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EFFECT OF Haemophilus influenzae POLYSACCHARIDE OUTER MEMBRANE PROTEIN COMPLEX CONJUGATE VACCINE ON MACROPHAGES

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Haemophilus influenzae type b polysaccharide-conjugate vaccines elicit protective antibody responses in young infants. One of these conjugates, polysaccharide linked to outer membrane protein complex (PRP-OMPC), is produced by linking the capsular polysaccharide to an outer membrane protein complex derived from group B Neisseria meningitidis. The outer membrane protein complex contains T cell carrier epitopes that elicit T cell-dependent antibody responses. OMPC also has been shown to increase the antibody response to other proteins administered concurrently that are not covalently linked (i.e., acts as an adjuvant). In this study PRP-OMPC immunized mice demonstrated significant increases in spleen size as well as in splenocyte number as compared to saline controls (p < 0.01, p < 0.001, respectively). No such increase was noted after immunization with another H. influenzae type b-conjugate vaccine, oligosaccharide linked to a variant of diphtheria toxin. By analytic flow cytometry, the mice immunized with PRP-OMPC demonstrated an increase in large splenocytes expressing the Ag Mac-1 (CD11b, CR3). Furthermore, the spleens on histologic examination were characterized by an increase in the red pulp area consisting predominantly of cells of macrophage morphology. By immunohistochemical staining, the cells were identified as macrophages due to expression of Mac-1 and p150,95 (CD11c) Ag. After PRP-OMPC immunization, severe combined immunodeficient mice also demonstrated significant splenomegaly with an increase in macrophages identified by expression of Mac-1 and MHC class II Ag. Thus PRP-OMPC vaccine resulted in T cell-independent splenomegaly with an increase number of macrophages. We propose that this unique property may confer increased immunogenicity to PRP-OMPC through macrophage activation and cytokine release. Furthermore, the effect on macrophages may explain the "adjuvant" capacity of OMPC.

Young children are unable to respond to T cell-independent Ag such as the capsular polysaccharides of HIB,3 Streptococcus pneumoniae, and Neisseria meningitidis (1–4) These encapsulated bacteria are responsible for most of the serious infections of childhood and until recently HIB was the leading cause of meningitis in young children (1). Although passively administered antibody directed to the capsular polysaccharide of HIB clearly protects children, attempts to immunize infants with pure polysaccharide vaccines have failed (1, 5).

More immunogenic vaccines have been produced by linking polysaccharides to protein carrier molecules (i.e., polysaccharide-conjugates) which results in the generation of Th cells that recognize carrier epitopes. In 1990 two conjugated vaccines for HIB were licensed for use in infants at 2 mo of age. One conjugate vaccine, PRP-OMPC, is produced by covalently linking the polysaccharide of HIB to an OMPC derived from group B N. meningitidis. The other vaccine, PRP-CRM197, links oligosaccharide to a variant of diphtheria toxin (CRM197). Both preparations have been shown to be highly efficacious in preventing invasive HIB disease (6, 7). However, the PRP-OMPC vaccine produces measurable responses in infants at 2 mo of age after one immunization whereas PRP-CRM197 does not (6, 8). The cellular mechanism for this early primary response to PRP-OMPC is not defined.

We report that PRP-OMPC results in T cell-independent splenomegaly with an increase number of macrophages. We speculate on the relevance of this PRP-OMPC effect to early primary responses and the significance for future vaccine development.

MATERIALS AND METHODS

Animals and immunization. The 8- to 10-wk-old BALB/c. C3H/HeN, or C.B.-17 SCID/SCID mice (Taconic Animal Farm, Germantown, NY) were immunized i.p. 7 days before the removal of their spleens with PRP-OMPC (Merck Manufacturing Division, Merck and Co. Inc., West Point, PA) containing 15 µg PRP and 250 µg OMPC. PRP-CRM197 (Praxis Biologics, Inc., Rochester, NY) containing 10 µg oligosaccharide and 25 µg CRM197 protein. LPS (Escherichia coli 0:11:B4 LPS, List Biologicals, Campbell, CA) at 50 µg was used and dilluents were 0.9% NaCl or aluminum hydroxide diluent containing 225 µg of aluminum as aluminum hydroxide (Merck Manufacturing Division).

Splenocyte preparations and analytical flow cytometry. Spleens were surgically removed, minced through wire mesh, and placed over Ficoll-Hypaque gradients (LSM, Organon Teknika Co., Durham, NC). Cells were harvested at the interface, washed, and stained with directly conjugated FITC mAb anti-B220 (Clone RA3-6B2, Coulter Immunology, Hialeah, FL) or anti-Mac-1 (Clone M1/70.15, Coulter).

