Neisserial Porins Induce B Lymphocytes to Express Costimulatory B7-2 Molecules and to Proliferate

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Summary

The neisserial porins are the major protein components of the outer membrane of the pathogenic Neisseria (N. meningitidis and N. gonorrhoeae). They have been shown to be able to enhance the immune response to poorly immunogenic substances (e.g., polysaccharides, peptides, glycolipids, etc.). To explore the basis of their potent adjuvant activity, the effect of the neisserial porins on T-B cell interactions and T cell costimulation was examined. Neisserial porins increased the surface expression of the costimulatory ligand B7-2 (CD86) but did not affect the expression of B7-1 (CD80). In addition, incubation with the neisserial porins increased the T lymphocyte costimulatory ability of B lymphocytes, which was inhibited by anti–B7-2 but not anti–B7-1 monoclonal antibodies. Upregulation of B7-2 on the surface of B lymphocytes may be the mechanism behind the immunopotentiating activity of neisserial porins.

Protein IA (PIA) and protein IB (PIB) of Neisseria gonorrhoeae and class 1, 2, or 3 proteins of N. meningitidis (C1, C2, and C3 respectively) are the most abundant neisserial outer membrane proteins (1). These proteins function as porins (2–4), share significant homology (5–9), and are members of the gram-negative porin superfamily (10). Neisserial outer membrane vesicle vaccines and purified neisserial porin vaccine candidates induce immune responses in humans and animals without the addition of exogenous adjuvants (11–14). Neisserial porin preparations augment the humoral immune response to poorly immunogenic substances, for example, peptides, and induce a T cell–dependent immune response for normally T cell–independent antigens, for example, polysaccharides (15–18). Meningococcal OMV, mainly consisting of the class 2 protein, are used as carriers to boost the immune response towards the Haemophilus influenzae polysaccharide capsule in the recently licensed H. influenzae type b vaccine (15). The inclusion of purified PIA, PIB, C1, or C3 proteins in noncovalent complexes containing group C meningococcal capsular polysaccharides (CPS) greatly improves the antibody response in immunized mice to the polysaccharide compared with the

anti-CPS response in mice immunized with CPS alone (17). Neisserial porins are used as adjuvants in anti–melanoma cancer vaccines to augment the immune response to GM2 and GD3, two gangliosides present at much higher levels on malignant melanoma cells compared with normal human melanocytes (18).

We postulated that the porins' adjuvant ability could be related to their effect on interactions between T and B lymphocytes, thereby increasing T cell involvement in the immune response. In the current model of T lymphocyte stimulation, two sets of signals between the APC and the T lymphocyte are required (19, 20). The first signal (signal 1) is delivered via the interaction of the MHC on APC (e.g., B lymphocytes, dendritic cells, macrophages, etc.) and the TCR on T lymphocytes. This interaction confers antigenic specificity. The second or costimulatory signal (signal 2) is delivered by the binding of two sets of counterreceptors during the interaction between the B and T lymphocytes.

The most significant B lymphocyte counterreceptors are B7-1 (BB1, CD80) (21, 22) and the more recently discovered B7-2 (CD86) (23–27). The costimulatory ligands of the B7 family are expressed on professional APC: activated B lymphocytes, macrophages and dendritic cells (21, 22, 28–32). The B7 family of ligands bind to two T lymphocyte counterreceptors, CD28 and CTLA-4 (19, 20). It appears that B7-2 has a greater role in stimulating T lymphocytes than B7-1 (19, 20). The expression of B7-2 occurs earlier than the expression of B7-1, and there is more B7-2 present on the surface of activated B lymphocytes than B7-1 (19, 20, 33, 34). Most importantly, the upregulation of B7-1

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¹Abbreviations used in this paper: CPS, group C meningococcal capsular polysaccharide; ICAM-1, intracellular adhesion molecule-1; PIA, PIB, protein IA, IB, respectively; Th, T-helper.

and B7-2 surface expression correlates with the induction of the ability of B lymphocytes to costimulate T lymphocytes (19, 20). The effect of neisserial porins on B lymphocyte stimulation and costimulatory ligand expression (B7-1 and B7-2) was examined as a possible mechanism by which porins enhance the immune response to other antigens.

Materials and Methods

Animal Strains. Lymphocytes were isolated from LPS-resistant mice, strain C3H/HeJ (35), or their LPS-responsive related strain C3H/HeOuJ. 7-8-wk-old mice were used in the experiments. Both strains were obtained from The Jackson Laboratory (Bar Harbor, ME). LPS-resistant mice were used in all experiments in order to prevent confounding results secondary to contaminating LPS.

Antibodies. The following mAbs were used in the B and T lymphocyte isolations: anti-Thy1.2 (mouse IgM, TIB 99, clone HO.13.4), anti-class II MHC, IA^k (mouse IgG2b, TIB 93, clone 10.2.16) and anti-CD8 (rat IgG 2b, TIB 211, clone 3.155). They were all obtained from myeloma cell lines purchased from American Type Culture Collection (Rockville MD). The following mAbs were used in flow cytometry analysis: anti-murine B7-1-FITC (24) [hamster F(ab)2; clone 16-10A1], anti-murine B7-2-FITC (Rat IgG2a; PharMingen, San Diego, CA; clone GL-1)

