Mechanisms of Monophosphoryl Lipid A Augmentation of Host Responses to Recombinant HagB from Porphyromonas gingivalis

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Porphyromonas gingivalis, a gram-negative, black-pigmented anaerobe, is among the microorganisms implicated in the etiology of adult periodontal disease. This bacterium possesses a number of factors, including hemagglutinins, of potential importance in virulence. Our laboratory has shown the induction of protection to P. gingivalis infection after subcutaneous immunization with recombinant hemagglutinin B (rHagB). The purpose of this study was to determine if humoral antibody responses are induced after intranasal (i.n.) immunization of rHagB and if monophosphoryl lipid A (MPL), a nontoxic derivative of the lipid A region of lipopolysaccharide, acts as a mucosal adjuvant and potentiates responses to rHagB. Further, the effects of MPL on the nature of the response to HagB and on the costimulatory molecules B7-1 and B7-2 on different antigen-presenting cells (APC) were evaluated. Groups of BALB/c mice were immunized three times (2-week intervals) by the i.n. route with HagB (20 µg) alone or with MPL (25 µg). A group of nonimmunized mice served as control. Serum and saliva samples were collected prior to immunization and at approximately 2-week intervals and evaluated for serum immunoglobulin G (IgG) and IgG subclass and for salivary IgA antibody activity by enzyme-linked immunosorbent assay. Mice immunized with rHagB plus MPL had significantly higher salivary IgA (P < 0.05) and serum IgG (P < 0.05) anti-HagB responses than mice immunized with rHagB alone. The IgG1 and IgG2a subclass responses seen in mice immunized with rHagB plus MPL were significantly higher (P < 0.05) than those seen in mice immunized with rHagB only. Further, the IgG2a/IgG1 ratio in the latter group was ~1, whereas in mice immunized with rHagB plus MPL the ratio was <1. These results provide evidence for the participation of T helper (Th) 1 and Th2 cells in responses to rHagB and that MPL potentiates a type 2 response to HagB. MPL was also shown to preferentially up-regulate B7-2 expression on B cells, whereas a preferential increase in B7-1 costimulatory molecule was seen on macrophages and dendritic cells. These results provide evidence that MPL exerts a differential regulation in the expression of costimulatory molecules on APC.

Periodontal disease is the result of interactions between periodontal pathogens such as Porphyromonas gingivalis and the host’s immune system. Interest in developing a vaccine against periodontitis has recently increased not only because about 25% of the adult population is affected by this infectious disease but also because of the possibility of an association between periodontitis and systemic diseases (3, 9, 48). Immunization studies with P. gingivalis whole cells or purified antigens in animal models have provided encouraging results that indicate a vaccine can be developed to protect against periodontal disease (27, 47, 54, 60).

Several virulence antigens of P. gingivalis have been identified, such as fimbriae, hemagglutinins, lipopolysaccharide (LPS), and proteases (23). The fimbriae and hemagglutinins appear to be involved in the attachment of P. gingivalis to host tissues (11, 22, 32, 51, 61). A number of hemagglutinins have been identified and their genes have been cloned (16, 36–38, 50, 51). Although evidence for a direct role of the hemagglutinins in host tissue binding has not yet been demonstrated, we have previously shown in an experimental rat model that systemic immunization with recombinant hemagglutinin B (rHagB) results in protection from P. gingivalis infection (27). These results suggest a role for HagB in periodontal disease pathogenesis.

Vaccines consisting of antigen alone are often not very effective in inducing the desired immune responses. Therefore, adjuvants are commonly used to enhance the host response to the vaccine antigen. Adjuvants can alter the avidity, affinity, kinetics, and specificity of the antibody response to the antigen, as well as affecting cell-mediated immunity (12, 25). Thus, it is essential to elucidate the cellular mechanisms by which adjuvants modulate host responses to an antigen. Monophosphoryl lipid A (MPL) is a detoxified derivative of the LPS of Salmonella enterica serovar Minnesota R595 that lacks the endotoxic properties but retains both the adjuvant and immunostimulatory activities of the parent LPS (5, 19, 46). Studies in humans have shown that systemic coadministration of MPL and antigen results in an increased immune response to the specific antigen without causing toxicity (56, 58). Although most studies with MPL have involved the systemic route of immunization, it has recently been shown to also be a mucosal adjuvant (2, 8, 42, 53). However, the mechanism(s) involved in MPL adjuvanticity has not been fully defined. MPL has been shown to induce interleukin-12 (IL-12) protein and IL-10 mRNA production (43, 52). It has also been suggested to exert an effect on the costimulatory molecules B7-1 (CD80) and B7-2.