3 Abbreviations used in this paper: HIB, Haemophilus influenzae type b; PRP-OMPC, polysaccharide linked to outer membrane protein complex; PRP-CRM197, oligosaccharide linked to a variant of diphtheria toxin; SCID, severe combined immunodeficiency; GM-CSF, granulocyte-macrophage CSF; MIEP, major immunoenhancing protein.
or unconjugated mAb anti-Thy-1 (Coulter), anti-MHC class II (clone M5/114,15.2, American Type Culture Collection (ATCC), Rockville, MD), anti-33D1 (ATCC) or control rat IgG2a followed by goat anti-rat Ig FITC second antibody (Coulter). Flow cytometric analysis was performed on an EPICS V Dual Laser Cell Sorter (Coulter) with a MDADS data acquisition package.

Immunohistochemical staining of frozen tissue sections. Cut sections of 4- to 6-μ thick were placed on poly-L-lysine-coated slides, fixed in 4% paraformaldehyde, and blocked with Tris saline (1% BSA (fraction V) and 5% normal mouse serum. Primary antibodies used included anti-B220 (RA3-6B2, ATCC), anti-Thy-1 (Becton Dickinson, Lincoln Park, NJ), anti-Mac-1 (Clone M1/70.15, Becton Dickinson), and anti-p150, 95 (clone 8C5, Becton Dickinson). Slides were washed and then incubated with mouse anti-rat Ig alkaline phosphatase (Jackson ImmunoResearch, West Grove, PA), developed with naphthol AS-Mx-phosphate in 0.1 M Tris, pH 8.2, with fast blue (Sigma), and counterstained with nuclear fast red (Sigma). A positive reaction is indicated by blue staining.

NK cell assays. NK cell activity was assayed by51Cr release assay using varying dilutions of effector splenocytes from a mouse immunized 7 days before with PRP-OMPC, PRP-CRM197, saline, or aluminum hydroxide diluent. The cells were mixed with 1 × 106 51Cr-labeled YAC lymphoma target cells in a final volume of 0.2 ml of Dulbecco’s modified Eagle medium supplemented with 10% BSA in V-bottom microtiter plates (Costar, Cambridge, MA). Plates were incubated for 4 h at 37°C in 10% CO2 incubator. 175 ul of cell free supernatant were removed and counted. Total releasable counts were determined by incubating target cells with Triton X100 for 4 h and spontaneous releasable counts were determined by incubating target cells in medium.

Cell proliferations. Splenocytes at 1 × 106 cells/0.2 ml in DMEM 10% FCS with penicillin (100 U/ml) and streptomycin (100 μg/ml) were incubated in 96-well microtiter round bottom wells (Costar) with 4 μg/ml Con A or medium for 72 h at 37°C in 10% CO2 incubator. During the last 12 h each sample was pulsed with 1 μCi of [3H]Tdr and then harvested onto glass fiber strips by using a cell harvester. The amount of incorporated [3H]Tdr was assessed by liquid scintillation counting and the results are expressed as the mean proliferation index of quadruplicate samples.

Mouse Ig ELISA. Serum IgG was measured by ELISA using mouse anti-mouse IgG capture antibody (Tago, Burlingame, CA) and goat anti-mouse IgG alkaline phosphatase conjugate (Tago). The assays were sensitive to approximately 1 ng/ml of mouse Ig.

RESULTS

Spleen size after immunization. We determined the spleen size of BALB/c mice 7 days after immunization with PRP-OMPC vaccine, PRP-CRM197 vaccine, or saline. The length and width of spleens were measured after surgical removal and the surface areas (length cm × width cm) were compared (Fig. 1A). The geometric mean surface area of 12 spleens of mice immunized with PRP-OMPC was significantly greater than that of the 11 saline controls (p < 0.01). The spleens of nine mice immunized with PRP-CRM197 vaccine were not larger than those of controls. To determine if the effect was due to the aluminum hydroxide diluent of PRP-OMPC vaccine, mice received injections with aluminum hydroxide diluent alone and no significant increase spleen size was demonstrated (Fig. 1A). In addition, mice received injections with PRP-CRM197 mixed with aluminum hydroxide diluent and no size increase was noted. Two mice were immunized with 50 μg of LPS and the surface area was the same as saline controls. Finally, mice received injections with PRP-OMPC vaccine diluted in saline and a significant increase of spleen size was again noted as compared to saline controls (p < 0.05) although the size increase was less than that observed with PRP-OMPC administered in aluminum hydroxide.

