beta 11$  and  $\text{V}\beta 17$  (Fig. 3B). Analysis of Sp1-stimulated T cells showed an increase of approximately half the TCR  $\text{V}\beta$  chains, with an overall pattern similar to that obtained for Con A, but different from that for SEA. Consistent with the in vitro proliferation kinetics data shown in Fig. 1, this strongly suggests that Zps triggers a distinct T cell-specific response that can be classified as polyclonal.

#### Generation and analysis of Sp1- and PS A-reactive T cell hybridomas

To further elucidate the T cell response to Zps at the single-cell level, hybridomas resulting from the fusion of Sp1- or PS A-activated T cells and the thymoma cell line BW5147.G.1.4 were generated. From >200 T cell hybridomas obtained, 10 reactive for Sp1 and 10 reactive for PS A were recovered by limiting dilutions. For obvious handling difficulties associated with T cell hybridoma screening, not all of them had been cloned to single cells when the repertoires were analyzed. The analysis was conducted with the same set of  $\text{V}\beta$  and constant primers as that used in the semiquantitative RT-PCR. The presence or absence of  $\text{V}\beta$  expression by T

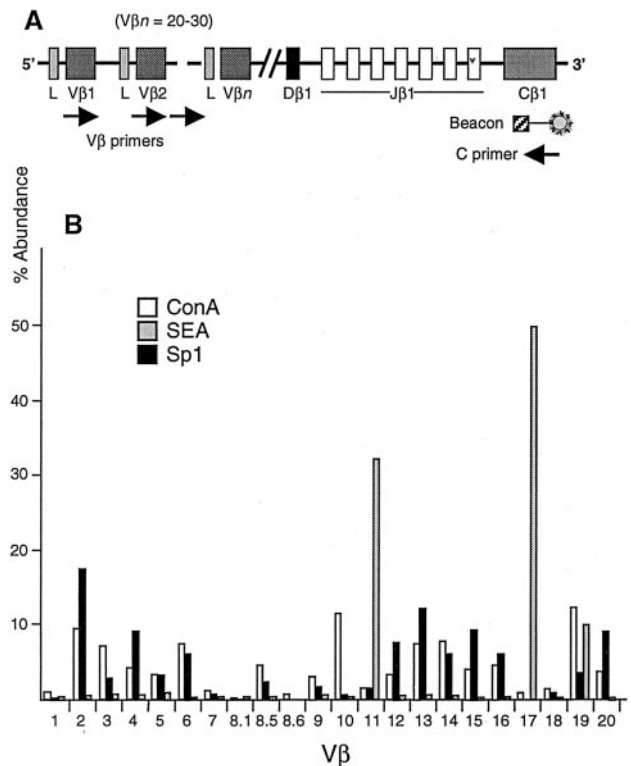
Table I. Primer sequences used for V $\beta$  repertoire determination

	Sequences
Vb1	5'-TCTAGTCATGGGAAGAGCAA-3'
Vb2	5'-CCAAGGTGGCGTCTGGTACC-3'
Vb3	5'-ACTCCACAGCATCTGCTCAG-3'
Vb4	5'-CTGGTGGCAGGTCCAGTCCGACCCTA-3'
Vb5	5'-CTGGGGTGGTCCAGTCTCCAAG-3'
Vb6	5'-GGACTTAGACTGATCTACTA-3'
Vb7	5'-GACCCAGATCTGGGGCTACGG-3'
Vb8.1	5'-TGAGTTGTAAGCAGAATAAT-3'
Vb8.5	5'-GTTCAACTGTCACCAAGTCC-3'
Vb8.6	5'-ATTGAGCTGTCGCCAGACTA-3'
Vb9	5'-GTTATGCAGAACCCAAGATAC-3'
Vb10	5'-TCTAAGCAATTGCTGAAGGT-3'
Vb11	5'-ACAAATGCTGGTGTTCATCCA-3'
Vb12	5'-AGGGCCACAATGATCTTTTC-3'
Vb13	5'-CCAGTCCCCAGATATGCAA-3'
Vb14	5'-GACTATCCATCAATGGCCAG-3'
Vb15	5'-GGAGCTCTCGTCCACCAATC-3'
Vb16	5'-GACCCAGTTTCTAATCACCT-3'
Vb17	5'-GTAACCCAGACTCCAAGATAC-3'
Vb18	5'-CTGTGTTCAAGTGTCTTCTC-3'
Vb19	5'-GAACAGAAAGCCAGAATGCA-3'
Vb20	5'-AGGGCAGCAAGTAGAGATGA-3'
C	5'-CATAGAATCCACTTGGCAGCGGAAGTGGT-3'