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(CD86), i.e., to induce B7-1 but not B7-2 expression on monocytes (10).

T-cell activation requires the recognition of the T-cell receptor (TCR) with the major histocompatibility complex (MHC)-peptide complex on antigen-presenting cells (APC) and the interaction between costimulatory molecules on APC and their respective receptors on T cells (35, 35). The receptor CD28 on T cells interacts with the costimulatory molecules B7-1 and B7-2 on APC (1, 40, 41). In the absence of costimulation, antigen-specific hyporesponsiveness, clonal T-cell anergy, or apoptosis may occur (6, 41). The CD28 receptor and the B7 ligands are type I transmembrane glycoproteins and are members of the immunoglobulin superfamily (20). Both B7-1 and B7-2 are induced by cell activation; however, they respond to different stimuli and exhibit different expression kinetics and receptor binding properties (26, 39). These molecules are mainly expressed on monocytes, dendritic cells, and activated B cells. The B7-1 and B7-2 costimulatory molecules have been shown to influence the immune response by skewing the CD4+ cell response towards either a Th1 or Th2 cell differentiation. Studies in a murine experimental autoimmune encephalitis model have shown that antibodies to B7-1 increased IL-4 (Th2 derived), while antibodies to B7-2 enhanced gamma interferon (IFN-γ) (Th1 derived) production (31). Moreover, in vitro studies with human T cells have shown that B7-2 transfectants preferentially activate type 2 cytokines, whereas B7-1 transfectants produced type 1 cytokines (14). However, other investigations have found that both B7-1 and B7-2 were involved in IL-4 and IFN-γ production in the mouse system (33).

The purpose of the present study was to determine the effectiveness of MPL as a mucosal adjuvant in potentiating a type 1 immune response (14). However, other investigators preferentially activated type 2 cytokines, whereas B7-1 transfectant produced type 1 cytokines (31). Moreover, in vitro studies with human T cells have shown that B7-2 transfectants preferentially activate type 2 cytokines, whereas B7-1 transfectants produced type 1 cytokines (14). However, other investigations have found that both B7-1 and B7-2 were involved in IL-4 and IFN-γ production in the mouse system (33).

**MATERIALS AND METHODS**

**HagB purification.** HagB was purified as previously described (29) with some modifications. Briefly, the *hagB* gene was cloned from *P. gingivalis* 381 into a PET vector with a lac promoter and histidine tag and expressed in *Escherichia coli* JM 109 (kindly provided by Ann Progulske-Fox and Thomas Brown, University of Florida, Gainesville). A culture of *E. coli* (30 ml) was grown overnight at 35°C in Luria-Bertani (LB) broth containing carbenicillin and kanamycin. The overnight culture was transferred to LB broth (2 liters) with antibiotics and incubated at 35°C with vigorous shaking. When the concentration of bacteria reached an optical density of 600 nm of 0.6, 0.36 mM isopropl-β-D-thiogalactoside was added and the culture was incubated for an additional 3 h. The culture was centrifuged and the pellet was resuspended in 5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9, and stored at −70°C. The next day, the cells were thawed at room temperature and sonicated (10 s) three times at 5-s intervals. The supernatant was allowed to stand overnight at 4°C. The blood samples were collected from the retroorbital plexus by using heparinized capillaries. The supernatant was obtained after centrifugation. All experimental samples were stored at −70°C until assayed.

**Anti-rHagB antibody (27).** The concentration of rHagB was estimated by the bicinchoninic acid protein determination assay (Pierce, Rockford, Ill.), using bovine serum albumin (BSA) as standard.

