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Parameters Underlying Distinct T Cell-Dependent Polysaccharide-Specific IgG Responses to an Intact Gram-Positive Bacterium versus a Soluble Conjugate Vaccine

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IgG anti-polysaccharide (PS) responses to both intact Streptococcus pneumoniae (Pn) and PS conjugate vaccines are dependent on CD4+ T cells, B7-dependent costimulation, and CD40-CD40-ligand interactions. Nevertheless, the former response, in contrast to the latter, is mediated by an ICOS-independent, apoptosis-prone, extrafollicular pathway that fails to generate PS-specific memory. We show that pre-existing PS-specific Igs, the bacterial surface or particulation, selective recruitment of B cell subsets, or activation and recruitment of Pn protein-specific CD4+ T cells do not account for the failure of Pn to generate PS-specific IgG memory. Rather, the data suggest that the critical factor may be the lack of covalent attachment of PS to protein in intact Pn, highlighting the potential importance of the physicochemical relationship of PS capsule with the underlying bacterial structure for in vivo induction of PS-specific Igs. The Journal of Immunology, 2009, 183: 1551–1559.

In this regard, studies from our laboratory have indicated that PS in the context of an intact Gram-positive extracellular bacterium, Streptococcus pneumoniae (Pn), is in fact not a classical TD Ag. Rather, it combines features of both an isolated PS Ag (TI) and a PS-protein conjugate vaccine (TD; Ref. 5). Thus, whereas the IgM anti-PS response to intact Pn is TI, both the IgG antiprotein and anti-PS responses are dependent on CD4+ T cell help, B7- and CD40-dependent costimulation, and comprise IgG of all four isotypes. However, in contrast to the antiprotein response, the IgG anti-PS response to intact Pn exhibits attenuated primary kinetics, fails to generate immunological memory, and is dependent on a shorter period of T cell help and B7-dependent costimulation. Furthermore, the IgG anti-PS response to Pn is ICOS independent and extrafollicular (6) and more apoptosis prone (7), in contrast to the IgG antiprotein response. The mechanism underlying these differences, as well as the essential difference between PS expressed by an intact bacterium vs a conjugate vaccine, is unknown.

Covalent linkage of pneumococcal PS type 14 (PPS14) to the immunogenic cell wall protein, pneumococcal surface protein A (PspA; Ref. 8), radically changes the nature of the in vivo IgG anti-PPS14 response relative to both purified capsular PPS14 as well as intact Pn. Thus, the IgG anti-PPS14 response to soluble PPS14-PspA conjugate is largely similar to the IgG anti-PspA responses to both conjugate and intact Pn14, including prolonged primary kinetics of induction and the generation of a CD4+ T cell-dependent, PPS14-specific IgG memory response (7, 9). Collectively, these data indicate that PPS14 in association with intact Pn14 is immunologically distinct from a soluble covalent conjugate of PPS14 and an immunogenic pneumococcal protein, despite the CD4+ T cell dependence of the IgG responses to both immunogens. In this report, we explore several parameters that may underlie the distinct nature of the IgG anti-polysaccharide responses to these two related immunogens. Our current data suggest that the physicochemical relationship of the capsular PS of an intact Gram-positive bacterium with its underlying immunogenic proteins, may critically determine the nature of the T cell-dependent IgG anti-PS response in vivo.
Materials and Methods

Mice

Lsc−/− mice (10) were generated in a C57BL/6 genetic background. Both Lsc−/− and control C57BL/6 mice were bred and maintained at the National Jewish Biological Resource Center (Denver, CO). RAG-2−/− (BALB/c background) mice were obtained from The Jackson Laboratory and were bred and maintained at the United States Uniformed Services University, 75 McNair Road, Bethesda, MD. Female BALB/c mice and athymic nude mice were purchased from the National Cancer Institute. Mice were used between 7 and 12 wk of age. These studies were conducted in accordance with the principles set forth in the Guide for Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, revised 1996, and approved by the USUHS and National Jewish Health institutional animal use and care committees.

Reagents

Recombinant PspA was expressed in Saccharomyces cerevisiae B31S05 and purified as described (9). Purified PPS14 was purchased from American Type Culture Collection. PPS14-PspA conjugates were synthesized as previously described (11). The molar ratio of PPS14 to PspA was ~1:3 except in Fig. 7, where conjugates containing PPS14-PspA ratios of 1:2.4 were used.

Antibodies

Mouse IgG2aκ antiphosphorylcholine (PC) mAb (clone PG2a2.2A1), mouse IgG1κ anti-PPS14 mAb (clone 44.1), and mouse IgG2aκ anti-PspA (DC10-IA5) were gifts from Dr. James Kenny (Beth Israel Deaconess Medical Center, Boston, MA), and Dr. Alex Lucas (Children’s Hospital Oakland Research Institute, Oakland, CA), and Dr. Rick Schuman (Antibody and Immunoassay Consultants), respectively. mAbs 44.1 and DC1-IA5 were both biotinylated using biotin-LC-hydrazide (Pierce), and the carboxylic acid succinimidyl ester (Invitrogen).