(25), anti–class II MHC–IA^k–PE (mouse IgG2a; PharMingen; clone 11-5.2), anti-murine CD23-FITC (rat IgG2a; PharMingen; clone B3B4), anti-murine heat-stable antigen–FITC (CD24) (rat IgG2b; PharMingen; clone M1/69), anti-murine intracellular adhesion molecule (ICAM)-1-FITC (CD54) (hamster IgG; PharMingen; clone 3E2). A mixture of nonspecific murine IgG mAbs was added to each reaction mixture to block nonspecific Fc receptor binding (Becton Dickinson Immunocytochemistry System, San Jose, CA). Rat IgG2a–FITC (PharMingen) or hamster IgG–FITC (PharMingen; clone UC8-4B3) were used as controls to check for nonspecific binding. Anti-CD3 mAb (hamster IgG/PharMingen; clone 145-2C11) was used to cross-link CD3 on purified T lymphocytes in the costimulation assay (see below). Anti-murine B7-1 [hamster F(ab)2; obtained from Dr. Hans Reiser) or anti-murine B7-2 (PharMingen; clone GL-1) were used in the costimulation experiments to investigate their ability to inhibit T lymphocyte costimulation.

Bacterial Porins and Products. Neisserial porins were purified by detergent extraction and column chromatography as described previously (36–38). Briefly, gonococci or meningococci were resuspended in 1 M sodium acetate, pH 4.0, and then a solution of 0.5 M $CaCl_2$, 5% Zwittergent was added, and the supernatant was obtained by centrifugation. The protein was precipitated by the addition of ethanol to a concentration of 80% and then resuspended in a 50-mM Tris, pH 8.0, 5%-Zwittergent buffer. It was
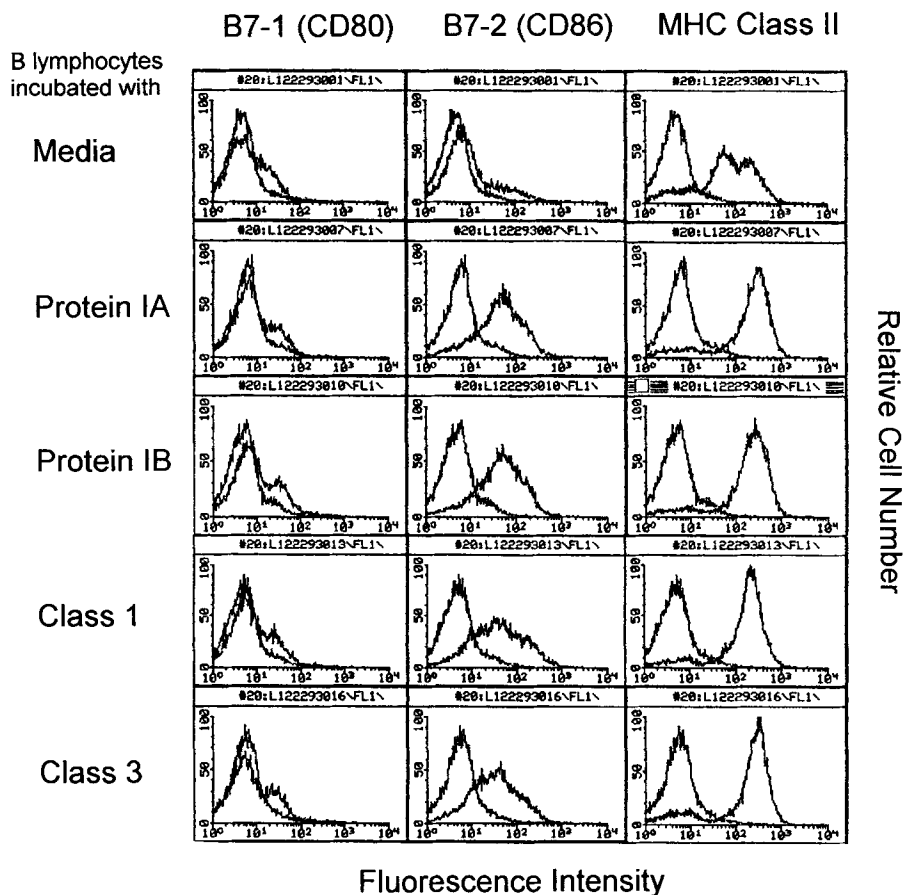
A

Figure 1. Neisserial porins induce B lymphocytes to express B7-2 molecules. Purified murine B lymphocytes isolated form C3H/HeJ mice were incubated with purified neisserial porins or medium alone for 48 h. The treated B lymphocytes were stained with nonspecific rat IgG–FITC or PE (*curve closest to vertical scale*), anti-B7-1–FITC, anti-B7-2–FITC, or anti–IA^k–PE (MHC class II) (*the second curve in each panel*). (*A*) Histograms of fluorescence intensity obtained for each set of B lymphocytes incubated with media alone or each of the neisserial porins at a concentration of 10 μg/ml, when analyzed by flow cytometry (10,000 cells were evaluated per sample). The data displayed are from a representative experiment. (*B*) Geometric mean cellular fluorescence intensity obtained for each set of B lymphocytes incubated with decreasing concentrations of PIB (10, 5, 2, 1, 0.5, or 0.1 μg/ml) upon labeling with anti–B7-2–FITC. The data displayed are from a representative experiment. The experiments shown in *A* and *B* were performed four times, essentially with identical results.

further isolated by passage over an ion exchange column and a molecular sieve column. PIA (34 kD) and PIB (35 kD) were purified from gonococcal strains lacking protein III (11, 38), and C1 (40 kD) and C3 (34 kD) proteins were purified from meningococcal strains lacking class 3 and 4 or class 1 and 4 proteins, respectively (39, 40). The porins were isolated from the mutant neisserial strains to avoid contamination of the purified porin preparations with other major outer membrane proteins. Negligible contamination by other proteins and LPS was demonstrated by gel electrophoresis (data not shown). The porins used for incubation with the B cells were formed into proteosomes (11), pure protein micelles, to eliminate all detergents that could be toxic to the lymphocytes. Neisserial LPS (3.6 kD) was kindly provided by Dr. Michael Apicella (University of Iowa Medical Center, Iowa City IA) (41).

