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*J Immunol* 2002; 169:6149-6153; doi: 10.4049/jimmunol.169.11.6149
http://www.jimmunol.org/content/169/11/6149

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Zwitterionic Polysaccharides Stimulate T Cells by MHC Class II-Dependent Interactions

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Polysaccharides of pathogenic extracellular bacteria commonly have negatively charged groups or no charged groups at all. These molecules have been considered classic T cell-independent Ags that do not elicit cell-mediated immune responses in mice. However, bacterial polysaccharides with a zwitterionic charge motif (ZPSs), such as the capsular polysaccharides of many strains of Bac teroides fragilis, Staphylococcus aureus, and Streptococcus pneumoniae type 1 elicit potent CD4+ T cell responses in vivo and in vitro. The cell-mediated response to ZPS depends on the presence of both positively charged and negatively charged groups on each repeating unit of the polysaccharide. In this study, we define some of the requirements for the presentation of ZPS to CD4+ T cells. We provide evidence that direct interactions of T cells with APCs are essential for T cell activation by ZPS. Monocytes, dendritic cells, and B cells are all able to serve as APCs for ZPS-mediated T cell activation. APCs lacking MHC class II molecules do not support this activity. Furthermore, mAb to HLA-DR specifically blocks ZPS-mediated T cell activation, while mAbs to other MHC class II and class I molecules do not. Immunoprecipitation of lysates of MHC class II-expressing cells following incubation with ZPS shows binding of ZPS and HLA-DR. Electron microscopy reveals colocalization of ZPS with HLA-DR on the cell surface and in compartments of the endocytic pathway. These results indicate that MHC class II molecules expressing HLA-DR on professional APCs are required for ZPS-induced T cell activation. The implication is that binding of ZPS to HLA-DR may be required for T cell activation.

M ost pathogenic extracellular bacteria produce large molecular mass surface polysaccharides, usually in the form of a capsule that coats the bacterial cell surface. Bacterial polysaccharides have been considered classic T cell-independent (TI-2) Ags that do not elicit cell-mediated immune responses (1) but rather elicit humoral immunity comprising low-affinity IgM and restricted classes of IgG Abs. The prevailing theory is that in humans these TI-2 Ags (with a minimum molecular mass of 80–90 kDa) bind to polysaccharide-specific B cells and stimulate production of specific Abs without recruitment of T cell help or induction of immunologic memory (2). The classification of polysaccharides as TI-2 Ags is based on the lack of T cell-dependent responses to these typically negatively charged or uncharged molecules (3, 4). The negatively charged capsular polysaccharides of group B Streptococcus, for example, fail to activate human T cells in vitro (5). Some polysaccharide Ags are internalized and localized in endosomes and lysosomes of APCs and inhibit T cell responses to peptides (6–8). Moreover, the carbohydrate component of glycolipids and glycoproteins can influence T cell responses (4, 9–11). Finally, nonpeptide Ags containing lipids and glycolipids can be recognized by T cells in conjunction with the CD1 molecule (12–15).

In view of their immunogenic characteristics, bacterial zwitterionic polysaccharides (ZPSs) isolated from strains of Bac teroides fragilis, Staphylococcus aureus, and Streptococcus pneumoniae type 1 (Fig. 1) represent an unusual group of bacterial carbohydrates. ZPSs which include both positively and negatively charged groups have unique immunological properties: molecules as small as 17 kDa elicit a potent CD4+ T cell response in vitro, and ZPS-activated T cells confer protection against experimental intraabdominal abscess formation (16). These activities clearly distinguish ZPS from TI-2 polysaccharides. Structure-function studies have shown that the proliferative response of T cells depends on free amino (positively charged) and carboxyl or phosphate groups (negatively charged) that are part of the repeating unit structure. Chemical neutralization of either charge on these groups results in loss of the ability of the polysaccharide to activate T cells (5, 17). Detailed nuclear magnetic resonance studies of one ZPS from B. fragilis suggested that the polysaccharide accommodates positive and negative charges in a groove that might be capable of binding α-helical peptides (18).

The studies we report herein begin to elucidate the fundamental mechanism by which ZPSs elicit a T cell-dependent immune response. We provide evidence for the requirement of direct contact between professional APCs bearing the MHC class II molecule HLA-DR and T cells to result in T cell proliferation in vitro.