cell hybridomas was determined after analysis of RT-PCR products on agarose gels. The frequency profile showed that several V $\beta$ -chains of the different hybridomas were preferentially reactive to PS A and Sp1 (Fig. 4). These T cell hybridomas were CD3<sup>+</sup>/CD4<sup>+</sup> cells and TCR  $\alpha\beta$ <sup>+</sup> as determined by flow cytometry (our unpublished observations). There was 65% similarity between the V $\beta$  repertoires exhibited by Sp1 and PS A. In addition, the V $\beta$  profile did not exactly reflect that obtained by semiquantitative RT-PCR of in vitro cultured T cells. This can be explained by technical constraints, such as the intrinsic poor stability of T cell hybridomas and the TCR bias resulting from selection of a single random fusion event. However, these data do support the polyclonal nature of the T cell response to Zps. Two stable T cell hybridomas, 4E8 and 3B5, were recovered after three additional cycles of limiting dilutions with Sp1 and PS A, respectively, and were used for further analysis.

#### Protective capacity and cross-reactivity of cloned T cell hybridomas

To assess the function of the Zps-reactive T cell hybridomas in vivo, T cell transfer experiments were performed in which recipient rats were challenged with *B. fragilis* and protection against intra-abdominal abscess formation was assessed. T cell hybridoma clone 4E8 was stimulated in vitro with Sp1 or Con A or was left unstimulated (PBS), and increasing numbers of cells were transferred to groups of animals as described in *Materials and Methods*. The transfer of T cells from Sp1-stimulated cultures conferred protection to naive recipient animals compared with control animals (Table II). Although protection increased as more T cells were used, as few as  $3 \times 10^2$  transferred cells led to protection of >50% of rats challenged with *B. fragilis* (Table II). The transfer of Con A-stimulated T cells also resulted in protection. Activation of clone 4E8 cells with Con A before transfer resulted in similar overall protection (Table II), further arguing for the mitogenic-like stimulation of Sp1 in the assay. In previous studies the transfer of a mouse hybridoma that was not specific for Zps did not confer protection (data not shown). Hence, in terms of functionality, this represents the first demonstration that a single-cell cloned, Sp1-



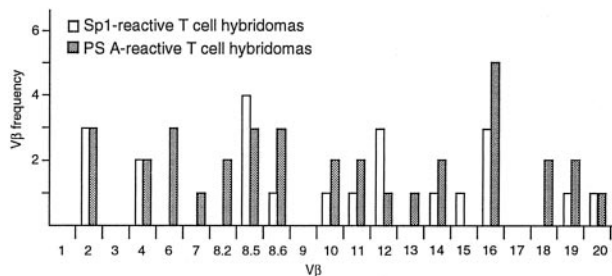
**FIGURE 3.** Analysis of the V $\beta$  repertoire usage of Zps-reactive T cells by semiquantitative RT-PCR. *A*, Schematic representation of the experimental PCR setting. Forward primers were derived from the variable region of different V $\beta$  and used in combination with a unique reverse primer (molecular beacon) corresponding to the constant region in PCR amplification (for details, see *Materials and Methods*). *B*, Relative abundance of mRNA transcripts recovered from a T cell population previously exposed to Con A (mitogen), SEA (superantigen), or Sp1 (Zps) for three proliferation periods, each for 6 days.

activated T cell hybridoma, is able to confer protection in rats similarly to polyclonal T cells previously stimulated by Zps.

As the V $\beta$  repertoires of PS A- and Sp1-reactive T cell hybridomas were very similar, the capacity of the PS A-specific T cell hybridoma 3B5 to cross-react with other Zps and non-Zps Ag was assessed by determination of IL-2 levels by ELISA after Ag stimulation. 3B5 produced IL-2 levels when stimulated with Sp1 or PS A yielding OD<sub>490</sub> of 0.50 and 0.58, respectively, compared with an OD<sub>490</sub> of 1.5 elicited by Con A. In contrast, the negatively charged GBS type III capsule and medium-stimulated cells yielded OD<sub>490</sub> of 0.2 and 0.25, respectively. This argues in favor of a broad, yet charge-specific, pattern of recognition by T cells.