Lymphocyte Isolation. Single-cell suspensions of spleens and mesenteric lymph nodes were depleted of red blood cells by treatment with PBS/NH_4Cl (42). B lymphocytes were obtained by negative selection through complement lysis using anti–T cell mAbs (anti–Thy 1.2, anti-CD4, and anti-CD8) with guinea-pig complement (Sigma Chemical Co., St. Louis, MO) followed by removal of adherent cells on a G10 sephadex column (42). Th cells (CD4$^+$) were obtained by enrichment over a nylon wool column and subsequent complement lysis using anti–class II MHC (anti-IA^k) and anti-CD8 mAbs with guinea pig comple-

ment (42, 43). Medium used was RPMI (pH 7.4) with 10% heat-inactivated FCS (HyClone Laboratories, Inc., Logan UT), 50 μM 2-ME, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (R10).

B Lymphocyte Incubation with Neisserial Porins. B lymphocytes (5 × 10^6/ml) were incubated with neisserial porins, PIA, PIB, C1, or C3 protein, formed into proteosomes, for 2 d in humidified 5% CO_2 at 37°C. Incubation with dextran (20 μg/ml) (Sigma Chemical Co.) was used as a positive control as this mixture has previously been shown to induce B7-1 or B7-2 expression on B lymphocytes (22, 44, 45). Incubation with R10 cell culture media alone was used as a negative control. The B cells were harvested and separated from dead cells and debris by density gradient centrifugation (Ficoll–Hypaque) (42, 43). The cells were viable as tested by trypan blue exclusion (43).
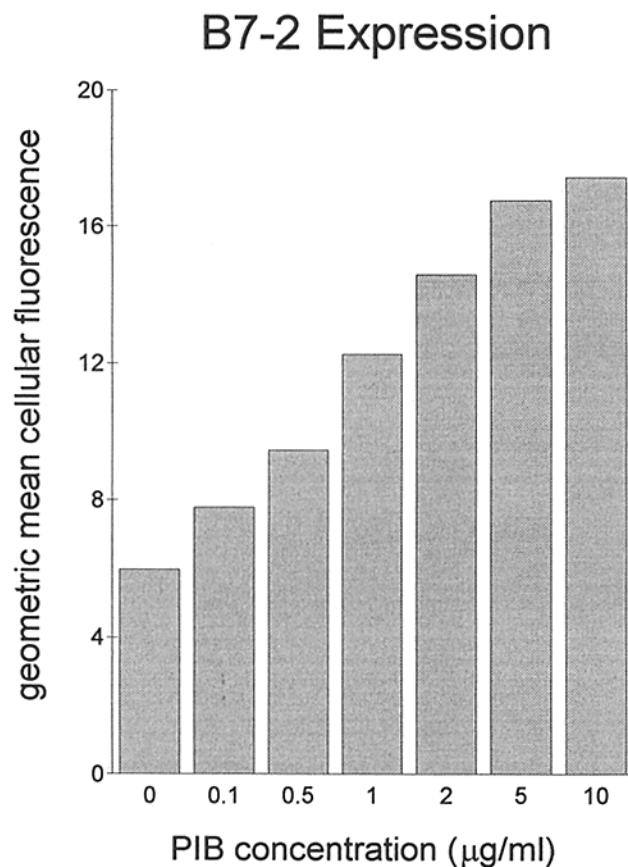
B Lymphocyte Proliferation Experiments. B lymphocytes from LPS-nonresponsive strain C3H/HeJ or LPS-responsive strain C3H/HeOuJ, or purified T lymphocytes from LPS-nonresponsive strain C3H/HeJ (see below) were incubated as above with PIB or purified neisserial LPS at a concentration of 10 μg/ml (3 nM LPS or 0.3 nM PIB) for 48 h. Control incubations contained only media. The B or T lymphocytes, 5 × 10^6/ml, 100 μl/well in triplicate, were incubated in humidified 5% CO_2 at 37°C with PIB, LPS, or media. After 2 d the cells were pulsed with 1 μCi, [^{3}H]thymidine per well. After an additional 18-h incubation, the wells were harvested onto filter paper discs, and the [^{3}H]thymidine incorporation, as a measure of PIB- or LPS-induced proliferation, was measured on a β scintillation counter.

Flow Cytometry Evaluation. The expression of B lymphocyte surface antigens was studied by flow cytometric analysis of single-cell suspensions using a FACScan® (Becton Dickinson & Co.). (46). Data were analyzed using LYSYS™ or CELL QUEST™ FACS® analysis software (Becton Dickinson, & Co.). The B lymphocytes, previously incubated with neisserial porins, were aliquotted and incubated with the anti–B cell surface ligand fluorochrome conjugate mAb mentioned above, per standard protocols (46). Specificity of the anti–B7-1–FITC and anti–B7-2–FITC binding was evaluated by the addition of unlabeled anti–B7-1 or anti–B7-2 mAb to reaction mixtures, which completely ablated any signal of their respective fluorochrome conjugate antibody conjugates (data not shown). Nonspecific binding via the Fc portion of the mAbs was blocked by the addition of a mixture of mouse mAbs (IgG1, IgG2a, IgG2b) to each reaction mixture. FITC-labeled rat or hamster IgG was used as isotype controls in certain reaction mixtures to determine the nonspecific binding of these species of antibodies to B lymphocytes.