Materials and Methods

Ags

Polysaccharide A1 (PS A1) from B. fragilis NCTC 9343 was prepared as described previously (19–22). In brief, the polysaccharide was isolated by
**Bacteroides fragilis** “PS A1”

![Zwitterionic bacterial polysaccharide](image)

**Streptococcus pneumoniae type 1 “Spl1”**

![Capsular polysaccharide](image)

**FIGURE 1.** Repeating unit structures of the zwitterionic bacterial polysaccharides PS A1 (from *B. fragilis*) and Spl1 (from *S. pneumoniae* type 1).

hot phenol/water extraction, enzyme digestion, gel-filtration and anion-exchange chromatography, and isoelectric focusing. *S. pneumoniae* type 1 capsular polysaccharide (Spl1) was obtained from the American Type Culture Collection (Manassas, VA) and treated with 2 M NaOH for 1 h at 80°C. Capsular polysaccharide was purified by gel-filtration, electrodialysis, and lyophilization in 0.15 M NaCl. Spl1 was concentrated by ultrafiltration, and lyophilized at 0.15 M NaCl.

The polysaccharide Ags were purified aseptically with sterile water. The instruments and devices used in the Ag purification process were deproteinized by treatment with sulfuric and chromic acid and depyrogenated by heat inactivation for 4 h at 240°C or by treatment with 1-2 M sodium hydroxide buffer.

The Ags were analyzed for protein by the BCA method (Pierce, Rockford, IL) and by UV absorbance at 280 nm; for endotoxin (LPS) by the Limulus amebocyte lysate test (Charles River Breeding Laboratories, Charleston, SC). In the *Limulus* test, the Ags were evaluated alone and in the presence of LPS; LPS alone served as a positive control. The purity of the B cell, monocyte, and dendritic cell populations was confirmed by flow cytometry (≥95%). Human B lymphoma lines Raji (27) and RJ2.2.5 (28), an EBV-transformed B cell line AN6-4 from a healthy donor, L cell fibroblasts, and L cell fibroblasts transfected with human HLA-DR2b (29) were used as APCs. These cells were grown in RPMI 1640 supplemented as described above.

**APCs and cell culture**

Mononuclear cells from healthy donors were isolated by centrifugation in Ficoll-Hypaque gradients (24). CD19+ B cells and CD14+ monocytes were negatively selected by immunomagnetic separation (Dynal Biotech, Lake Success, NY). Immature dendritic cells were generated from CD14+ monocytes by the addition of IL-4 (1500 U/ml of culture medium) and GM-CSF (500 U/ml) every 48 h for 5 days in RPMI 1640 supplemented with 1-glutamine, sodium pyruvate, penicillin-streptomycin, nonessential amino acids, 2-ME, and 10% FBS (Life Technologies, Gaithersburg, MD) (25, 26). The purity of the B cell, monocyte, and dendritic cell populations was confirmed by flow cytometry (≥95%). Human B lymphoma lines Raji (27) and RJ2.2.5 (28), an EBV-transformed B cell line AN6-4 from a healthy donor, L cell fibroblasts, and L cell fibroblasts transfected with human HLA-DR2b (29) were used as APCs. These cells were grown in RPMI 1640 supplemented as described above.

**T cell proliferation assays**

T cells were isolated by centrifugation in Ficoll-Hypaque gradients and purified with nylon wool and immunomagnetic beads (24). The purity of the CD3+ and CD4+ CD8- cell populations was confirmed by flow cytometry (≥95%). T cells (5 × 10⁴ per well, unless otherwise specified) were incubated in quadruplicate with gamma-irradiated APCs (1 × 10⁵ per well for all cells except dendritic cells, which were at 5 × 10⁴ per well), Ags, Abs, or medium alone (37°C, 5% CO₂) in a 96-well plate in RPMI 1640 supplemented as described above. Freshly isolated APCs were irradiated with 2500 rad; B lymphoma cells were irradiated with 5000 rad. The number of APCs was shown to be optimal in preliminary experiments, as were the concentrations of Ags and Abs and the incubation period (4–7 days).

In proliferation assays with transwells, 24-well plates and inserts with a 0.1-μm pore size were used. These assays were performed with 10⁶ gamma-irradiated mononuclear cells as APCs in the lower compartment and 5 × 10⁴ CD4+ T cells in the upper compartment. In proliferation assays in the presence of blocking Abs, the following purified mAbs without azide and endotoxin were used: HLA-A, -B, -C (W6/32) and HLA-DP (B7/21) obtained from Leinco Technologies (Ballwin, MO); HLA-DQ (SPVL3) obtained from Immunotech (Miami, FL); HLA-DR (L243) and isotype controls obtained from BD Pharmingen (San Diego, CA). The Abs were added 30 min before the Ags. T cell proliferation was quantitated by [³H]thymidine incorporation (1 μCi/well) for 6 h. Assays were performed at least three times at independent time points. The results were expressed as counts per minute or stimulation index.

