T Lymphocyte Response to *Neisseria gonorrhoeae* Porin in Individuals with Mucosal Gonococcal Infections

Scott D. Simpson,† Yu Ho, Peter A. Rice, and Lee M. Wetzler

T lymphocytes from a majority of patients with urogenital gonococcal disease (67%–80%) proliferated on incubation with gonococcal porin (Por), compared with minimal induced proliferation of T lymphocytes from normal volunteers. A significant increase in Por-specific interleukin (IL)–4–producing CD4+ T helper lymphocytes was seen in patients with mucosal gonococcal disease and not in normal controls. Similar results were observed in CD8+ T lymphocytes from these patients. There was no measured increase in IL-2, IL-10, IL-12, interferon-γ, or tumor necrosis factor–α production by T lymphocytes from infected subjects on incubation with Por. Concomitant increases in IL-4 production in T lymphocytes from infected subjects expressing the mucosal addressin VLA-α/β, on their surface were also observed on Por incubation, but the increases were similar in T lymphocytes that were VLA-α/β negative. In conclusion, mucosal gonococcal disease can induce Por-specific circulating T lymphocytes with a Th2 phenotype, and a portion of these Por-specific T lymphocytes can potentially traffic to mucosal surfaces.

Gonorrhea is one of the most common sexually transmitted diseases. Manifestations include urethritis and epididymitis in men and urethritis, cervicitis, endometritis, and pelvic inflammatory disease (PID) in women. PID is a major cause of fallopian tube scarring, which can lead to ectopic pregnancies and infertility [1]. In addition, in a small percentage of patients, disseminated gonococcal infections can occur, characterized by arthritis, tenosynovitis, and other end-organ involvement [2].

*Neisseria gonorrhoeae* is a gram-negative diplococcus that lacks a polysaccharide capsule. The outer membrane is composed of lipooligosaccharides (LOSs) and a variety of outer membrane proteins (OMPs). The gonococcal porin, termed protein I, or Por, comprises 60% of the OMP content [3]. Por is a 34- to 35-kD transmembrane protein whose native configuration is a homogeneous trimer that functions as a pore for water and small ions [3, 4]. Por is an allelic protein consisting of two main isoforms, protein IA (PIA) and protein IB (PIB) [5, 6]. There is moderate antigenic variation between the Por isoforms, which accounts for a variety of gonococcal serotypes [7, 8]. In addition, Por has extensive sequence homology to the meningococcal porins and class 1, 2, and 3 proteins [7, 9–11] and moderate sequence homology to the lipopolysaccharide porins from *E. coli* and *Salmonella enterica* [12]. PIA is frequently associated with *N. gonorrhoeae*, which causes disseminated disease, whereas PIB has a higher association with organisms that cause local urogenital disease and PID [5, 6, 13].

Most studies that have investigated antigonococcal immune responses have focused predominantly on humoral responses [13–18]. Anti-PIA, -PIB, -LOS, and –protein III (PIII or RMP, the reduction modifiable OMP)–specific antibodies have been detected in the sera of patients with local [16–20] or disseminated disease [21, 22]. Furthermore, the presence of serum antibodies to Por and LOS may correlate with protection against disease transmission and PID, whereas antibodies to RMP may correlate with increased susceptibility to infection [14, 15, 18, 23].

The generation of an antibody response to protein antigens on a pathogen entails a series of coordinated interactions between antigen-specific B cells and T cells, involving the exchange of costimulator signals and cytokines necessary for cellular growth, proliferation, and immunoglobulin production [24–27]. Importantly, the generation of a humoral response to most protein antigens requires T cell help and the presence of antigen-specific T cells. As previously stated, the presence of antigonococcal antibodies has been demonstrated in patients with gonococcal disease. However, there is a paucity of data that examines the T cell response to *N. gonorrhoeae* in humans.

In this study, the human T cell response to *N. gonorrhoeae* in patients with mucosal gonococcal disease was examined—
particular, the immune response to Por. On the basis of proliferative assays and flow cytometric (FACS) analysis, the characteristics of the anti-Por T cell response are detailed, including cytokine production and the mucosal homing ability of Por-specific T cells. The baseline information presented here will enable investigators to better interpret future results, defining antigonococcal T cell responses and their correlation with protection from disease and the ability to interrupt transmission.