**MPL preparation.** MPL-AF, an aqueous formulation containing MPL at a 4:1 molar ratio with dipalmitoylphosphatidyl choline and water, was obtained from Corixa Corporation (Hamilton, Mont.) and is referred to as MPL in this study.

**Experimental design.** Normal BALB/c mice used in this study were obtained from Corixa Corporation (Hamilton, Mont.) and is referred to as MPL in this study.

Groups of normal female BALB/c mice (6 to 8 weeks old; six mice/group) were immunized three times on days 0, 14, and 28 by the i.n. route with either rHagB alone (20 μg group A) or with a combination of MPL and rHagB (20 μg MPL plus 25 μg rHagB group B). A group of nonimmunized mice (group C) served as control. The vaccine was applied slowly into each nare. Serum and saliva samples were collected prior to immunization and at approximately 2-week intervals following the initial immunization. Briefly, saliva samples (100 μl) were collected over a 20-min interval after stimulation of the saliva flow by intraperitoneal injection of carbachol (5 μg in 0.05 ml). Saliva samples were clarified by centrifugation at 16,000 × g for 10 min at 4°C. The blood samples were collected from the retroorbital plexus by using heparinized capillaries. The sera were obtained after centrifugation. All experimental samples were stored at −70°C until assayed. The levels of sera IgG, IgG subclasses, and salivary IgA anti-HagB antibody activity were determined by enzyme-linked immunosorbent assay (ELISA).

**Evaluation of antibody responses.** Antibody activity to rHagB in serum and saliva samples was assessed by ELISA as previously described (24, 27). Briefly, individual flat-bottom Maxisorp microtiter plates (Nunc International, Roskilde, Denmark) were coated with rHagB (1 μg/ml) or with optimal amounts of goat antimouse a or γ heavy chain antibody or affinity-purified goat anti-mouse IgG1 or IgG2a (Southern Biotechnology Associates, Inc., Birmingham, Ala.) in borate buffer saline (BBS; 100 mM NaCl, 50 mM boric acid, 1.2 mM Na2B4O7, pH 8.2). Non-specific binding sites were blocked with 1% BSA and 0.1% sodium azide in BBS for 1 h at room temperature. From a starting dilution of serum (1:100 [groups A and C] and 1:500 [group B]) for IgG, and 1:500 for IgG1 and 1:500 for IgG2a subclasses) or saliva (1:10 for specific or 1:100 for total IgA) prepared in BBS containing 1% BSA and 0.1% sodium azide, five twofold dilutions were added in duplicate to individual wells. After incubation (overnight at 4°C) and washing of plates, horseradish peroxidase-conjugated goat anti-mouse IgA, IgG, or IgG subclass antibody (Southern Biotechnology Associates, Inc.) was added to appropriate wells. After 4 h of incubation at room temperature, plates were washed and o-phenylenediamine substrate with hydrogen peroxide was added to wells. Color development was recorded at 490 nm. The concentrations of anti-rHagB antibodies in all serum and saliva samples were determined by interpolation on standard curves generated using a mouse immunoglobulin reference serum (ICN Biomedicals, Costa Mesa, Calif.) and constructed by a computer program based on a four-parameter logistic algorithm (Softmax/Molecular Devices Corp., Menlo Park, Calif.). Data were logarithmically transformed and statistical analysis was done by using the InStat program (GraphPad Software, San Diego, Calif.). The data were transformed and presented as the geometric mean ± the standard error of the mean (SEM) for ease of interpretation.