**Immunoprecipitation**

MHC class II-expressing cell lines (Raji and L cell fibroblasts transfected with human HLA-DR2b) and MHC class II-negative cell lines (RJ2.2.5 and L cell fibroblasts) were cultured at a concentration of 5 × 10⁴ cells/ml in medium for 1 h before the addition of Sp1 (50 μg/ml). After incubation for 8 h (37°C, 5% CO₂), cells were washed and lysed (5 × 10⁵ cells/ml) in lysis buffer (0.5% Nonidet P-40, 50 mM Tris, pH 7.4, 5 mM MgCl₂ and 1 mM PMSF, Sigma-Aldrich, St. Louis, MO) on ice for 45 min. Debris was removed by centrifugation, and the supernatant was preclarified by two successive incubations with 3 μl of normal rabbit serum and recombinant protein A (RepliGen, Needham, MA), performed according to a standard method (30), one incubation with 3 μl of normal mouse serum and recombinant protein A, and one incubation with protein A alone. MHC class II and class I were immunoprecipitated with recombinant protein A from the lysates with mAb TU36 to HLA-DR (BD Pharmingen) (31, 32) and mAb W6/32 to MHC class I (Leinco Technologies) (33). Class II and class I molecules were released from protein A by incubation in sample buffer (0.0625 M Tris, pH 6.8, 5% 2-ME, 10% glycerol, and 4% SDS) at room temperature for 20 min or by boiling in sample buffer for 5 min. The preparations were run overnight on 12.5% SDS/30 × 25-cm polyacrylamide gels at 80–100 V.

**Western analyses of immunoprecipitates**

Immunoprecipitates were run on SDS/polyacrylamide gels as described above. The gels were soaked in transfer buffer, and the Ags were transferred to polyvinylidene difluoride membranes with a semidy transfer apparatus (Trans-blot SD; Bio-Rad, Hercules, CA) for 2 h at 100 V. Completion of transfer was measured by observing the transfer of the low-m.w. marker. After blocking, the membranes were exposed at room temperature first to an Ab to Sp1 at a dilution of 1/1000 for 2 h. The polyclonal serum to Sp1 was prepared by immunization of rabbits with a Sp1-TT conjugate vaccine. This vaccine was made by oxidation of purified Sp1 with periodate treatment to create reactive aldehyde groups. The oxidized

**FIGURE 2.** Direct contact of APCs and T cells is required for ZPS-induced T cell stimulation. T cell proliferation assays in 24-well plates with transwells (pore size, 0.1 μm) were performed with 10⁶ gamma-irradiated mononuclear cells as APCs in the lower compartment and 5 × 10⁴ autologous CD4+ T cells in the upper compartment. Cells were incubated for 6 days in the presence of Sp1 (20 μg/ml) or medium alone. Prevention of contact between APCs and T cells significantly decreased T cell proliferation; *, p < 0.0001.
FIGURE 3. Professional APCs support ZPS-induced T cell proliferation. T cell proliferation assays with B cells, monocytes, and dendritic cells as APCs were performed as described above except that dendritic cells were incubated with T cells at a concentration of $5 \times 10^4$ per well. The wells contained the APCs, autologous CD3$^+$ T cells, and PS A1 (20 μg/ml), Sp1 (20 μg/ml), or medium alone.

Sp1 was conjugated to monomeric TT by reductive amination (34). After incubation with the first Ab, the membranes were exposed to a HRP-conjugated goat anti-rabbit Ab (Southern Biotechnology Associates, Birmingham, AL) at a dilution of 1/5000 for 1 h. Bands bound by anti-Sp1 were revealed with a Western blot chemiluminescence reagent (NEN, Boston, MA) and photographic film (Hyperfilm ECL; Amersham Pharmacia Biotech) with various exposure times.