Methods

Patient population. Peripheral blood mononuclear cells (PBMC) and sera were obtained from subjects with urogenital gonococcal disease who were seen in the Public Health Clinic at the Boston Medical Center and who had given informed consent. Men with signs and symptoms of urethritis and women with cervicitis or urethritis accompanied by a gram stain of urethral or cervical exudate that demonstrated gram-negative intracellular diplococci were initially included in the study. Urogenital specimens for gonococcal cultures were obtained from all subjects, and the diagnosis of gonorrhea was confirmed by a positive culture in all study participants. All subjects were aged ≥18 years; 6 women and 24 men participated. Individuals with a previous diagnosis of human immunodeficiency virus infection or other concomitant medical illnesses were excluded from the study. Control sera and PBMC were also obtained from normal volunteers who had no history of neisserial or other systemic gram-negative infections.

Antibodies. The following antibodies were used for FACS analysis: anti-human CD3–fluorescein isothiocyanate (FITC), anti-human CD4–FITC, anti-human CD8–FITC, anti-human CD14–FITC, and anti-human CD19–phycoerythrin (PE), all from Becton Dickinson (Mountain View, CA); anti-human CD3–CyChromeC, anti-human interleukin (IL)–2–PE, anti-human IL–4–PE, anti-human IL–10–PE, anti-human interferon (IFN)–γ–PE, and anti-human tumor necrosis factor (TNF)–α–PE, all from Pharmingen (San Diego, CA); and anti-human VLAα4/β1–biotin (Act-1), kindly provided by Dr. D. Ringler (Leukosite, Cambridge, MA). To detect VLAα4/β1 staining of human T lymphocytes, streptavidin–PerCP (Pharmingen) was used. Mouse IgG1–PE (Pharmingen) and rat IgG2b–PE (Pharmingen) were used as isotype controls for intracellular cytokine staining.

Gonococcal porins. Gonococcal porins were purified by detergent extraction and column chromatography, as described elsewhere [28–30]. PIA and PIB were purified from well-characterized gonococcal mutant strains lacking reduction modifiable protein (PIII or RMP), UU1ΔPIII and PghΔPIII, respectively, to improve the purity of the porin preparations [31, 32]. Negligible contamination by other proteins and lipopolysaccharide was demonstrated by gel electrophoresis and silver staining [33] (data not shown). Recombinant gonococcal GroES, an 11-kDa heat-shock protein [34], was used as a fusion protein with glutathione-S-transferase (GST), which was kindly provided by Dr. William Shafer (Emory University, Atlanta) and was included as a negative control. The GST obtained from Dr. Shafer was also used as a negative control protein. The porins and control antigens, GroES–GST and GST, used to stimulate the PBMC and T cells were assembled into proteosomes, which are pure protein micelles free of detergents [35]. This was performed to eliminate all detergent that could be potentially lymphotoxic.

ELISA and antibody quantification. The concentrations of anti-PIA– and anti-PIB–specific IgG present in the sera of subjects infected with N. gonorrhoeae and those of normal uninfected volunteers were determined by use of a quantitative ELISA, as described elsewhere [35], with the following alterations. In place of murine anti-murine reagents, human IgG and anti-human IgG alkaline phosphatase (Sigma, St. Louis) were used to quantitate anti-Por–specific human IgG in patient sera. The concentration of IL-4 in PBMC culture supernatants was determined by use of an Immunotek enzyme immunoassay kit according to the manufacturer’s instructions (Immunotek, Marseille, France).

PBMC preparation. PBMC from individuals with laboratory-proven gonococcal infections and noninfected controls were isolated as described elsewhere [36]. Briefly, PBMC were obtained by centrifugation of heparinized whole blood over Ficoll-Hypaque (Sigma). Cells were subsequently washed twice with Hanks’ buffered saline solution and then resuspended in XVIVO-15 (a culture medium that does not require the addition of fetal calf serum for adequate growth and stimulation of PBMC; BioWhittaker, Walkersville, MD). T cells were purified from PBMC by immunomagnetic bead sorting (Dynal, Lake Success, NY). PBMC were suspended to a concentration of 2 × 10⁶ cells/mL in XVIVO-15 and then incubated with anti-CD14 and anti-CD19 immunomagnetic beads at a ratio of 5:1 (beads: target cells) for 1 h at 4°C. Rosette cells were separated by placement of the reaction tube adjacent to a magnet, and nonrossetted T cells were collected, washed, and resuspended in XVIVO-15 to a concentration of 1 × 10⁶ cells/mL. Subsequently, they were analyzed for T cell purity by use of FACS analysis [37]; this was found to be ≥95% (see below).