PspA-specific CD4+ T cell hybridomas

CD4+ T cell hybridomas specific for PspA peptide in association with MHC class II (MHC-II)b were produced and screened according to a previously published protocol (13). One BALB/c (MHC-II−/−-restricted, clone BALD4) and one C57BL/6 (MHC-IIb-restricted, clone B6D2) CD4+ CD8− T cell hybridoma was selected for use in these studies.

Bacterial strains

Ph1 was provided by Sam Wilson (USUHS). The isogenic noncapsulated mutant of D39 (Pn, capsular type 2, strain R36A) was provided by Dr. David Briles (University of Alabama, Birmingham, AL). Strain R6J (R6cp:Janus), a variant of a descendant of R36A (strain R6) in which the capsule locus is substituted by a Janus cassette, was provided by Drs. Krzysztof Trzcinski and Marc Lipsitch (Harvard School of Public Health, Boston, MA). An isogenic variant of R6 expressing capsular PS type 14 (R6-14) was constructed by transformation with chromosomal DNA as described (14). DNA from one isolate was purified and used to retransform R6 into a Cps14-expressing strain. R6-14 expresses quantitatively identical levels of Cps14 relative to the donor strain (Ph1). The PspA-expressing strain R6-14 is serologically identical with the PspA of R36A and the recipient R6 strain.

Growth and preparation of Pn strain stocks

Frozen stocks of Ph1 strain were thawed and subcultured on BBL premaide blood agar plates (VWR International). Isolated colonies in blood agar were grown in Todd Hewitt broth (BD Biosciences) to midlog phase, collected, and either heat killed or UV inactivated. Sterility was confirmed by subculture on blood agar plates. Bacteria were frozen at −80°C until their use.

Preparation of R36A depleted of choline-binding proteins (CBP)

Bacterial cultures were pelleted by centrifugation and treated for 20 min at room temperature with 2% choline chloride (Sigma-Aldrich) in PBS containing 0.25 M NaCl to release CBPs from the bacterial cell wall (8). The resulting CBP-depleted bacteria (R36Ach) were washed by centrifugation, heat killed, and stored as indicated previously. The PspA content of CBP-depleted R36A preparations was ≤60 ng/10^9 CFU.

Preparation of R36Ach coated with PPS14-PspA conjugate vaccine

R36A, 1 × 10^6 CFU depleted of native PspA (R36Ach), were coated with PPS14-PspA conjugate by incubation overnight with 30–100 μg of PPS14-PspA in 30 mM acetate buffer, pH 5.0. After incubation, coated bacteria were washed five times with PBS, pH 7.2, and used directly for immunizations or stored at −20°C in PBS containing 50% glycerol.

Preparation of latex beads coated with PPS14-PspA

Fifty micrograms of PPS14-PspA in 0.1 M phosphate buffer, pH 6.5, were covalently linked to 10^6 surfactant-free aldehydesulfate latex beads ( Molecular Probes; Invitrogen), 0.96 μm in diameter, by incubation overnight in an orbital shaker. Free binding sites in the latex beads were blocked using 0.1% mouse serum albumin (MSA) in PBS, by incubation for 1 h at room temperature, or using 0.2% normal mouse serum previously adsorbed with an excess of R6-14. Blocked beads were washed six times with 40 volumes of blocking buffer and resuspended in blocking buffer. Beads coated with MSA alone were used as controls. Bead density after coupling was determined by densitometry at 630 nm. For some experiments, PPS14-PspA was attached to the beads through noncovalent linkage. Sulfactant-free carboxymethylated latex beads (10 μm; Molecular Probes; Invitrogen), 1.2 μm in diameter were incubated overnight with 100 μg of PPS14-PspA in 0.1 M phosphate buffer, pH 6.0. After incubation, the beads were treated as above for the aldehyde-sulfate latex beads.

Quantitation of PspA content

PspA content was determined by competitive inhibition ELISA. Serial dilutions of bacteria or latex beads were incubated overnight at 4°C in PBS plus 1% BSA containing 1 ng of mAb DC10-IA5, specific for PspA, per ml. Similar mixtures of bacteria not expressing PspA or control latex beads were included as positive controls, and mixtures lacking the anti-PspA mAbs included as negative controls. Standard curves were generated using serial dilutions of CBPs purified from Ph1 strain R36A, and of known PspA content, or of recombinant PspA, mixed with 1 ng/ml DC10-IA5. After incubation, suspensions were transferred to ELISA microtiter plates (Costar 96-well 4HBX; Thermo Electron) previously coated with 4 ng/well recombinant PspA and blocked with 2% BSA. The ELISA plates were then incubated overnight at 4°C and washed five times with PBS-Tween, and the amount of bound DC10-IA5 was detected using alkaline phosphatase-conjugated polyclonal goat IgG anti-mouse IgG (γ chain) in combination with p-nitrophenyl phosphate disodium (Sigma-Aldrich) as enzyme substrate. The limit of detection was 3 ng/ml PspA.