Cell Cycle Evaluation. To evaluate the percentage of B lymphocytes in the various stages of the cell cycle (G1, S, G2, and M), the following experiments were performed. B lymphocytes were incubated in medium containing PIB or neisserial LPS at various concentrations for 2 d as described above. Freshly isolated B lymphocytes and B lymphocytes incubated in plain media (R10) were included in the evaluations as controls. All cells were fixed and permeabilized by washing with ice-cold 70% ethanol. DNA was stained with propidium iodine (50 μg/ml) in PBS, 2% FCS for 1 h. The cells and DNA content were analyzed by flow cytometric methods as previously described (46). As the ploidy of the DNA increases, indicating cells entering the synthetic and mitotic phases of the cell cycle, each cell incorporates greater amounts of propidium, whose fluorescence can be measured and quantitated.

T Lymphocyte Costimulation. B lymphocytes, activated by porins, were incubated with CD3–cross-linked CD4$^+$ T lympho-

B

B7-2 Expression

PIB concentration (μg/ml)

A

B

Freshly Isolated B cells   B Cells Incubated with Media   B Cells Incubated with PIB

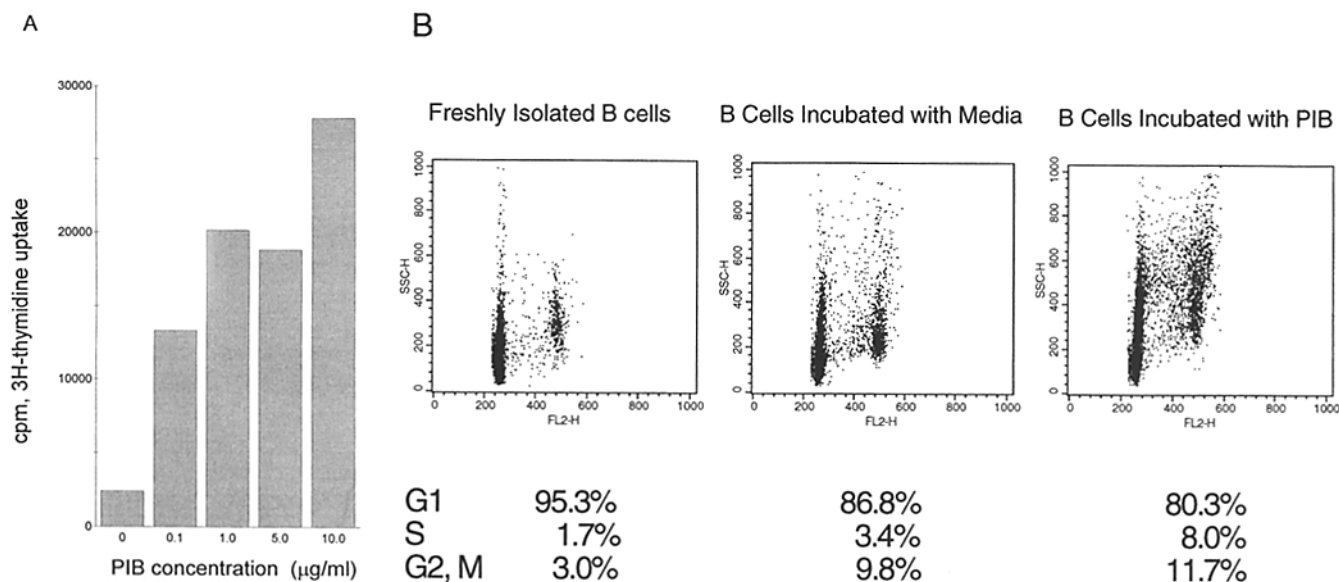|	Freshly Isolated B cells	B Cells Incubated with Media	B Cells Incubated with PIB
G1	95.3%	86.8%	80.3%
S	1.7%	3.4%	8.0%
G2, M	3.0%	9.8%	11.7%

Figure 2. Neisserial porins induce B lymphocytes to proliferate. (A) Purified B lymphocytes from C3H/HeJ mice were incubated, at a concentration of 5 × 10⁶/ml, with purified neisserial PIB (10, 5, 1, or 0.1 μg/ml; 0.3, 0.15, 0.03, or 0.003 μM, respectively) or medium alone for 48 h and then pulsed with [³H]thymidine. [³H]Thymidine incorporation (cpm, on the y-axis) was measured as an indicator of lymphocyte proliferation. The data displayed are from one typical experiment; experiments were performed three times. (B) The DNA content of freshly isolated B lymphocytes or B lymphocytes incubated with medium alone or PIB (10 μg/ml, 0.3 μM) was determined by propidium iodine (PI) staining and measuring the PI retention by the DNA using flow cytometry. The B lymphocytes were isolated from C3H/HeJ mice. As the amount of DNA per cell increases, the greater each cell incorporates PI after permeabilization by ice-cold ethanol. The greater the DNA content, the more cells are in the cell cycle. The area of the plot to the far left shows cells in the first gap phase (G1), the area to the far right shows cells in the second gap phase (G2) or mitotic phase (M), and the area in between shows cells in the synthesis phase (S). B cells from LPS-responsive murine strain C3H/HeOuJ all had similar profiles when incubated either PIB or LPS (data not shown). The experiments were performed three times, with essentially identical results.