Spleocyte number after immunization. We also examined the number of splenocytes obtained from individual spleens. Mice immunized with PRP-OMPC demonstrated significantly increased numbers of splenocytes as compared to saline controls (Fig. 1B) (p = 0.0001). A significant increase in number of splenocytes was also noted for mice receiving PRP-OMPC in saline although the number of splenocytes were less than those immunized with PRP-OMPC in aluminum hydroxide diluent. Again no significant increases were noted for mice receiving PRP-CRM197 vaccine, LPS, or aluminum hydroxide diluent alone.

Analytic flow cytometry and immunohistochemical staining. By flow cytometry we examined the splenocytes after immunization to determine the cell type[s] that were increased. The cell size distribution were compared for mice of each group by determining 90° angle light scatter vs forward angle light scatter by flow cytometry. The 90° scatter is indicative of granularity and forward scatter is increased by size. Thus large cells with increased granularity are located in the upper right hand quadrant. Mice immunized with PRP-OMPC demonstrated an increased number of large cells as compared to saline or PRP-CRM197 immunized controls (Fig. 2). The splenocytes were stained for surface markers of B cells (B220), T cells (Thy-1), dendritic cells (33D1), and macrophages (Mac-1). Consistently, mice immunized with PRP-OMPC had an increased percentage of cells expressing Mac-1 Ag as compared to saline or PRP-CRM197 immunized controls (Fig. 3). PRP-OMPC immunized mice also demonstrated a consistent decrease in the percentage of cells expressing B220 and Thy-1 (Fig. 3). No increase in 33D1 expression was noted suggesting the cells were not dendritic cells (data not shown) (9). Mice immunized with aluminum hydroxide diluent, PRP-CRM197 vaccine in saline, or PRP-CRM197 vaccine in aluminum hydroxide, did not demonstrate increases in Mac-1 expression as compared to controls (data not shown).

To confirm the flow cytometry results we examined the spleens histologically and by an immunohistochemical method stained frozen sections for B cell (B220), T cell (Thy-1), and macrophage-specific (Mac-1 and p150, 95) cell surface protein expression. Mice immunized with PRP-OMPC had a marked decrease in the number of splenic follicles with an increase in the red pulp area as compared to controls (Fig. 4, a and c). An overall decrease
in the number of B cells and an increase in Mac-1+ cells in the red pulp area were noted (Fig. 4, c and g). A decrease in the number of Thy-1+ cells was also noted (data not shown). The splenocytes were stained with B220 mAb that recognizes p150.95 Ag (CD11c) found on macrophages and granulocytes. The PRP-OMPC-immunized mice demonstrated an increase in CD11c+ cells as compared to the saline injected control (Fig. 4, d and h). In addition to macrophages, an increase number of granulocytes and megakaryocytes were noted in the PRP-OMPC-immunized mice (Fig. 4e).

Functional assays. Inasmuch as the relative number of B cells and T cells were decreased after immunization with PRP-OMPC, we examined the functional activity of the remaining T cells. To control for differences in number of T cells, we first nylon wool-depleted splenocytes obtained from mice immunized with either PRP-OMPC, PRP-CRM197, or saline. Proliferation assays were then performed on the T cell-enriched populations and no functional impairment of the T cells from PRP-OMPC-immunized mice was observed (Fig. 5). NK activity was examined using splenocytes for mice immunized with PRP-OMPC, PRP-CRM197, or saline. No purification for NK cells was used and thus the assay for NK cell activity had limited sensitivity. No increase in NK activity was shown although those injected with poly-IC (an IFN inducer) demonstrated increased killer activity as expected (data not shown).

Effect of booster dose, alteration of dose, and mouse strain. To determine if the effect of PRP-OMPC on increasing spleen size was transient, we examined spleens at day 21 as well as at day 28 after a booster dose at day 21. Mice immunized with PRP-OMPC had increased spleen size 21 days after the primary dose and at 28 days after a booster dose as compared to saline controls (data not shown). The effect was observed at a 1/5 and 1/10 dilution of the PRP-OMPC dose, but not at a 1/100 dilution (0.15 µg PRP and 2-25 µg OMPC). Mice of a different mouse strain, C3H/HeN (H2h), also had an increase in spleen size with a mean surface area of 156 after PRP-OMPC immunization as compared to an area of 42 for both saline and PRP-CRM197-immunized controls.