Quantitation of PPS14 content

The content of PPS14 was determined by a quantitative sandwich ELISA in which mAb 44.1 specific for PPS14 was used as both capture and detection Ab. Purified PPS14 was used as a standard. The limit of detection of the assay was 60 pg/ml PPS14.

Quantitation of PPS14-PspA conjugate

The content of PPS14-PspA on bacteria and particles was determined by two different quantitative approaches. ELISA essentially identical with that described above for the quantitation of PPS14, except for the use of PPS14-PspA conjugate as standard; (2) a capture ELISA in which particles or bacteria were captured with 5 μg/ml PPS14-PspA specific mAb DC10-IA5 and detected with 1 μg/ml biotinylated 44.1 (anti-PPS14 mAb). PPS14-PspA was used as a standard. Wells incubated with R36Ach or control (MSA-coated) beads were used as negative controls. The limit of detection of this assay is 0.9 ng/ml PPS14-PspA. Both methods resulted in similar quantitative values of the PPS14-PspA content, validating the quantitative determinations. The content of PPS14-PspA on the particles was expressed as the average of the value obtained using these two approaches.

Detection of the conjugate on the bacterial cell wall

The presence of conjugate on the bacterial cell wall was further established by the detection of PPS14 and increased levels of PspA in R36Ach coated with PPS14-PspA by a capture ELISA. Briefly, bacteria were captured using wells coated with 5 μg/ml PC-specific mAb PG2a2.2A1. PC is expressed on the Pn cell wall teichoic acid and membrane lipoteichoic acid (4). Captured bacteria were detected with biotinylated 44.1 μm (specific for PPS14) or DC10-IA5 (specific for PspA). R36Ach, not exposed to PPS14-PspA, and free PPS14-PspA were used as negative controls.
Flow cytometric detection of PPS14-PspA conjugate attached to latex beads

Beads (2.5 × 10⁷) were incubated overnight at 4°C with 5 μg of 44.1 mAb labeled with Alexa Fluor 488, with 1 μg of DC10-IAS labeled with Alexa Fluor 633, or with a mixture of both in PBS plus 2% BSA. MSA-coated latex beads were used as negative controls. Bead fluorescence was analyzed on a BD LSRII (BD Biosciences). Bead singlets and multiplets were acquired and analyzed. PPS14-specific Ab and latex beads were used in excess to reduce the agglutination of beads coated with PPS14-PspA.

Immunizations

Mice were immunized and boosted i.p. with immunogen suspended in saline or adsorbed on 13 μg of alum (Brenntag Biosector) mixed with 25 μg of a stimulatory 30-mer CpG-containing oligodeoxynucleotide (CpG-ODN) (15). Serum was prepared from blood obtained through the tail vein.

Cell purification and adoptive transfer of B cells and CD4⁺ T cells into RAG-2⁻/⁻ mice

Purified splenic B and CD4⁺ T cells were obtained by positive selection using anti-B220 and anti-CD4 MACS beads, respectively (Miltenyi Biotec). Cell purities of ~90% were typically observed. RAG-2⁻/⁻ mice were injected i.p. with a mixture of 2 × 10⁷ B cells and 1 × 10⁷ CD4⁺ T cells. Mice were then immunized 18 h later.

Measurement of serum titers of Ag-specific Ig isotypes

Serum titers of PPS14- and PspA-specific IgM and IgG were determined by ELISA as described in detail previously (6).

Bone marrow dendritic cell (BMDC) preparation and Ag presentation assay using a PspA-specific CD4⁺ T cell hybridoma

BMDCs were prepared as described (16) and plated at 2 × 10⁷ BMDCs/well in 96-well flat-bottom tissue culture plates (Costar), containing serial dilutions of conjugate or heat-killed bacteria. After 2 h of pulse with Ag at 37°C, the T cell hybridoma was added to 10³ cells/well and cocultured with BMDCs for 24–48 h in the continual presence of free Ag. Alternatively, BMDCs were loaded for 2 h with the same amount of conjugate or bacteria but at a 10-fold higher concentration. After the pulse, free Ag was removed by centrifugation and washed with at least 100 volumes of PBS. A fraction of the Ag-loaded BMDCs and Ag controls was lysed to determine the content of internalized PspA by inhibition ELISA. BMDCs pulsed with Ag were then cocultured with the T cell hybridoma in the absence of free Ag. Ag presentation was determined by measuring IL-2 concentrations in cell culture supernatant by ELISA (BD Pharmingen). Serial dilutions of rIL-2 were used as standard.