**Proliferative responses.** Single-cell suspensions were prepared from spleens obtained from normal BALB/c mice. Erythrocytes were removed by suspending the cells in lysis buffer (buffer ammonium chloride) for 5 min at 4°C. The spleen cells were then washed three times with Hank’s balanced salt solution (Cellgro Mediatech, Washington, D.C.). In addition, purified B cells were isolated from spleen cell suspensions by using CD43 MicroBeads (Miltenyi Biotec, Sunnyvale, Calif.). CD43 MicroBeads were incubated with the cell suspension for 15 min at 6°C in PBS containing 0.05% BSA and 2 mM EDTA. The cell suspension was then washed by adding a 20-fold excess of PBS containing 0.5% BSA and 2 mM EDTA. After centrifugation, cells were resuspended in 1 ml of PBS-0.5% BSA and added to the magnetic depletion column. CD43 B cells were eluted with 15 ml of PBS containing 0.5% BSA and 2 mM EDTA. Cells were then washed twice in PBS. The viability of the spleen cells and the purified B cells was assessed by trypan blue exclusion. The cells were then suspended in complete medium (RPMI 1640 [Cellgro] supplemented with 10% fetal bovine serum [FBS], 50 μM 2-ME, 0.1% sodium pyruvate, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 10 mM HEPES, 50 μg of gentamicin/ml, 50 U of penicillin/ml, and 50 μg of streptomycin/ml) and added to sterile 96-well flat-bottom plates (Falcon Labware, Oxnard, Calif.) in triplicate at a concentration of 4 × 10^5 spleen cells or 2 × 10^5 B cells/well. Cells were incubated (37°C, 5% CO2) with or

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without various concentrations of MPL (0.001, 0.01, 0.1, 1, 2.5, 5, and 10 μg/ml) for 48 h (a single-cell suspension) or 72 h (B cells). Cultures were pulsed with [3H]Tdr (Amersham Corp., Arlington Heights, Ill.) (0.5 μCi/well) during the last 18 to 20 h of incubation. Cells were harvested onto a glass fiber filter with a MASH II cell harvester (Microbiological Associates, Walkersville, Md.), and the amount of [3H]Tdr incorporation was measured in a liquid scintillation counter.

**Flow cytometry.** Single spleen cells, prepared as described above, were cultured at a concentration of 10^6 cells/ml in sterile 24-well plates (Falcon Labware) for 48 h in the presence or absence of MPL (0, 0.01, 0.1, 1, and 10 μg/ml). Cells were harvested by low-speed centrifugation and suspended in fluorescence-activated cell sorter (FACS) buffer (PBS containing 1% FBS and 0.1% NaCl). The B cells (B220^+^) and macrophages (CD11b^-^) were assessed for the presence of B7-1 (CD80) and B7-2 (CD86). Briefly, aliquots of the cell suspension (10^6^ cells) were incubated with allopolyocyanin-conjugated anti-B220 or -CD11b antibody for 30 min on ice. The cells were washed twice with FACS buffer and then aliquots of each preparation were incubated with fluorescein isothiocyanate (FITC)-labeled anti-CD86 or phycoerythrin (PE)-labeled anti-CD80 antibody (Caltag Laboratories, Burlingame, Calif.) for 30 min on ice. The cells were washed, suspended in 2% paraformaldehyde, and analyzed using a FACStar flow cytometer (Becton Dickinson, Mountain View, Calif.). The B220^-^ cells were further divided into B220^-^B1 and B220^-^B2, since differences have been observed among these cell populations (45).

For the preparation of enriched dendritic cell cultures, spleens were teased apart using a 21-gauge needle and incubated with 1 mg of collagenase/ml and 20 μg of DNase/ml in complete RPMI 1640 medium for 30 min at 37°C. The single-cell suspension was then passed through a wire mesh (200 μm) and the leukocyte population was isolated by Histopaque (1083). The cells were washed three times with Hank's balanced salt solution, suspended in complete medium, and then plated in polypropylene tubes at ~2 × 10^6^ cells/ml with or without MPL (2.5 μg/ml) for 24 h. The dendritic cells (CD11c-allophycocyanin conjugated; BD Pharmingen) were assessed for the presence of B7-1 (CD80) and B7-2 (CD86) as described above.