cytes to measure the B cells' ability to costimulate the T cells (22, 25, 44, 45, 47, 48). The CD3 antigens on T lymphocytes were cross-linked by incubation with anti-CD3 mAb, 1 μg/ml, for 1 h before the addition of the B lymphocytes. B lymphocytes, previously incubated with the neisserial porins or media alone, were fixed with mitomycin C, 50 μg/ml, for 30 min at room temperature. Various numbers of these fixed B lymphocytes (10³ to 2 × 10⁵) were added to 96-well tissue culture plates containing CD3–cross-linked CD4⁺ T lymphocytes, 2 × 10⁵/well. The mixtures of B and T cells were incubated in humidified 5% CO₂ at 37°C, and after 2 d they were pulsed with 1 μCi [³H]thymidine per well. 18 h later, the cells from each well were harvested onto filter paper discs, and [³H]thymidine incorporation was measured on a gamma counter. Anti–B7-1 or anti–B7-2 mAbs or appropriate isotype controls (hamster IgG or rat IgG 2a) were added to wells containing porin-incubated B lymphocytes and CD3–cross-linked T lymphocytes to determine the specificity of the B lymphocyte costimulatory activity.

Results

To examine if neisserial porins affect the surface expression of B7-1 and B7-2 on B lymphocytes and increase B lymphocyte costimulatory ability, murine B lymphocytes were purified from spleens of LPS-nonresponsive mice, strain C3H/HeJ (35, 49), and incubated with neisserial porins formed into proteosomes (11). The porin-treated B lymphocytes were labeled with anti–B7-1, anti–B7-2, or anti-IAᵏ (class II MHC) fluorochrome conjugates, and the level

of surface expression was determined by flow cytometric analysis. 48 h after incubation with the neisserial porins PIA, PIB, C1, or C3, B lymphocytes expressed more B7-2 on their surface compared with B cells incubated with media alone (Fig. 1 A). The upregulation of surface expression of B7-2 occurred in a dose-dependent manner (Fig. 1 B). Importantly, the effects of the neisserial porins were very specific. The porins also induced the expression of MHC class II and, to a small degree, ICAM-1 (CD54) molecules. In contrast, neisserial porins did not induce the expression of B7-1, heat-stable antigen (CD24), or CD23 at all concentrations tested (0.1–10.0 μg/ml) (data not shown).

The ability of the porins to induce B lymphocyte proliferation was measured by [³H]thymidine incorporation experiments. PIB was able to induce proliferation of purified B lymphocytes from strain C3H/HeJ in a concentration-dependent manner (10.0–0.1 μg/ml, 0.3–0.003 μM) (Fig. 2 A). PIB could not induce T cell proliferation (Fig. 3 A). In addition, PIB was able to induce progression of the B lymphocytes into the cell cycle. This was performed by analyzing the DNA content of freshly isolated B lymphocytes and B lymphocytes incubated in media or with PIB (10 μg/ml, 0.3 nM) and measured by propidium iodine uptake using flow cytometric methods (46). PIB was able to increase the percentage of B cells into the synthesis phase (S) and the second gap and mitotic phase (G2/M) by two- to fourfold over freshly isolated B cells or B cells incubated with media alone.

A series of experiments were performed to rule out that contamination of the neisserial porin preparations by LPS was responsible for B lymphocyte proliferation and induced B7-2 expression. Purified B lymphocytes from LPS-nonresponsive murine strain C3H/HeJ or from equivalent but LPS-responsive murine strain C3H/HeOuJ were incubated with media, PIB (10 µg/ml, 0.3 nM), or neisserial LPS (10 µg/ml. 3.0 nM) separately. PIB was able to induce proliferation of C3H/HeJ B cells, whereas LPS or media alone could not (Fig. 3 A). Both PIB and LPS were able to induce proliferation of B lymphocytes from the LPS-responsive strain C3H/HeOuJ (Fig. 3 A). Moreover, incubation of B lymphocytes from both strains with PIB induced a greater number of cells to enter the cell cycle compared with media-incubated B cells. In contrast, LPS incubation of B lymphocytes only from strain C3H/HeOuJ (and not strain C3H/HeJ) induced a greater num-