Determination of the intracellular content of PspA in Ag-pulsed BMDCs

BMDCs loaded with PPS14-PspA or bacteria were extensively washed, and the pelleted BMDCs were lysed by resuspending 1–2 × 10⁶ cells in 100 μl of 1 mM EDTA, 0.05% Tween 20, 1% TX-100 in PBS, pH 7.2, supplemented with protease inhibitors (final concentration, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, and 10 mM PMSF; Sigma-Aldrich). Dilutions of Ag at the same concentrations as that used during the Ag loading of BMDCs were incubated, washed, and resuspended in the same amount of lysis buffer to determine the amount of free Ag contaminating the cell lysates. After 30 min of incubation in an ice bath, the cell lysate was cleared by centrifugation at 12,500 rpm at 4°C, and the content of PspA in the cell extract supernatant was determined by inhibition ELISA.

Statistics

Data were expressed as the geometric mean ± SEM of the individual values. Levels of significance of the differences between groups were determined by the Student t test. Values of p < 0.05 were considered statistically significant.

Results

Priming and boosting with PPS14-PspA, but not live or inactivated Pn14, results in marked enhancement in the secondary IgG anti-PPS14 response

We initially compared the ability of heat-killed S. pneumoniae, capsular type 14 (Pn14) with a soluble, covalent conjugate of pneumococcal type 14 capsular polysaccharide (PPS14) and pneumococcal surface protein A (PspA; PPS14-PspA; conjugate) to elicit primary and secondary IgG anti-PPS14 and IgG anti-PspA responses in vivo. Conjugate was administered in alum plus a stimulatory TLR9 ligand, CpG-ODN (17). Pn14 and conjugate both induced an enhanced IgG response...
were obtained 21 days later, and B and CD4+ T cells were separately purified. Purified cells (2 × 10^7 B cells plus 1 × 10^7 CD4+ T cells/mouse) were injected i.p. into RAG-2-/− mice (5 mice per group). One day later (day 0), recipient mice were immunized i.p. with the same corresponding dose of Pn14 or PPS14-PspA conjugate. Serum titers are expressed as the geometric mean ± SEM of the individual values. *, Significant p < 0.05 between donor cells from naive vs Pn14-primed BALB/c mice.

Anti-PspA response after boosting (Fig. 1A). In contrast, conjugate, but not Pn14, induced an enhanced secondary IgG anti-PPS14 response. Similar results were obtained after i.p. injection of UV-inactivated or live Pn14 (Fig. 1B), with heat-killed Pn14 injected either i.v. or s.c., or with heat-killed Pn14 suspended in alum plus CpG-ODN and injected i.p. (data not shown). A delay in boosting with Pn14 by up to 6 wk after initial priming also failed to elicit an enhanced secondary IgG anti-PPS14 response, although a boosted IgG anti-PspA response was still observed (6). Finally, mice immunized i.p. with varying doses of intact, heat-killed Pn14, generating dose-dependent differences in relative serum titers that were preserved during bead coupling.

PPS14- or double-stained beads (26%; rectangle in Fig. 3A). This result of cross-linking, by PPS14-specific IgG1 mAb, of multivalent beads, or noncovalently linked to 1.2-m-μm diameter aldehyde-sulfate latex beads. Attachment of the conjugate was stable and homogeneous. Thus, for the aldehyde-sulfate latex beads, >98% of the conjugate-coated beads were doubly stained with anti-PPS14 and anti-PspA mAbs (Fig. 3A; 375 ± 127 ng of conjugate per 1 × 10^8 beads), with no detectable staining observed for control beads coated with MSA. The content of PPS14 (202 ± 25 ng) and PspA (140 ± 17 ng) per 1 × 10^8 beads was close to the chemical composition of the conjugate, suggesting that intact conjugate, and not traces of free Ag that could be present, were attached to the beads. The content of free conjugate was <0.07% of the total conjugate in the bead preparation. Multimerization of the double-stained, conjugate-coated beads (Fig. 3A) was the sole result of cross-linking, by PPS14-specific IgG1 mAb, of multivalent PPS14. Thus, conjugate-coated beads stained only with anti-PspA mAb yielded 62% of beads as singlets, similar to the number of singlets in control beads (74%), but considerably higher than in the PPS14- or double-stained beads (26%; rectangle in Fig. 3A). This further indicates that the multivalent structure of the PPS14 was preserved during bead coupling.