SCID mice. To further document that the macrophage was the cell type expanded after immunization, as well as to determine the role of T cells, mice homozygous for the SCID defect were immunized with PRP-OMPC, aluminum hydroxide diluent, or saline. The PRP-OMPC injected SCID mice demonstrated increased spleen size and cell number (Fig. 6). Furthermore, when the splenocytes were examined by flow cytometry, an increase in Mac-1 and MHC class II expression occurred after immunization with PRP-OMPC vaccine (Fig. 7). As expected, no cells expressing B220 (B cells) were noted although a small percentage of Thy-1+ cells were observed. Others have also noted Thy-1+ cells in SCID mice (10). After PRP-OMPC immunization, the Thy-1+ cells could no longer be detected (data not shown). To document that the mice with increased spleen size had no lymphocyte function, mouse serum Ig was measured by ELISA. The three SCID mice with increased spleen size all had less than 0.010 µg/ml of serum IgG with a control BALB/c mouse serum measuring 125 µg/ml.

In this study, mice immunized with PRP-OMPC vaccine developed marked increases in spleen size and splenocyte number that were not observed after immunization with another ribb-conjugate vaccine, PRP-CRM197. By analytic flow cytometry and immunohistochemical methods we demonstrated that the increased cell population consisted of large cells expressing Mac-1 and p150.95 Ag and the cell morphology was characteristic of splenic macrophages. An increase in the number of granulocytes and megakaryocytes was also noted but not quantitated. The PRP-OMPC effect on macrophages was enhanced by administration of PRP-OMPC vaccine with the supplied aluminum hydroxide diluent. Finally, SCID mice developed splenomegaly with an increase in Mac-1 and MHC class II expression after PRP-OMPC immunization. Thus, the macrophage effect occurred via a T cell-independent pathway.

Macrophages express the CD11-CD18 family of integrins including LFA-1, Mac-1 (CR3), and p150.95. Each of these cell surface molecules consists of a common β chain (CD18) and an unique α chain (CD11a, 11b, 11c, respectively) expressed as a heterodimer (12). In addition, macrophages express MHC class II molecules with splenic macrophages having a high level of expression as compared to alveolar macrophages (13). Thus, the finding of
Figure 3. Flow cytometric analysis of splenocytes from BALB/c mice immunized with PRP-OMPC vaccine. Splenocytes were stained from mice immunized 7 days before removal of spleen with saline, PRP-CRM197, or PRP-OMPC vaccine. The solid line represents staining with an IgG2a rat control antibody followed by a goat anti-rat Ig FITC antibody. The dotted line represents staining for B cells (B220), T cells (Thy-1) and macrophages (Mac-1). B220 and Mac-1 antibodies were directly FITC-labeled reagents.

Figure 4. Histology and immunohistochemical staining of spleens from mice immunized with PRP-OMPC vaccine or saline. Spleen sections from mice immunized with saline are shown stained with H&E (a), by immunohistochemical staining for B220 (b), Mac-1 (c), and p150.95 Ag (d). Spleen sections from mice immunized with PRP-OMPC vaccine are shown stained with H&E (e), for B220 (f), Mac-1 (g), and p150.95 Ag (h). Positive staining of cells is indicated by black. The arrows in b demonstrate the positive staining of B cells expressing B220 Ag in the marginal zone of a follicle. The m in e identifies a megakaryocyte within the splenic red pulp.

Figure 5. Proliferation of T cells from mice immunized with PRP-OMPC vaccine. The geometric mean cpm (±SEM) for nylon wool-depleted splenocytes from one mouse immunized with either saline, PRP-CRM197, or PRP-OMPC vaccine are displayed for two experiments. The open bars represent cells incubated with media and the solid bars represent cells incubated with Con A.

increased Mac-1 and p150.95 expression by flow cytometry as well as the results of immunohistochemical staining identify the cells as macrophages.