ber of cells to enter the cell cycle compared with media-incubated B cells (data not shown). Finally, the expression of B7-2 on B lymphocytes, upon incubation with PIB, LPS, or media alone, was analyzed (Fig. 3 B). PIB was able to increase B7-2 expression using B lymphocytes from either C3H/HeJ or C3H/HeOuJ mice. However, LPS was only able to increase B7-2 surface expression on B lymphocytes from C3H/HeOuJ mice. Control incubation with media alone did not increase B7-2 surface expression on either set of B lymphocytes (Fig. 3 B). B7-1 surface expression did not increase on any set of B lymphocytes (data not shown). Importantly, LPS was used at a 10-fold higher molar concentration than PIB in these experiments. Taken together, these data strongly suggest that the induction of B cell proliferation and B7-2 expression on the surface of B lymphocytes by the neisserial porins is due to the protein itself, and not to LPS contamination.
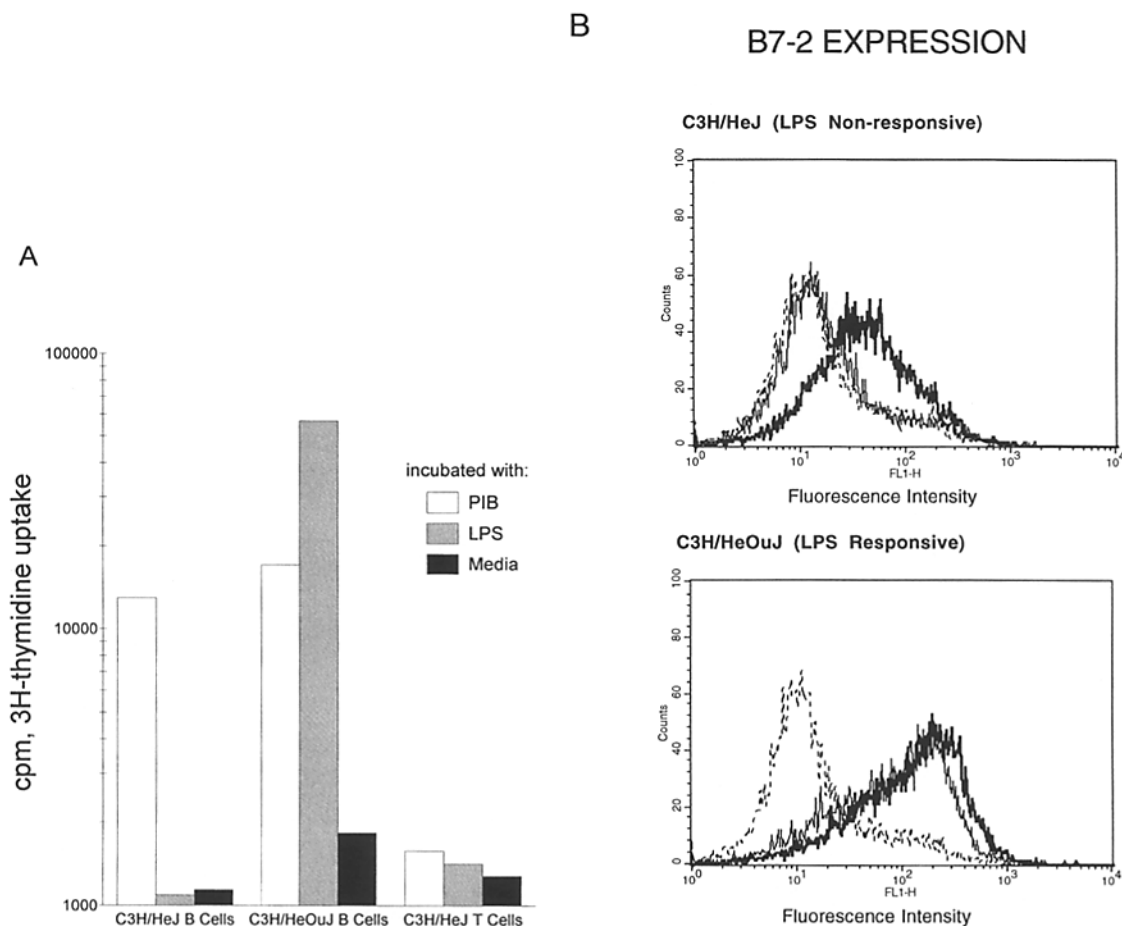

Figure 3. The B lymphocyte stimulatory activity of neisserial porins is not due to contamination with LPS. (A) Purified B lymphocytes from either LPS-nonresponsive murine strain C3H/HeJ or from the LPS-responsive strain C3H/HeJ were incubated, at a concentration of 5 × 10⁶/ml, with purified neisserial LPS (10 µg/ml, 3 µM), PIB (10 µg/ml, 0.3 µM) or medium alone for 48 h and then pulsed with [³H]thymidine. T lymphocytes were obtained from strain C3H/HeJ and were used at a concentration of 5 × 10⁶/ml. [³H]Thymidine incorporation (cpm, on the y-axis) was measured as an indicator of PIB- or LPS-induced B lymphocyte proliferation. The data displayed are from one typical experiment; experiments were performed three times. (B) Purified B lymphocytes from either the LPS-nonresponsive murine strain C3H/HeJ or the LPS-responsive strain C3H/HeOuJ at a concentration of 5 × 10⁶/ml were incubated with purified neisserial LPS (10 µg/ml, 3 µM), PIB (10 µg/ml, 0.3 µM), or medium alone for 48 h. The surface expression of B7-2 (CD86) was then determined by labeling with anti–B7-2–FITC conjugate and subsequent analysis by flow cytometry. 10,000 cells were analyzed per sample. Media incubated B lymphocytes (*dashed line*), LPS-incubated B lymphocytes (*thin solid line*), PIB-incubated B lymphocytes (*thick solid line*). The data displayed are from one typical experiment; experiments were performed three times.

Next, the ability of B lymphocytes incubated with neisserial porins to costimulate autologous CD4$^+$ T lymphocytes was measured. CD4$^+$ T lymphocytes were incubated with anti-CD3 mAb to cross-link the TCR and mimic signal 1 in the model of T cell costimulation (50–52). Increasing numbers of mitomycin C–treated B lymphocytes preincubated with PIA, PIB, or media were incubated with CD3–cross-linked T lymphocytes. B lymphocytes incubated with the porins were able to stimulate T lymphocytes, whereas B lymphocytes incubated with media alone were not able to stimulate the T lymphocytes (Fig. 4 *A*). Importantly, the ability of B lymphocytes to costimulate T lymphocytes also increased with the concentration of PIB with which the B lymphocytes were incubated (Fig. 4 *B*). The increase in costimulatory activity correlates with the upregulation of B7-2 expression noted above (Fig. 1 *B*). This indicates that the ability of the neisserial porins to induce B cell costimulatory activity is related to the amount of porin the B lymphocyte encounters. T lymphocyte stimulation by the B cells incubated with PIA or PIB was inhibited by the addition of anti–B7-2 mAb to the reaction mixtures (>80%) and only minimally inhibited by the addition of anti–B7-1 to the reaction mixtures (<20%) (Fig. 5). The addition of both mAbs totally inhibited the costimulatory ability of the B lymphocytes. It is not surprising that the anti–B7-1 mAb could inhibit a small amount of the B lymphocyte costimulatory ability since B7-1 expression increases on all incubated B lymphocytes (including media-incubated B lymphocytes) compared with freshly isolated B lymphocytes (see Fig. 1). The addition of control nonspe-