Mice were immunized i.p. with 0.75 μg of free conjugate (Fig. 4A) or varying amounts of conjugate-linked aldehyde/sulfate latex beads (maximal 2 × 10^9 particles; Fig. 4B) in alum plus CpG-ODN and similarly boosted on day 14. Mice immunized with conjugate covalently attached to aldehyde-sulfate latex beads elicited a dose-dependent primary, and a significantly boosted secondary IgG anti-PPS14, as well as IgG anti-PspA response, similar to that of Pn14 or conjugate-primed mice, in contrast to cells from naive mice, elicited a comparable and significant induction of IgG anti-PspA (Fig. 2). Collectively, these data indicate that Pn14 fails to generate a boosted secondary IgG anti-PPS14 response, regardless of the presence or absence of primary anti-PPS14 Abs.
observed using free conjugate. Latex beads, by themselves, elicited no detectable response. Conjugate attached to beads were 5-fold more immunogenic on a per weight basis than free conjugate. Similarly, an enhanced secondary IgG anti-PPS14 and IgG anti-PspA response, although no detectable primary response, was elicited by conjugate that was noncovalently attached to the carboxylate-modified latex bead 1.2 μm in diameter (C), or adsorbed to S. pneumoniae strain R36A, previously depleted of native PspA by treatment with choline chloride (R36Ach; E). The highest dose used contained 1 × 10^8 latex particles or 2 × 10^8 CFU of R36A. Groups of mice immunized with aldehyde-sulfate latex beads coated with MSA (B) and untreated R36A (D) were used as controls. Alum plus CpG-ODN was used as adjuvant for all immunizations.

In a second approach, we adsorbed PPS14-PspA conjugate noncovalently to the surface of intact, heat-killed Pn strain R36A, an unencapsulated variant of the capsular type 2 D39 strain, which was depleted of native PspA using choline chloride treatment (R36Ach-PPS14-PspA). Conjugate was stably attached to the bacterial surface (~4 ng/1 × 10^8 CFU). Free conjugate was <0.4% of the total R36Ach-PPS14-PspA preparation. The presence of PPS14-PspA conjugate on the bacterial surface was established by a capture ELISA assay. Both R36Ach and R36Ach-PPS14-PspA were captured with an anti-PC mAb and detected with mAbs specific for PC, PPS14, or PspA (Fig. 3B). PC is a component of the teichoic and lipoteichoic acid present within the Pn cell wall and membrane, respectively (4), that is not detectable in conjugate. R36Ach and R36Ach-PPS14-PspA were captured and detected equally well with anti-PC mAb, regardless of the presence of coating conjugate (Fig. 3B). In contrast, detection with anti-PspA, and especially anti-PPS14 mAbs were significantly higher for R36Ach-PPS14-PspA than for R36Ach (Fig. 3B), demonstrating the presence of conjugate on the bacterial surface.

Since R36A does not express PPS14 and was depleted of native PspA (<6 ng PspA/10^9 CFU), the IgG responses induced to both Ags would be elicited almost exclusively by the conjugate on the bacterial surface. As expected, mice immunized i.p. with untreated R36A failed to induce a detectable IgG anti-PPS14 response but did elicit both a primary IgG anti-PspA response, and an enhanced secondary IgG anti-PspA response following boosting (Fig. 4D). In contrast, mice immunized with R36Ach, without adsorbed conjugate, made no detectable IgG anti-PPS14 or IgG anti-PspA response (data not shown). When mice were immunized i.p. with R36Ach-PPS14-PspA, both a primary IgG anti-PPS14 response and a significantly enhanced secondary response following boosting was observed (Fig. 4E). A secondary, although not primary, IgG anti-PspA response was also elicited. The secondary, boosted, responses were obtained with doses as low as 1 ng of PPS14-PspA attached to R36Ach. Collectively, these data indicate that neither the particulate nature of the immunogen or unrelated cell surface components of the bacterial surface are determining factors for the lack of PPS14-specific IgG memory generation in response to Pn14. On the contrary, these factors enhance the PPS14-specific IgG response.

**Conjugate adsorbed to intact Pn, in contrast to free conjugate, induces an IgG anti-PPS14 memory response that depends on marginal zone B cell migration**

Intact Pn14 vs conjugate could selectively engage different splenic B cell subsets (i.e., marginal zone B cells (MZB) and/or B1 cells vs follicular B cells (FB), respectively), for eliciting the IgG anti-PPS14 response. Selective utilization of B cell subsets by intact Pn14 vs conjugate could determine whether PPS14-specific IgG memory is generated (21–23). MZBs in mice genetically deficient in *lsc* (*lsc*−/−) are defective in detaching from the marginal zone and receiving CD4+ T cell help for TD Ig responses (10). Using these mice, we confirmed our previous observation (7) that the T cell-dependent primary and secondary IgG anti-PPS14 responses to intact Pn14 are virtually abrogated in *lsc*−/− mice, whereas the T1 IgM anti-PPS14 response is unaffected. However, the IgG anti-PspA response, presumed to be mediated by FBs, was similar between *lsc*−/− and wild-type (WT) mice. In contrast to intact Pn14, both the IgM and IgG anti-PPS14, as well as IgG anti-PspA, responses to conjugate were normal in *lsc*−/− mice (Fig. 5B). When conjugate was adsorbed to intact Pn (i.e., R36Ach-PPS14-PspA), it induced a boosted IgG anti-PPS14 secondary response in WT mice (Fig. 5C) as demonstrated earlier (Fig. 4). However, in striking contrast to free conjugate, the IgG anti-PPS14 response to R36Ach-PPS14-PspA was completely abrogated in *lsc*−/− mice, whereas the IgM anti-PPS14 and IgG anti-PspA responses were similar to WT mice.