In vitro functional assay. Single spleen cell suspensions, prepared as described above, were separated into CD4^-^ T cells and a T-cell-depleted population of APC. The CD4^-^ cells were purified by using magnetized polystyrene Dynabeads coated with rat anti-mouse CD4 (L3T4) antibody (Dynal ASA, Oslo, Norway). Cells were incubated with the Dynabeads for 20 min at 4°C. Positive selection of CD4^+^ cells was done with the Dynal magnetic particle concentrator. The resulting CD4^-^ cell population was then washed three times in PBS containing 1% FBS and 2 mM EDTA. The purified CD4^-^ cells were detached from the beads with Detachabead mouse CD4 antigen provided by the manufacturer, washed three times, and suspended in complete medium. The remaining spleen cell suspension was further depleted of T cells by incubation with Dynabeads coated with mouse pan T antibody (Thy 1.2). This T-cell-depleted population of APC was incubated with or without MPL (1 μg/ml) for 24 h, washed with PBS, and fixed with 0.5% paraformaldehyde. The APC (10^6^ cells/ml) were then cocultured with the purified CD4^-^ cells (0.5 × 10^9^/ml), suboptimal amounts of anti-CD3 antibody (100 ng/ml), and with or without anti-B7^-1 or anti-B7^-2 (1 μg/ml) for 5 days (37°C, 5% CO2). Cultures were pulsed during the last 18 to 20 h of incubation with [3H]Tdr, harvested, and the amount of [3H]Tdr uptake was determined using a scintillation counter.

**Statistics.** Statistical analysis (analysis of variance and Student’s t test) of differences between groups were determined using the InStat computer program (GraphPad Software).

**RESULTS**

**Specific serum responses.** We were initially interested in determining the effectiveness of MPL as a mucosal adjuvant in potentiating responses to rHagB. Therefore, groups of mice were immunized by the i.n. route with rHagB plus MPL or rHagB alone and samples of serum and secretion were collected at various times. Serum IgG anti-HagB responses were seen in mice by week 4 following the initial immunization with rHagB and MPL (group B), and they were significantly higher (P < 0.05) than those observed in mice immunized with rHagB alone (Fig. 1). The response in group B mice persisted through week 10 (Fig. 2C). The presence of IgG2a and IgG1 antibodies suggest the participation of Th1 and Th2 cells. It has been shown that MPL potentiated a Th2-like response to HagB. The results further demonstrate the effectiveness of the mucosal route of immunization, i.e., the i.n. route, in inducing serum responses.

**Specific salivary responses.** In order to determine the effectiveness of rHagB in inducing mucosal IgA responses following i.n. immunization and of MPL in potentiating these responses, individual saliva samples collected throughout the experiment were assessed for the level of IgA antibody activity to rHagB.
Mice immunized with rHagB and MPL (group B) had significantly higher \((P < 0.05)\) salivary IgA anti-HagB responses than those seen in mice immunized with rHagB alone (Fig. 3). The response peaked at 4 and 8 weeks following the initial immunization and persisted through week 12. Essentially no salivary IgA antibody activity was seen in mice immunized with rHagB alone. These results indicate that MPL has the ability to augment the salivary response to HagB antigen following i.n. immunization, and they thus provide evidence that MPL is an effective mucosal adjuvant.

**Cell stimulation by MPL.** In order to determine the mechanism of MPL adjuvanticity, we next investigated the ability of MPL to stimulate murine spleen cell proliferation. Murine spleen cells or purified splenic B cells were incubated with various concentrations of MPL and pulsed with \(^{3}H\)TdR for the last 18 to 20 h of a 48- or 72-h incubation, respectively. MPL was a potent inducer of cell proliferation (Fig. 4). A stimulation index of 5 was seen in spleen cell cultures incubated with only 10 ng of MPL/ml (Fig. 4A). An approximately fivefold increase was seen with 1 \(\mu\)g of MPL/ml. Maximum stimulation was observed in cultures incubated with 2.5 \(\mu\)g of MPL/ml. Purified B cells were also stimulated by MPL in a dose-dependent manner (Fig. 4B). Maximum stimulation was seen with 10 \(\mu\)g of MPL/ml. The higher sensitivity of spleen cells compared to purified B cells to MPL stimulation is likely due to the various types of APC present in the former cultures. These results provided evidence that MPL, like the parent LPS molecule, is a potent stimulator of cell proliferation.