#### Mechanism of protection against abscess formation

To gain insight into the mechanism underlying protection by Zps-activated T cells, these cells were isolated from PBS- or PS A-treated rats, and their reactivity was tested in an in vitro proliferation assay as illustrated in Fig. 5*A*. In the absence of s.c. treatment with PS A, splenic cells from rats responded to Zps and a zwitterionic peptide (KD20) as expected (Fig. 5*B*). Conversely, prophylactic s.c. administration of PS A prevented proliferation of T cells from whole spleen when assayed in vitro (Fig. 5*B*). Purified T cells isolated from PS A-vaccinated rats lost their proliferative capacity when mixed with irradiated spleen or thymocytes, whereas they remained inducible when the animals were administered PBS (Fig. 5*B*). The response pattern showed significant similarity for PS A and Sp1 ( $p < 0.04$ ) and less homogeneity when KD20 was used



**FIGURE 4.** The V $\beta$  repertoire usage of T cell hybridomas that were reactive with Sp1 or PS A. To map the V $\beta$  repertoire, mRNA preparations were subjected to conventional RT-PCR with the primers schematically depicted in Fig. 3A, replacing the molecular beacon with a conventional primer of the same sequence. V $\beta$  frequency is expressed as the absolute number of analyzed T cell hybridomas expressing the indicated V $\beta$ -chain.

( $p < 0.08$ ), suggesting a general down-regulation of T cell activity. When IL-2 at 500 pg/ml was added exogenously to the proliferation assay, immune modulation pertaining to the s.c. treatment was abolished (Fig. 5C). Thus, after s.c. Zps administration the protective function of T cells results from anergy, as indicated by the possibility to reverse the effect upon addition of IL-2.

To address whether treatment with Zps was inducing a generalized anergic response rather than an Ag-specific suppression, a one-way MLR assay was performed using autologous Lewis or allogeneic Wistar rat APC (Fig. 6). The proliferative capacity of T cells recovered from PS A-treated Lewis rats was markedly reduced (77% for spleen and 36% for LN T cells) when incubated with APC from Wistar rats. Consistent with this finding, no difference was observed with autologous Lewis rat APC (Fig. 6). This implies that a pronounced general T cell anergy caused by s.c. PS A administration represents the dominant immune mechanism by which protection against abscess formation takes place.

## Discussion

Although T cell-mediated responses to microbial polysaccharides have been well documented (18, 27–29), the mechanism by which T cells recognize such nonpeptide Ag remains largely undefined. In vitro, studies with mycobacterial glycolipids have shed light on the importance of T cells in controlling immune responses to foreign Ag other than peptides. Mycobacterial glycolipid-specific T cells recognize their Ag presented by nonpolymorphic MHC class I-like CD1 proteins (30, 31). However, the relevance of microbial polysaccharides to the host response remains poorly defined, and the role of the TCR in this process is currently unknown.

As a model for host response, the experimental intra-abdominal abscess has been shown to closely mimic the clinical course of this pathology and is a valuable tool to assess the effector function of T cells stimulated by Zps. Using this animal model, the functionality of a T cell hybridoma (4E8, V $\beta$ 16) selected for Sp1 responsiveness was demonstrated, indicating the central role of T cells in the host response to Zps. After transfer to rats, this clone was able to confer protection against intra-abdominal abscess formation following *B. fragilis* infection. As few as 300 Sp1-activated T cells could significantly reduce the number of animals that developed abscesses. This suggests that T cell hybridoma 4E8 was able to provide the level of IL-2 required for protection against abscess formation (15). Although we have shown that multiple V $\beta$ -chains can recognize different Zps, our data show that few cells of only a single hybridoma activated in vitro with Sp1 can confer protection.

The mechanism of protection was further analyzed in ex vivo proliferation experiments with rats that had been previously treated

**Table II.** Effect of transfer of in vitro-activated T clone hybrid 4E8 on intra-abdominal abscess formation by *B. fragilis*<sup>a</sup>

In Vitro Stimulation of Hybrid 4E8 <sup>b</sup>	Number of T Cell Transferred <sup>c</sup>	Number of Animals Positive for Abscess/Total
Con A	$3 \times 10^2$	4/9
Sp1	$3 \times 10^2$	4/9
Con A	$3 \times 10^4$	2/5
Sp1	$3 \times 10^4$	4/9
Con A	$3 \times 10^6$	3/8
Sp1	$3 \times 10^6$	3/10
PBS	$3 \times 10^6$	8/9

<sup>a</sup> Activated T hybridoma clone 4E8 protects rats against abscess formation. In vitro Con A- or Sp1-activated T cell clone dilutions were transferred to the rat via the intracardiac route. The rats were challenged 24 h later with *B. fragilis* and protection against abscess formation was analyzed 6 days later.