When *lsc*−/− mice were immunized with conjugate covalently attached to aldehyde-sulfate latex beads, a significant, though partial reduction in the IgG anti-PPS14 response was observed, whereas the
IgM anti-PPS14 and IgG anti-PspA responses were similar to WT (Fig. 5D). These data strongly suggest that the failure of intact Pn14 to elicit PPS14-specific IgG memory is not due to its dependence on MZB, as opposed to FB, per se. Further, the complete and partial dependence on MZB cells in the IgG anti-PPS14 responses to PPS14 associated with bacteria vs latex beads, respectively, highlights the unique immunological features of the intact bacterium, besides its particulate nature.

The total amount of internalized PspA, but not its antigenic form, determines the efficiency of APC to activate PspA-specific T cells in vitro

Differential endocytic routing and phagosome maturation (24–26) of Pn- and conjugate-associated PspA could result in an altered efficiency in its processing and presentation to CD4+ T cells. Thus, we initially determined the ability of BALB/c-derived BMDCs to activate a syngeneic PspA-specific T cell hybridoma (BALD4) in the continuous presence of varying doses of either PPS14-expressing Pn (strain R6-14) or conjugate (PPS14-PspA; Fig. 6A). BMDCs continuously exposed to intact Pn were >5000 times more efficient in presenting PspA to BALD4 than those exposed to a similar amount of PspA in the form of soluble conjugate. BMDCs cultured with purified (unconjugated) PspA had an efficiency for activating BALD4 T cells similar to those exposed to conjugate (data not shown), indicating that the PPS14 in the conjugate did not significantly interfere with processing or presentation of the PspA. Further, the conjugate contains a PspA serologically identical with the PspA expressed by R6-14, excluding that the effect was due to differences in the affinity of the T cell hybridoma for peptide variants derived from the PspA of these two immunogens.

The differences in the efficiency of presentation of R6-14- and conjugate-derived PspA may relate to the more concentrated exposure of PspA to BMDCs when expressed within the particulate bacteria, relative to soluble conjugate. To determine this, we pulsed BMDCs for 2 h, using a dose response containing the same total amounts of PspA as in the previous experiment, but at 10-fold higher concentrations. The potency of BMDCs presentation of conjugate-associated PspA increased, whereas that of Pn-associated PspA decreased, in response to the 2 h, high concentration pulse, relative to the lower concentration, continuous Ag exposure (Fig. 6B). This resulted in R6-14 being only 10–50 times more efficient than conjugate. However, when BMDCs containing similar amounts of internalized PspA were compared, there were no significant differences in the efficiency of PspA presentation to BALD4 T cells by R6-14- and conjugate-pulsed BMDCs (Fig. 6C). These results indicate that the total amount of protein internalized by dendritic cells is the major factor determining Ag-specific T cell activation, regardless of the major pathway of internalization, the form, or complexity of the Ag, with particulation favoring overall uptake. These data strongly suggest that the failure of intact Pn, in contrast to conjugate vaccine, to promote PS-specific IgG memory, is not due to a relative inefficiency in presenting bacteria-derived protein for CD4+ T cell activation.
Free and Pn-adsorbed conjugate induce PPS14-specific IgG memory under conditions of limiting CD4+ T cell help

Despite data presented in Fig. 6, it is possible that other factors, operative in vivo, could lead to a more limited recruitment of CD4+ T cell helper function for PPS14-specific B cells responding to Pn14, resulting in a failure to generate PPS14-specific IgG memory. To test this hypothesis, we created two PPS14-PspA conjugates containing different molar ratios of PPS14 to PspA (i.e., 1:2.4 and 1:2.4) and immunized separate sets of WT mice with the two conjugates, either free or adsorbed to R36Ach. For each conjugate, 1 μg of PPS14/mouse was injected. As illustrated in Fig. 7A, conjugate (1:2.4) elicited significantly lower IgG anti-PPS14 and IgG anti-PspA responses than conjugate (1:24), but an equivalent IgM anti-PPS14 response. Nevertheless, secondary immunization with conjugate (1:2.4) elicited a highly boosted IgG anti-PPS14, as well as IgG anti-PspA response. Although Pn14 elicited higher primary IgG anti-PPS14 and IgG anti-PspA responses relative to conjugate (1:2.4), it failed to generate a boosted IgG anti-PPS14 secondary response (Fig. 7A). Using athymic nude mice, which are markedly deficient in T cells, we confirmed that the IgG anti-PPS14 responses to both types of conjugate, and to intact Pn14 were in fact T cell dependent (Fig. 7A).