Macrophage activation and proliferation occur in response to cytokines including IFN-γ, IL-1, IL-3, CSF-1, TNF-α, and GM-CSF (13-17). These cytokines are produced by fibroblasts and endothelial cells (GM-CSF, CSF-1), by T cells (IFN-γ, IL-3, CSF-1, GM-CSF), NK cells (IFN-γ, GM-CSF, IL-3), and macrophages (TNF-α, IL-1, GM-CSF, CSF-1) (15, 18-21). Macrophage activation occurs through both T cell-dependent and T cell-independent pathways (13). The T cell-independent pathway has been recently defined in the SCID mouse model of chronic infection with *Listeria monocytogenes* (13). Mice homo-
zygous for the recessive SCID gene have an almost complete absence of functional T and B cells due to lack of somatic rearrangement of either Ig or TCR genes (10). A small percentage of splenic cells do express Thy-1 although these cells may be myeloid precursors (10, 22). Even though SCID mice lack functional T and B cells, they do have normal NK and macrophage cell number and function (23). In SCID mice T cell-independent activation of macrophages by *Listeria* organisms requires both IFN-γ (produced by NK cells) and IL-1 (13, 14). Those studies suggest that after an infection, macrophages are stimulated to release TNF and other product(s). NK cells are then activated to produce IFN-γ which results in increased MHC class II expression by the SCID macrophages (24).

In our study, PRP-OMPC immunization of SCID mice resulted in an increase number of splenic macrophages. Although we cannot exclude the possibility that macrophages were recruited to the spleen, we propose that macrophage proliferation occurred. Inasmuch as NK cells can mediate macrophage activation, we suggest that T cell-independent proliferation can occur as well. TNF-α and CSF-1 (produced by macrophages) and GM-CSF (produced by macrophages and NK cells) have all been shown to induce macrophage proliferation in vitro (15-18). In addition, the increased number of granulocytes and monocytes could result from NK-derived cytokines, as IL-3 and GM-CSF stimulate progenitors of these cell types (20, 25, 26).

The components of PRP-OMPC vaccine that elicit macrophage proliferation are not defined in this study. Recently, the major membrane protein of OMPC has been identified as the class 2 porin protein (27). This protein has been termed MIEP and contains Th cell carrier epitopes. MIEP was also found to be mitogenic for both human and mouse lymphocytes (27). When purified MIEP was conjugated to polysaccharide, the PRP-MIEP conjugate vaccines demonstrated immunogenicity similar to PRP-OMPC preparations. However, MIEP did not retain the adjuvancy characteristics of OMPC. When OMPC was administered with a nonlinked soluble protein (e.g., tetanus toxoid), an increase response to the protein occurred. In contrast, mixture with MIEP did not result in an increase response to the protein. Thus, components of OMPC other then MIEP may be important in generating protective responses to PRP-OMPC.

MIEP could be the component of the vaccine that induced increases in the number of macrophages. However, the decrease in T cell and B cell percentages noted in the spleens, is not suggestive of a lymphocyte mitogenic response. Furthermore, because the effect on macrophages occurred in a T cell-independent fashion, a MIEP-induced lymphocyte secondary effect can be excluded. OMPC also contains small amounts of LPS and liposomal structures of other lipids (28). Macrophages are known to bind LPS and other lipids through Mac-1 and Mo-2(CD 14) molecules (28, 29). We doubt that LPS in PRP-OMPC is inducing macrophage proliferation because LPS derived from *Escherichia coli* at a dose of 50 µg had no effect on splenic macrophage number or spleen size in our study. However, we did not study meningococcal LPS and thus cannot completely exclude an LPS effect. Alternatively, cytokine release from macrophages may occur after exposure to so called “super Ag” such as staphylococcal exotoxin B and toxic shock syndrome toxin (30). These Ag cause T cell proliferation through direct binding to TCR (31). If PRP-OMPC acted by a similar mechanism, one might expect an increase in T cell numbers that was not observed in our study. Thus the mechanism for T

![Figure 6](image1.png)

**Figure 6.** Spleen size and cell number after immunization with PRP-OMPC vaccine in SCID mice. A. Geometric mean surface area of spleens (+SEM) from four SCID mice immunized with saline and four SCID mice immunized with PRP-OMPC vaccine. B. geometric mean cell number (+SEM) obtained per spleen as described in A.

![Figure 7](image2.png)

**Figure 7.** Mac-1 and MHC class II expression by flow cytometry of SCID mice after immunization with PRP-OMFC vaccine or saline. The darker lines represent cells stained with Mac-1 or MHC class II mAb and the lighter lines represent cells stained with rat IgG2a control antibody.
cell-independent PRP-OMPC induced macrophage proliferation remains unknown.

The T cell-independent effect of PRP-OMPC on murine splenic macrophages may have relevance to the immune response to polysaccharide conjugate vaccines in children. PRP-OMPC produces a response characterized by IgGl and IgM antipolysaccharide antibodies. IgGl and IgM responses to polysaccharide immunizations are present in mice with severe combined immunodeficiency (SCID) mice. Nature 301:327.