**FACS analysis.** The costimulatory molecules B7-1 and B7-2 play an important role in T-cell activation. Therefore, we next assessed by FACS analysis the splenic B-cell and macrophage populations for changes in B7 expression following stimulation with MPL. Analysis of the B220\(^{−}\)low and B220\(^{−}\)high B-cell subpopulations revealed no or only slight changes in the mean fluorescence intensity of B7-1 and B7-2 expression on the B220\(^{−}\)low B cells (Table 1). However, an MPL dose-dependent increase was seen in the expression of B7-2 on the B220\(^{−}\)high subpopulation. The mean fluorescence intensity of B7-1 expression on this cell population decreased with increasing amounts of MPL. Since the mean fluorescence intensity of B7-1 on B220\(^{−}\)low cells was lower than that of B7-2 with and without MPL stimulation and since an increase in mean fluorescence intensity was only seen in relation to B7-2 expression on the B220\(^{−}\)high subpopulation of cells, it appears that the adjuvant MPL preferentially up-regulates B7-2 expression on B cells. The percentage of cells expressing either B7-1 or B7-2 costimulatory molecules increased only in the B220\(^{−}\)low B-cell subpopulation after MPL stimulation.

Analysis of the CD11b\(^{+}\) splenic macrophage population revealed an increase in the mean fluorescence intensity of B7-2 and especially B7-1 costimulatory molecules after MPL stim-

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**FIG. 2.** Time course of the serum anti-HagB IgG1 (A) and IgG2a (B) responses and the IgG2a/IgG1 ratios (C) in mice immunized by the i.n. route with rHagB or rHagB plus MPL. Values for serum IgG1 and IgG2a antibody activities are expressed as the geometric mean \(\times / \pm\) SEM. *, value is significantly different \((P < 0.05)\) from those obtained in the HagB-only immunized group.
ulation (Table 2). Furthermore, analysis of the CD11c+ dendritic cell population following stimulation with MPL revealed an increase in the mean fluorescence intensity of B7-1 (683) and B7-2 (1,486) expression above that seen in unstimulated cultures (B7-1, 397; B7-2, 726). These results demonstrate that MPL can stimulate B7-1 and B7-2 on macrophages, as has been shown for LPS (18, 28). Taken together, our results suggest a differential regulation of B7-1 and B7-2 on B cells and on macrophages and dendritic cells by MPL. That is, MPL induced an increase in B7-2 but not B7-1 expression on B cells (B220+ high), whereas an increase was especially seen in B7-1 expression on macrophages.

Assessment of cocultured cells. Although FACS analysis indicated that MPL stimulation affects the expression of the costimulatory molecules B7-1 and B7-2, we wanted to further assess the importance of this effect of MPL in a functional assay using cocultures of APC and T cells. The addition of anti-B7-1 or -B7-2 antibody to cultures of APC and T cells resulted in a 73 or 81% reduction in proliferative responses, respectively, compared to control cultures (Fig. 5). These results indicate the involvement of B7-1 and especially B7-2 in the immunoadjuvant effect of MPL in this system.

**DISCUSSION**

With the advent of more sophisticated and refined techniques, the current trend is to use purified recombinant, subunit, or synthetic antigens for mucosal vaccine development. However, these antigens when given alone are generally poorly immunogenic. Therefore, the success of a future mucosal vaccine will be contingent upon the use of an appropriate adjuvant. MPL is a promising adjuvant for vaccine development. MPL is a nontoxic derivative of LPS which has retained the ability to enhance immune responses (58). In the present study, we investigated the immunoadjuvant properties of MPL in conjunction with the *P. gingivalis* hemagglutinin antigen.
HagB as a potential mucosal vaccine against periodontal disease. Furthermore, we have investigated the role of the co-stimulatory molecules B7-1 and B7-2 on APC in the adjuvant activity of MPL. We have demonstrated that i.n. immunization of mice with rHagB and MPL resulted in a significantly higher serum IgG anti-HagB response than that seen in mice immunized with antigen alone. Furthermore, the level of specific salivary IgA antibody observed was also significantly higher when rHagB was given with MPL. These data demonstrate that MPL is an effective adjuvant when coadministered with the potential virulence antigen HagB. To our knowledge this is the first study in which MPL has been used in combination with an antigen derived from a periodontal pathogen.