As demonstrated in Fig. 5, free conjugate appears to recruit FB cells for the IgG anti-PPS14 response, whereas intact Pn14 elicits this response from MZB cells. Thus, it is possible that MZB cells require a higher degree of T cell help for inducing PPS14-specific IgG memory, relative to FB cells. To address this possibility, we adsorbed the two conjugates (1:2.4 and 1:2.4) to R36Ach. In this way, we could elicit the IgG anti-PPS14 response to both conjugates through MZB cells, instead of FB cells. As illustrated in Fig. 7B, R36Ach conjugate (1:2.4) elicited a significantly lower IgG anti-PPS14 response than R36Ach conjugate (1:24), whereas the IgM anti-PPS14 responses were again equivalent. Whereas R36Ach conjugate (1:24) induced a significantly boosted secondary IgG anti-PspA response, no detectable PspA-specific boosting was observed using R36Ach conjugate (1:2.4). Nevertheless, R36Ach conjugate (1:2.4) still elicited a boosted IgG anti-PPS14 secondary response, albeit lower than that observed for R36Ach conjugate (1:24; Fig. 7B). Collectively, these data indicate that even under conditions of highly limiting CD4+ T cell help, conjugate can still elicit PPS14-specific IgG memory through FB or MZB.

Discussion

The IgG anti-PPS14 response to Pn exhibits several characteristics associated with responses to classical TD Ags, such as dependence on CD4+ T cell help, B7- and CD40-dependent costimulation, and synthesis of all four IgG subclasses (5). However, this response also has features associated with purified PS (i.e., TI) Ags, including a shortened period of primary development, a failure to generate a boosted IgG response following secondary immunization, and independence on ICOS costimulation (6). The partial TD properties of intact Pn contrast with the full transition from TI to TD behavior of PS Ags covalently attached to an immunogenic carrier protein (i.e., conjugate vaccine) (2). This functional dichotomy, however, is puzzling because capsular PS expressed by Gram-positive extracellular bacteria such as Pn, is covalently linked to the underlying cell wall to which a number of immunogenic proteins are also bound through covalent and noncovalent linkages (3, 4). Thus, one might have predicted that immunologically, Gram-positive bacteria would be a particulate version of a soluble conjugate vaccine.

Our data demonstrate that the inability to generate memory appears solely related to the physicochemical properties of the intact bacterium as an inert immunogen. Particulation (27, 28) as well as components of the bacterial surface interacting with scavenger receptors (29) and complement components (30) could affect both the cell type-specific internalization and cellular trafficking of Ag within the secondary lymphoid organ, with a consequent impact on the functional outcome of the immune response. The propensity of particulate Ags to be sequestered within the marginal zone (MZ) (28), and the observation that MZBs in relation to FB are programmed to favor plasma cell over memory cell differentiation.

FIGURE 7. The induction of PPS14-specific IgG memory in response to the conjugate is independent of the ratio of PPS14 to PspA. BALB/c or athymic nude mice were immunized on days 0 and 14 with 1 μg/mouse of one of two PPS14-PspA conjugates differing in the indicated molar ratios of PPS14 to PspA (A) or with 119 ng of PPS14-PspA (1:24) or 106 ng of PPS14-PspA (1:2.4) adsorbed to 1 × 10^7 CFU R36Ach (B). A, left, *, significant p < 0.05 between WT and athymic (1:24); +, significant p < 0.05 for 1:2.4. A, right, *, significant p < 0.05 between WT and athymic. B, *, significant p < 0.05 between peak primary vs boost.
(22), led us to hypothesize that the failure of intact Pn to generate PPS14-specific IgG memory was secondary to its selective usage of MZBs to generate this response. Indeed, we show that the PPS14-specific IgG response to intact Pn14, but not conjugate, is markedly reduced in Lsc−/− mice, in which MZBs are defective in migrating from the MZ and are thus unable to receive CD4+ T cell help (7, 10). These data strongly suggest that intact Pn and soluble conjugate differentially utilize MZBs and FBs, respectively, to generate the PPS14-specific IgG response. A previous study also implicated MZBs, as well as B1 cells, in the TI anti-PC response to intact Pn (31). However, we demonstrate that this differential usage of MZBs and FBs, by intact Pn and conjugate, respectively, fails to account for the inability of intact Pn to generate PPS14-specific IgG memory. Thus, adsorption of conjugate to the surface of intact Pn, or covalent or noncovalent attachment to ~1 μm beads, induces a complete or partial switch, respectively, from FB to MZB usage for the IgG anti-PPS14 response but still results in the generation of highly boosted PPS14-specific IgG titters following secondary immunization.