Transmission electron microscopy

Transmission electron microscopy was performed as described previously (35). B cells of the EBV-transformed AN6-4 line were incubated for different intervals ranging from 30 min to 16 h with PS A1 (50 μg/ml), III-PS (50 μg/ml), or medium alone. After extensive washing and fixation, cells were sectioned at $-120^\circ$C and transferred to Formvar/carbon-coated copper grids. After blockage of nonspecific binding sites, the primary mAbs to PS A1 (CE3) (36) at a dilution of 1/5, to III-PS (37) at dilutions of 1/5 to 1/100, to lysosome-associated membrane protein-1 (LAMP-1) (H4A3) (BD PharMingen) at a dilution of 1/10, to HLA-DR (TU36) (BD PharMingen) at a dilution of 1/100, followed by rabbit anti-mouse IgG (at a dilution of 1/100) (DAKO, Carpinteria, CA) and protein A-gold, were applied. The grids were examined in a JEOL 1200EX transmission electron microscope (Peabody, MA) at 80 kV.

Statistical analysis

Results for the various groups in T cell proliferation assays were compared by Student’s $t$ test.

Results

HLA-DR mediates T cell proliferation by ZPS

Previous studies had shown that APCs are required for ZPS-induced T cell proliferation (17). To determine whether T cell proliferation depends on direct contact between APCs and T cells or on soluble factors released by APCs, T cell proliferation assays were performed with transwells. Fig. 2 shows the result of one representative transwell experiment. Prevention of direct contact between APCs and T cells eliminated T cell proliferation.

FIGURE 4. MHC class II mutant cell line does not allow ZPS-induced T cell proliferation. Shown are the results of a representative T cell proliferation assay with Raji and class II mutant RJ2.2.5 cells as APCs; CD3$^+$ T cells from a donor haplotyped DR4, DR16, DR53 as responders; and Sp1 in different concentrations ($p < 0.05$).

FIGURE 5. Ab to HLA-DR blocks ZPS-induced T cell proliferation. In this T cell proliferation assay in the presence of blocking Abs to HLA-DR, HLA-DR, HLA-DQ, and HLA-A, -B, and -C (1 μg/well) as well as their isotype controls, mononuclear cells served as APCs and autologous CD3$^+$ cells as responders (donor haplotyped DR1, DR16). Sp1 was added at a concentration of 20 μg/ml; $*, p < 0.05$.

To identify the surface molecule on APCs that mediates T cell proliferation and might be specific to a professional APC subpopulation, CD19$^+$ B cells, CD4$^+$ monocytes, and monocyte-derived dendritic cells were tested for their indispensability in induction of T cell proliferation in the presence of PS A1 and Sp1. APC subpopulations mediated T cell activation with a stimulation index of 4- to 8-fold (Fig. 3), despite the fact that these APCs were derived from different donors.

Characterization of the role of surface molecules common to all professional APCs-MHC class I and MHC class II-was pursued in subsequent studies. The human Burkitt lymphoma cell line (Raji) expressing MHC class II and MHC class I molecules supported ZPS-induced T cell proliferation when incubated with CD4$^+$ cells, whereas its MHC class II transcriptional mutant cell line (RJ2.2.5) did not (Fig. 4). T cell proliferation assays with mononuclear cells as APCs and CD3$^+$ T cells as responders in the presence of blocking Abs to the HLA class I molecules HLA-A, HLA-B, and HLA-C, the MHC class II molecules HLA-DR, HLA-DP, and HLA-DQ, and their isotype controls demonstrated that the MHC class II molecule HLA-DR mediated ZPS-induced T cell proliferation (Fig. 5). T cell proliferation was reduced significantly only in the presence of blocking Abs to HLA-DR and not by Abs specific for HLA-DP, HLA-DQ, or HLA-A, -B, or -C.

ZPSs bind to HLA-DR and localize on the APC surface and in endolysosomes

It is possible that physical interaction of ZPS with HLA-DR is required for the mediation of ZPS-induced T cell proliferation by
HLA-DR. Western blot analysis with an Ab to ZPS showed a band at 100 kDa of HLA-DR immunoprecipitated from MHC class II-positive cells treated with ZPS; in contrast neither MHC class II-positive cells incubated in medium alone nor MHC class II-negative cells incubated with and without ZPS showed the band (Fig. 6). Western blot analysis of immunoprecipitated MHC class I did not show binding of ZPS with MHC class I molecules.