cific rat IgG mAb did not affect the costimulation of T lymphocytes by the B lymphocytes incubated with PIA or PIB.

Discussion

There is evidence presented by various investigators that microbial products can stimulate B lymphocytes. LPS, mitogenic influenza virus, and an antigen that mimics viral infection (polyinosinic–polycytidylic acid), have all been shown to stimulate B lymphocytes, which in turn costimulate T lymphocytes (53). Purified *Salmonella typhi* porins (free of LPS) have been demonstrated to be potent B cell stimulators, but have minimal effect on T lymphocytes (54, 55). In addition, meningococcal outer membrane preparations, mainly consisting of the meningococcal porins, act as B cell mitogens and do not stimulate T lymphocytes (56–58). The data presented in this study are consistent with these previous findings. Neisserial porins are potent B cell stimulators and have minimal, if any, effects on T lymphocytes, including T lymphocytes that have been activated by TCR cross-linking by anti-CD3 mAb (data not shown). More specifically, the neisserial porins can stimulate B lymphocytes and induce upregulation of the costimulatory ligand B7-2 on the surface of B lymphocytes. B lymphocytes treated with neisserial porins can stimulate T lymphocytes whose TCRs have been cross-linked by anti-CD3 mAbs. The CD3 cross-linking mimics engagement of TCR by class II MHC whose peptide-binding clefts are occupied by antigen-derived oligopeptides (T cell epitope).

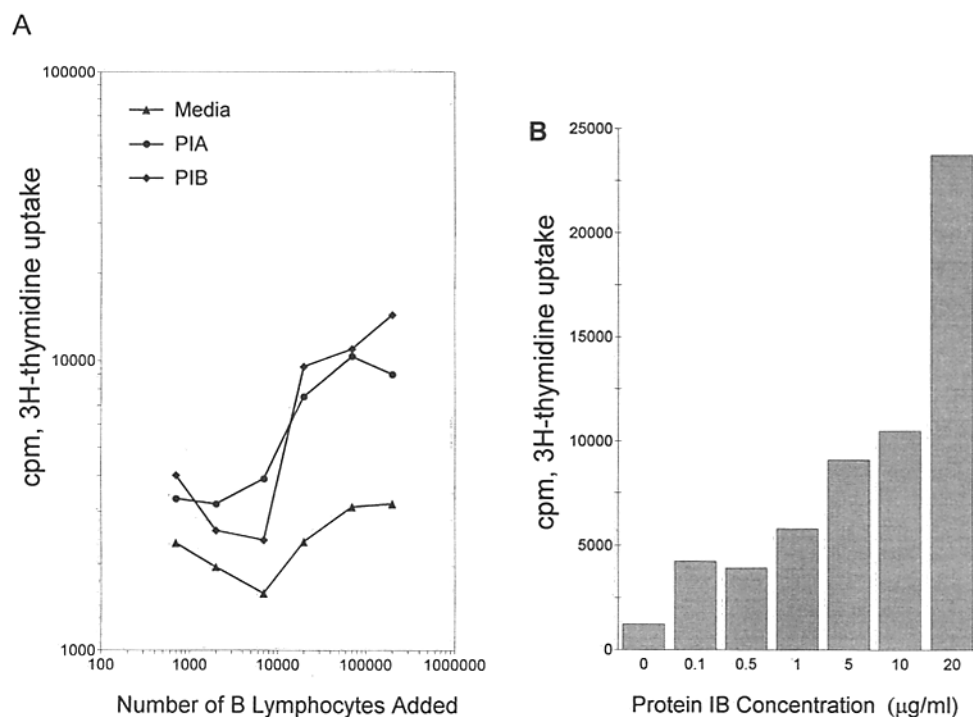LPS, including neisserial LPS, is an extremely effective B

Figure 4. Neisserial porins induce B lymphocytes to express costimulatory activity. (*A*) Cultures were constructed with anti-CD3–activated CD4$^+$ T lymphocytes and mitomycin C–treated B cells preincubated with PIA, PIB, or medium alone. The *x* axis represents the number of B lymphocytes added to each reaction mixture containing 2 × 10^5 T lymphocytes per well. Stimulation of T lymphocytes by B lymphocytes preincubated with neisserial porins was quantitated by [^{3}H]thymidine incorporation (*y* axis). All cultures were constructed in triplicate. (*B*) T lymphocyte costimulation experiments were performed using B lymphocytes incubated with increasing concentrations of PIB as noted in the figure. After 2 d of incubation, the B lymphocytes were fixed with mitomycin *c* and used in T lymphocyte costimulation assays. The number of T lymphocytes and B lymphocytes used was 2 × 10^5/well. The amount of T lymphocyte stimulation induced by the porin-treated B lymphocytes was measured by [^{3}H]thymidine uptake as in Fig. 3 and is displayed on the *y* axis.