Previous studies have provided evidence suggesting that MPL can act as a mucosal adjuvant with various potential vaccine antigens (8, 42, 53). In one study (42), we reported that i.n. immunization of mice with MPL and the recombinant salivary-binding region (SBR) of *Streptococcus mutans* AglII adhesin induced higher serum and mucosal antibodies than the response seen in mice immunized with SBR alone. Moreover, the responses peaked 2 weeks after the last (third) immunization and were maintained for about 17 weeks. Our present findings indicate that both serum IgG and salivary IgA responses reached peak levels 2 weeks after the second immunization, and while the serum response was maintained at a high level until the termination of the experiment (~22 weeks), salivary IgA antibody activity decreased to background levels by week 14. The regimen of immunization used in our studies was based on those reported by Wu and Russell (62), who showed an increase in both serum IgG and salivary IgA antibody activities after a third i.n. immunization with AglII. It is interesting that following i.n. immunization of rats with MPL and rHagB, using the same basic protocol, a serum IgG response but not a notable salivary IgA response was induced (unpublished observation). Therefore, it is likely that the differences seen in the salivary IgA responses in other studies (8, 42, 53) compared to the present one are due to differences in the nature of the antigens.

With regards to the subclass of the serum IgG antibody responses induced following immunization, the presence of IgG2a and IgG1 antibodies are indicative of a mixed type 1 and type 2 immune response (15, 44). A number of studies have reported a shift to a type 1 response following immunization with MPL as adjuvant (2, 43, 46, 53). Other studies by DeBecker et al. (10) demonstrated in vivo and in vitro that MPL drives the development of cells that secrete type 1 and type 2 cytokines and the induction of IgG2a and IgG1 antibodies.

### TABLE 1. Effect of MPL on B7-1 and B7-2 expression on murine splenic B cells

<table>
<thead>
<tr>
<th>MPL (ng/ml)</th>
<th>Mean fluorescence intensity</th>
<th>B220&lt;sup&gt;+&lt;/sup&gt;&lt;sup&gt;low&lt;/sup&gt;</th>
<th>B220&lt;sup&gt;+&lt;/sup&gt;&lt;sup&gt;high&lt;/sup&gt;</th>
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<td></td>
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<td>B7-1</td>
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* Single spleen cell cultures were incubated in the presence or absence of MPL for 48 h. Cells were harvested and stained with allophycocyanin-conjugated anti-B220 and FITC-labeled anti-CD86 or PE-labeled anti-CD80 antibody and analyzed by flow cytometry.

### TABLE 2. Effect of MPL on B7-1 and B7-2 expression on murine splenic macrophages

<table>
<thead>
<tr>
<th>MPL (ng/ml)</th>
<th>Mean fluorescence intensity&lt;br&gt;Mean ± SEM</th>
<th>B7-1</th>
<th>B7-2</th>
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<td>259 ± 47</td>
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<td>1,844 ± 196**</td>
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<td></td>
<td>1,644 ± 144**</td>
<td>339 ± 24</td>
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<td>10,000</td>
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<td>779 ± 123</td>
<td>342 ± 21*</td>
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* Significantly different from control (no MPL) at *P* < 0.01; **, significantly different from control (no MPL) at *P* < 0.001.
respectively. In our study, we observed the induction of a type 1 and type 2 response following immunization with HagB, as suggested by the subclass of the IgG anti-HagB response, whereas immunization with HagB plus MPL resulted in an increase in the level of serum IgG1 antibody activity. These findings suggest that the nature of the response was mainly influenced by the HagB antigen and that MPL amplified a type 2 response. One possibility that a type 2 response is favored may relate to the ability of MPL to up-regulate B7 expression on APC. Studies have reported that B7-1 and B7-2 have differential effects on T-cell differentiation (31, 35, 57), whereas other evidence does not support these findings (7, 33). Thus, the increase in the type 2 response observed in the present study may not be related to the differential increase in the B7 molecules on different APC. Interestingly, Broeren et al. (4) demonstrated that Th cell activation by either B7-1 or B7-2 leads to the production of Th2-like cytokines, e.g., IL-4, IL-10, and IL-13. Another possibility for the augmented type 2 response is the ability of MPL to enhance the production of IL-10, which contributes to a decreased production of IL-12 and IFN-γ mRNA (52). Finally, another possibility could involve the interplay between MPL and specific Toll-like receptors. Current studies in our laboratory are investigating these possibilities.