To identify the cell compartment in which binding of ZPS with HLA-DR occurs, we incubated APCs with ZPS, III-PS, or medium alone for different time intervals and then examined the cells with an electron microscope. ZPS had already bound to the APC surface after incubation for 30 min (data not shown) and remained bound during incubation for 16 h (Fig. 7A). These data were confirmed by FACS analysis (data not shown). In addition, ZPS was found after an 8-h stimulation period in endo/lysosomes, where it colocalized with HLA-DR (Fig. 7B) and LAMP-1 (Fig. 7C). In B cells treated with the nonzwitterionic polysaccharide III-PS and stained with an Ab to III-PS and to HLA-DR, the polysaccharide Ag was not detected either intracellularly or on the APC surface. HLA-DR was detected in lysosomes exclusively. B cells incubated in medium alone and stained with an Ab to the ZPS and to HLA-DR had HLA-DR in lysosomes (Fig. 7D). These data demonstrate that ZPSs bind to HLA-DR and that binding can occur on the cell surface or in compartments of the endocytic pathway.

Discussion

In previous studies, incubation of human CD4+ T cells with ZPS in the absence of irradiated feeder cells failed to elicit T cell proliferation—a finding that established a requirement of APCs in this system (38). In this study, we document that direct interaction of T cells with APCs is essential to the mediation of T cell activation by ZPS. APCs lacking MHC class II molecules do not support this activity. Blocking studies using an HLA-DR-specific mAb have revealed a requirement of the MHC class II molecule HLA-DR in ZPS-mediated T cell activation.

Physical interaction of ZPS with HLA-DR may be required for the mediation of T cell proliferation by ZPS. We detected SDS-stable binding of HLA-DR with ZPS. In our hands, immunoblotting of immunoprecipitated HLA-DR and other control molecules was a more sensitive method for detection of HLA-DR-ZPS immune complexes than autoradiography of 35S-labeled immunoprecipitated HLA-DR-complexes (data not shown). Immunoblotting showed a band representing the HLA-DR-Sp1 complex at ~100 kDa. Crystallographic studies have revealed that the Ag-binding groove of HLA-DR is “open-ended” so that Ags are bound in an extended conformation projecting from both ends (39). Modeling of a ZPS from another B. fragilis strain, PS A2, demonstrated that the polysaccharide forms an extended right-handed helix in which two repeating units per turn form geometrical grooves with positive and negative charges exposed on the outer surface (18, 40). It is possible that a conformational zwitterionic epitope on a short oligosaccharide fragment of ZPS forms a complex with HLA-DR by accommodating the insertion of α-helices in its grooves. The observation that APCs of different HLA-DR haplotypes stimulated proliferation of unrelated donor T cells suggests that ZPS may offer several different binding sites and consequently promote promiscuous binding to HLA-DR. The physical-chemical basis of the interaction between ZPS and HLA-DR may depend both on ZPS charge and conformation.

Clearly, ZPSs rapidly bind to the surface of APCs. The high density of charge on these molecules facilitates electrostatic interaction. It is easy to envision ionic or Schiff base interactions causing ZPSs to stick to the APC surface at many sites, including the HLA-DR molecule.

ZPSs also localize with HLA-DR in compartments of the endocytic pathway. Earlier studies revealed the localization of nonzwitterionic TI-2 Ags in endosomes and lysosomes (6). Ongoing studies are seeking to identify the mechanism by which ZPS interaction with HLA-DR results in T cell proliferation. It is possible that these molecules bind on the cell surface and that ZPSs are presented by a superantigen-like mechanism without trafficking through the intracellular pathway. In this scenario, the presence of HLA-DR and ZPS together in intracellular vesicles may be a result of ZPS binding to HLA-DR on the cell surface followed by internalization of recycling class II molecules (41). In yet another scenario, ZPSs may be endocytosed nonspecifically or by a specific receptor, proceed through the endocytic pathway (whether processed or not) by a novel mechanism, and are presented on the cell surface by HLA-DR. It remains to be seen which form of presentation induces the ZPS-mediated T cell activation.

In summary, we provide evidence that the MHC class II molecule HLA-DR is required for ZPS-mediated T cell proliferation. The demonstration of colocalization of HLA-DR and ZPS might indicate a novel mechanism of Ag presentation of these polysaccharides.

Acknowledgments

We thank April Arrasate, Ronald Panzo, and Brian Hyett for their excellent technical assistance, Maria Ericson for electron microscopy, Drs. Thomas Berger and Anamaria Lennon for technical advice, Drs. Kai Wucherpfennig and Martin Krönke for critical discussions, and Julie McCoy for editorial services.

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