<sup>b</sup> T cell hybridoma 4E8 was obtained as described in *Materials and Methods*.

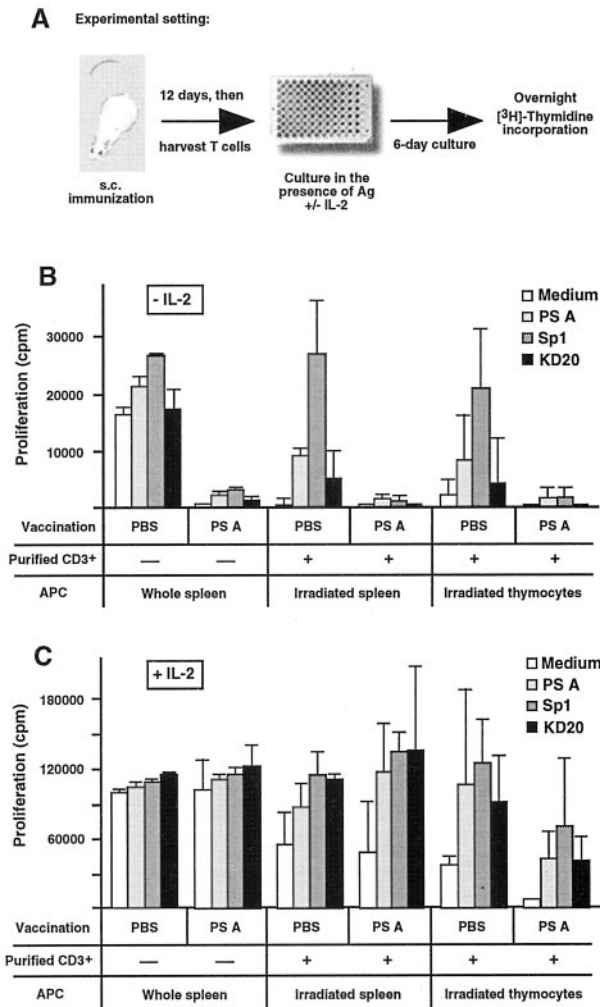
<sup>c</sup> Activated T cells were administered via the intracardiac route.

with PS A. The experiments clearly showed that T cells recovered from rats that had been treated with Zps alone became unresponsive to further Ag stimulation. This unresponsiveness could be reversed by exogenous addition of IL-2; this implies that anergy plays a role in protection (32). This is further underscored by results from the MLR assays, in which splenic T cells recovered from rats treated with Zps were unresponsive. Consistent with our data, this may imply a mechanism relying on down-regulation of IL-2 production (33). The regulatory mode of action of IL-2 might be responsible for the persistence of T cell populations such as CD4<sup>+</sup>CD25<sup>+</sup> or CD4<sup>+</sup>CD45RB<sup>low</sup> cells with known suppressive function via the secretion of IL-10 (34, 35). It is conceivable that the down-regulation of T cells by this mechanism is responsible for limiting the onset of proinflammatory cytokines required for abscess formation (8).

Consistent with previously published findings, the cytokine profile of Zps-stimulated T cells comprises an initial burst of IL-2, followed by the release of IFN- $\gamma$  and IL-10 (15). This cytokine profile is reminiscent of that induced by superantigens (36) even though the relative amount of cytokines secreted by Zps-exposed T cells is much lower. In vivo injection of SEA to mice rapidly elicits production of Th1-related proinflammatory cytokines IL-2, TNF- $\alpha$ , and IFN- $\gamma$  and later on promotes the development of immunoregulatory IL-10-producing cells (37), leading to suppression of Th1 cytokine production through what is known as a shut-down loop. Given the similar cytokine secretion profiles, we initially hypothesized that the mechanism underlying T cell activation in vitro by Zps could be similar to that known for superantigens. To evaluate this hypothesis, the V $\beta$  repertoire of T cells activated by Zps was analyzed by semiquantitative RT-PCR. In contrast to SEA superantigen, which expands rat T cells expressing V $\beta$ 11 and V $\beta$ 17, no distinct profile could be observed for T cells responding to Zps (Fig. 3B). We showed that multiple TCR  $\beta$ -chains were involved in Zps-mediated T cell activation, which most likely reflects a natural Zps-specific host response. This polyclonal response is in agreement with the finding that the T cell hybridoma 3B5 showed cross-reactivity between PS A and Sp1, but not with a negatively charged polysaccharide from GBS.