Previous studies in rats have also reported the induction of a mixed type 1 and type 2 serum IgG anti-HagB response following subcutaneous immunization with rHagB in complete Freund's adjuvant (27). This was somewhat surprising, since complete Freund's adjuvant favors the induction of type 1 responses to coadministered antigens and, thus, further implicates the role of HagB in the nature of the response. Studies by Kohler et al. (30) have shown that oral administration of a Salmonella strain expressing the cloned HagB to mice results in the induction of a predominant systemic IgG2a anti-HagB response. These results were similar to those obtained by others who used a Salmonella vector vaccine system (49, 59). Since Salmonella induces a type 1 response, these investigators suggested that the vector influenced the response to the expressed cloned antigen. However, our laboratory reported a mixed type 1 and type 2 response to a cloned antigen of S. mutans expressed by a Salmonella strain (17). These finding suggests that the property of the cloned antigen influenced the response and that the nature of the response was not entirely determined by the Salmonella vector.

It is well known that at least two signals are necessary for an immune response, i.e., antigen presentation to T cells in the context of peptide-MHC class II molecule(s) and the interaction of costimulatory molecules on APC with T cells. Based on our results and those of other investigators (10), it appears that the immunoadjuvanticity of MPL is associated with its potential to activate APC. We have shown in the present study that MPL stimulates proliferation of purified murine B cells and, as previously shown (21), of whole spleen cell cultures. Furthermore, FACS analysis showed a preferential up-regulation of B7-2 expression on the B220<sup>hi</sup> subpopulation of B cells. Lenschow et al. (34) demonstrated that cross-linking of surface immunoglobulin on B220<sup>−</sup> cells induced B7-2 but not B7-1 expression and that these cells were able to stimulate T-cell proliferation. However, these investigators did not separate the B220<sup>−</sup> cells into high- and low-intensity subpopulations. In the present study, we also observed an increase in the percentage of cells expressing either B7-1 or B7-2 only in the B220<sup>lo</sup> subpopulation of cells. Interestingly, the B220<sup>lo</sup> cells have been shown to be the subpopulation of B cells involved in immunoglobulin secretion and proliferation (45).

Converse to the preferential up-regulation of B7-2 on B cells, we observed an up-regulation of B7-2 and especially of B7-1 on macrophages following incubation with MPL. Studies by De Becker et al. (10) have reported that the expression of B7-2 on macrophages remained unchanged after MPL stimulation. However, these investigators did not observe an up-regulation of B7-2 on B cells. This difference in results may reflect the culture conditions used, since the present study shows differences in the expression of B7-1 and B7-2 not only between B-cell subpopulations but also between B cells and macrophages. Thus, the ability of both the B7-1 and B7-2 antibodies to inhibit the proliferative activity of purified CD4<sup>+</sup> T cells (Fig. 5) can be explained by the various types of APC present in the cultures, including CD11c<sup>+</sup> dendritic cells, which exhibited an increase in the mean fluorescence intensity of B7-1 and B7-2 following MPL stimulation.

In the present study we have shown that MPL is an effective adjuvant in promoting mucosal and serum antibody responses when given with rHagB from the periodontal pathogen _P. gingivalis_ by the i.n. route. Furthermore, we have shown that MPL differentially modulates the expression of the B7 co-stimulatory molecules on subpopulations of B cells and macrophages. Current studies are further investigating the mechanisms of MPL adjuvanticity in order to better design vaccines which will potentiate optimal protective responses against infectious agents.

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B7–1 do not deliver identical costimulatory signals, since B7–2 but not B7–1 preferentially costimulates the initial production of IL-4. Immunity 5:253–262.


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