In light of the previous data, the failure of intact Pn to induce PPS14-specific IgG memory raised the possibility that the complex antigenic structure of Pn itself, as opposed to particulation or the bacterial surface per se, led to suboptimal recruitment of CD4+ T cells, irrespective of B cell subset usage, and thus an inability to drive the memory response. This could be a consequence of a differential effect of Pn-expressed PPS14 on the processing and presentation of proteins by APCs (32). However, our results strongly argue against any direct involvement of PPS14 itself in limiting Pn-induced T cell activation. Thus, we find no significant differences in the ability of BMDCs loaded with PspA, unencapsulated Pn, PPS14-PspA conjugate, or Pn14, to present the resulting PspA-derived peptides to a PspA-specific T cell hybridoma, under conditions in which the total amount of internalized PspA is similar. These results are in agreement with previous work showing that neutral PSs do not affect MHC-II-restricted Ag presentation (33). Alternatively, as a result of their particulate nature, BMDCs internalized bacteria more rapidly than conjugate in vitro and thus were more efficient in inducing protein-specific T cell help. Finally, a PPS14-PspA conjugate preparation exhibiting poor immunogenicity due to a relatively low molar ratio of PspA to PPS14 was still able to recruit sufficient T cell help to induce a secondary anti-PPS14 IgG memory response, irrespective of whether this conjugate was injected as free (soluble) or Pn associated. This argues against the notion that limiting T cell help specific for bacterial proteins is the mechanism responsible for the failure of bacteria to generate PPS14-specific IgG memory responses.

On the contrary, we show that the adsorption of Ags onto particles enhanced Ag-specific IgG responses. Thus, the PPS14-PspA conjugate covalently attached to aldehyde-sulfate latex particles increased at least 5× its efficiency in inducing IgG anti-PPS14 and anti-PspA responses and >100× when it was noncovalently attached to unencapsulated Pn, depleted of native PspA. The greater efficiency of the PPS14-PspA conjugate attached to the bacterial surface may have been due in part, to the physical linkage of TLR ligands within intact Pn, in contrast to latex beads, which were merely suspended in alum plus CpG-ODN (25). Noncovalent linkage per se was unlikely to account for this enhanced efficiency of Pn-associated conjugate, because conjugate covalently linked to latex particles was more efficient than those noncovalently linked in inducing anti-PPS14 and anti-PspA IgG responses.

The underlying mechanism involved in the adjuvant effect of particulation is at least in part the targeted delivery of Ags to APCs in a concentrated form. BMDCs were >5000 times more efficient in the uptake and presentation of PspA to Ag-specific T cells, when delivered on the bacterial surface, than when in soluble form as a PPS14-PspA conjugate. This likely reflected the fact that ingestion of a single bacterial particle by an APC effected the uptake of multiple copies of PspA. In contrast, the amount of soluble PspA internalized via pinocytosis depends more heavily on the local concentration of Ag. Thus, the same total amount of soluble PPS14-PspA conjugate delivered at a higher concentration was internalized 10–50 times more efficiently by the BMDCs.

Collectively, our data suggest that CD4+ T cell help for PPS14-specific B cells in response to intact Pn, in contrast to conjugate, is not cognate for the associated bacterial proteins. This potential dichotomy between intact Pns and conjugate may reflect a requirement for covalent attachment of PPS14 to immunogenic protein, as is present in conjugate but not Pns, to elicit PPS14-specific IgG memory. In this regard, it was recently demonstrated that, in response to intact vaccinia virus, CD4+ T cell help for an in vivo IgG response to a specific viral protein was mediated strictly by CD4+ T cells with the same protein specificity as the B cell, despite there being a number of distinct immunogenic proteins expressed by the intact virion (34). This dependence on intramolecular help suggests the possibility that B cell samples isolated or covalently linked Ags following dendritic cell and/or macrophage transport, processing and/or membrane expression of intact pathogen. This may then explain the requirement for covalent linkage of protein and PS to recruit protein-specific, cognate CD4+ T cell help for a PS-specific B cell response, a condition apparently not present within intact Pns. Although other, nonconventional forms of T cell help for PS-induced Ig responses have been proposed (1), the nature of T cell help for the IgG anti-PS response to intact Pn14 remains to be determined. In summary, these data emphasize the critical importance of the physicochemical context of the polysaccharide Ag in governing the nature of the IgG anti-PS response in vivo.

Disclosures

The authors have no financial conflict of interest.

References


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ANTI-PS RESPONSE TO INTACT BACTERIA

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