number of B lymphocytes added
20,000
70,000
200,000

0   500   1000   1500   2000   2500   3000
cpm, ^{3}H-thymidine uptake

B lymphocytes   T and B lymphocytes
treated with: incubated with:

☐ media   nothing
■ PIB   rat IgG control
☐ PIB   anti-B7-1
▨ PIB   anti-B7-2
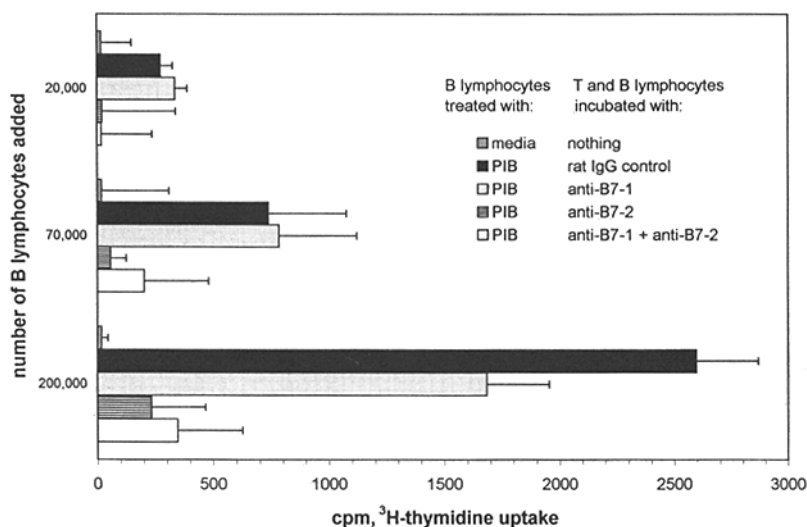☐ PIB   anti-B7-1 + anti-B7-2

Figure 5. The costimulatory activity of porin-stimulated B lymphocytes is inhibited by anti-B7-2 antibody. T lymphocyte costimulation experiments were performed as described in Materials and Methods and the legend to Fig. 4 using B lymphocytes incubated with PIA or PIB, with additional reaction mixtures that contained the following mAbs: anti-B7-1 (clone 1G10, rat IgG2a), anti-B7-2-(clone Gl-1, rat IgG2a), or non-specific rat IgG2a mAb, 10 μg/ml. All data points were performed in triplicate. Data are reported as the average [^{3}H]thymidine uptake (cpm) of wells containing no antibody, rat IgG control antibody, anti-B7-1, or anti-B7-2 mAb, minus the average [^{3}H]thymidine uptake (cpm) in wells only containing anti-CD3–activated CD4$^+$ T lymphocytes (n = 10). The number of T lymphocytes used was 2 × 10^5/well each, and the number of B lymphocytes per well is labeled on the y-axis. This is the average of three experiments.

cell mitogen and can increase expression of B7-1 and B7-2 on B lymphocytes, inducing potent costimulatory activity (28, 53). For a number of reasons, porin-induced B cell proliferation and increased B7-2 expression are most likely not due to LPS contamination of the neisserial porin preparations. B lymphocytes were purified from phenotypically LPS-nonresponsive mice (strain C3H/HeJ). LPS at equimolar or greater concentrations compared with PIB could not induce B7-2 expression (Fig. 3 B) and could not induce B cell proliferation as measured by [^{3}H]thymidine incorporation using lymphocytes from LPS-nonresponsive mice (Fig. 3 A). The LPS used was a potent stimulator of B lymphocytes from the related LPS-responsive strain C3H/HeOuJ. Finally, the purified porin preparations described and used in these studies had minimal contamination by LPS as demonstrated by gel electrophoresis and silver staining, and by limulus lysate assays (<0.01%) (11, 40, 59).

It has been previously shown that the neisserial porins are immunogenic without additional adjuvants and that they are potent stimulators of the immune response to otherwise poorly immunogenic substances (11–14, 17, 18). It is likely that the ability of the porins to augment the immune response to other antigens is related to their induction of B lymphocyte costimulatory ability and subsequent T lymphocyte costimulation. In individuals immunized with vaccines containing neisserial porins complexed with specific potentially protective antigens, the antigen of interest would

at first bind to surface immunoglobulin of antigen-specific B lymphocytes. This allows the porin present in the vaccine complex to come in close proximity to the B lymphocyte, stimulate the B lymphocyte, and increase surface expression of B7-2. The B lymphocytes, with increased T lymphocyte costimulatory ability, then stimulate T lymphocytes. Once activated in this manner, T lymphocytes release various cytokines, including IL-2 and IL-4, which positively affect antibody production. IL-2 and IL-4 induce B lymphocytes to become antibody-secreting cells, induce isotype switching from IgM to IgG, and allow T cell memory to occur, which is especially important if the antigen of interest is T cell independent, e.g., polysaccharide (60–63).

Expression of B7-2 has been demonstrated on cell types other than B lymphocytes, including dendritic cells and macrophages (28, 32). It remains to be determined whether neisserial porins have any effect on the activity of these APC. It is unclear by which mechanisms the porins stimulate B lymphocytes. Possibilities include direct intercalation of the porins into the membrane of the B lymphocyte. Another possible mechanism is that the porins bind and stimulate B lymphocytes via specific unrecognized surface receptors. Work is in progress exploring both such hypotheses. The better the understanding of the mechanism of adjuvants, the better we will be able to use their immune-augmenting activity in future vaccines.

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