Zps represent a new class of Ag because they cannot, by essence, function as protein Ag and do not behave as known T cell-independent polysaccharides. They also do not share properties with classical superantigens as we initially hypothesized. These polysaccharides exhibit a particular charge pattern (38), which is characterized by alternating positive and negative charges and a minimal  $M_r$  of  $\sim 17$  kDa (39). How the Zps are sensed by the TCR

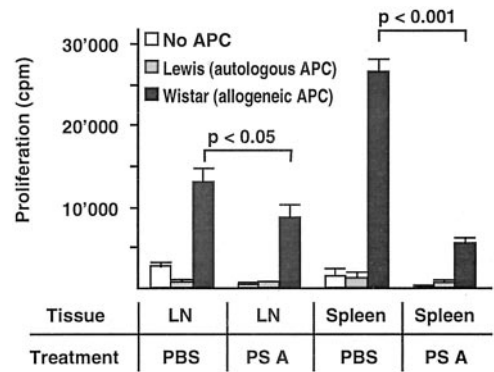




**FIGURE 5.** Analysis of ex vivo T cell response to PS A, Sp1, or KD20 after s.c. administration of Zps. *A*, Schematic representation of the experimental setting. Proliferative response of T cells recovered from rats that had been treated with PS A or PBS in the absence (*B*) or the presence (*C*) of IL-2.

and why MHC class II molecules on APC are required for activation are currently under investigation. However, Ag-pulsing experiments (Fig. 2*C*) revealed that preincubation of Zps for only 30 min with either APC or T cells elicited a proliferative reaction. In addition, direct binding of <sup>3</sup>H-labeled Zps showed similar association/dissociation kinetics (Fig. 2, *A* and *B*). The association/dissociation kinetics are relatively fast compared with classical proteins presented by MHC class II, which take ~2 h (40).

As recently described by Slifka and Whitton (41), TCRs are able to diffuse laterally in the plasma membrane and build clusters on the surface of the T cell that will affect avidity and the outcome of the TCR-triggered signal transduction. This mechanism would be especially sensitive to the size of the bridging polymer. Any Zps molecule with a size >17 kDa would therefore be able to cross-link several TCRs, but also possibly anchor MHC class II molecules (42), thus explaining why these latter molecules are as essential as the TCR to yield a productive response by the T cell. Given the polyclonal nature of the T cell response to Zps, this argues in favor of a limited specificity for polysaccharides as long as they are zwitterionic. Furthermore, it confirms that the ternary complex comprising T cell-APC-Zps is necessary and sufficient to lead to production of IL-2, the expression of which is tightly con-



**FIGURE 6.** Generalized T cell unresponsiveness in one-way Lewis/Wistar MLR. After s.c. Zps administration to Lewis rats, spleen and LN were recovered and cultured with autologous (Lewis) or allogeneic (Wistar) APC.

trolled through signals from the TCR (43). Whether different recognition of V $\beta$ -chain by Zps leads to various signaling events, as is the case for glycolipids and CD1 molecules (44), has to be established. Alternative mechanisms may include direct cross-linking of TCR-associated surface proteins or induction of other ligand-receptor couples, such as CD86-CD28, CD40-CD40 ligand, and CD5-CD72. Zps by themselves would maintain low expression level of these costimulatory markers, thus explaining the requirement for sterile cecal contents to be used as a proinflammatory adjuvant to trigger abscess formation. In addition to direct cross-linking, it remains possible that Zps could also follow endosomal processing; these two pathways are not mutually exclusive.

We conclude that this unconventional T cell presentation of Zps could account for the fact that Zps stimulate IL-2 production, which is followed by T cell anergy. The T cell proliferation and associated IL-2 secretion are probably the initial signals leading to the inflammatory reaction, which culminates in abscess formation when naive rats are challenged with *B. fragilis*. In contrast, prophylactic s.c. Zps immunization induces generalized T cell anergy, ultimately resulting in protection against abscess formation. It is possible that IL-2 release after Zps treatment leads to the subsequent production of IL-10, a key mediator in the induction of anergy (45). IL-10 has also been detected subsequent to the expression of IL-2 in splenic CD4<sup>+</sup> T cells harvested from rats treated with PS A (15), and we are now focusing on the role of this cytokine in Zps-mediated protection.

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