Lymphocyte proliferation. PBMC from infected and noninfected individuals were plated onto 96-well flat-bottomed plates (Becton-Dickinson, Mountain View) in 100-μL aliquots (1 × 10⁶ cells/mL). Serial dilutions of the appropriate antigens (PIA, PIB, GroES-GST, GST, or phytohemagglutinin [PHA]) were added to each well in triplicate. Control wells, in triplicate, contained medium alone. The cells were incubated with each of the antigens for 7 days in a humidified 5% CO₂ incubator at 37°C. Eighteen hours prior to harvesting, 1 μCi of [3H]-thymidine (New England Nuclear Life Sciences Products, Boston) in a 10-μL volume was added to each well. For some representative patients, purified T cells (see above) were plated onto 96-well plates (1 × 10⁶ cells/mL; 100 μL/well) containing 2 × 10⁻³/mL or 1 × 10⁻³/mL purified irradiated (3000 rad in a Cobalt irradiator) syngeneic PBMC used as antigen-presenting cells (APCs) [36]. Proliferation is reported either as [3H]-thymidine uptake in counts per minute (cpm) or as a stimulation index (SI; average cpm of wells with PBMC incubated with antigen divided by average cpm of wells with PBMC incubated with medium alone for each individual patient).

Cytokine analysis. The cytokine profiles of PIB-stimulated T cells isolated from N. gonorrhoeae–infected subjects or noninfected control subjects were analyzed by use of intracellular staining and FACS analysis [37–39]. PBMC (1 × 10⁶ cells/mL) were stimulated with PIB (10 μg/mL) for 7 days in a bulk culture. During the final 4 h of incubation, brefeldin A (10 μg/mL; Sigma), a protein secretion inhibitor, was added to PBMC to maximize the sensitivity of intracellular cytokine staining. Brefeldin was omitted when super-
natants were analyzed for the presence of cytokines. After incubation with antigen, PBMC were washed in PBS, then aliquotted and stained with fluorochrome-conjugated monoclonal antibodies (mAbs) specific for CD3 (PerCP), along with mAbs specific for either CD4 (FITC) or CD8 (FITC). After extracellular staining, PBMC were permeabilized with 0.1% saponin (Sigma) in PBS. The cells were then stained with PE-conjugated anti-cytokine mAb (anti-IL-2, -IL-4, -IL-10, -IFN-γ, and -TNF-α) to detect the presence of intracellular cytokines. Expression of the different surface markers and intracellular cytokines was then examined by first gating on CD3+ cells (T cell-specific) and then determining CD4 or CD8 staining versus cytokine staining in the CD3+ population by FACS analysis (FACScan; Becton Dickinson, San Jose, CA) flow cytometer and CellQuest acquisition and analysis software (Becton Dickinson, San Jose). Determination of cytokine staining for VLAα/β-positive or -negative T lymphocytes was performed as follows. PBMC were labeled after antigen incubation with anti-CD4 or -CD8 conjugated with FITC and anti-VLAα/β, labeled with biotin/streptavidin-PerCP. After extracellular staining, the cells were permeabilized as described above and probed with anti-cytokine mAbs conjugated with PE. Cytokine responses were determined by first gating on either CD4 or CD8+ T cells in FACS analysis and determining VLAα/β staining versus cytokine staining in either the CD4 or CD8 population.

Statistical analysis. Differences in antibody levels and proliferative and cytokine responses in the patient populations were compared by nonparametric ranked sum analysis (Wilcoxon ranked sum analysis for statistical significance) using SigmaStat (SPSS, Chicago) computer software. Standard deviations or errors were also determined using SigmaStat (SPSS) computer software.

Results

Anti-Por IgG response in patients with urogenital gonococcal infections. Previous reports have shown that antigonococcal porin antibodies can be detected in the sera of subjects with urogenital gonococcal infections [13–15]. To determine whether infected individuals in this study were mounting a B cell response against gonococcal porins, their sera were screened for anti-PIA and anti-PIB IgG antibodies by use of a quantitative ELISA. A mean of 5.31 ± 5.62 µg/mL of anti-PIA IgG (range, 0.27–23.2 µg/mL; median, 3.04 µg/mL) was detected in the sera of infected subjects (figure 1). Similarly, a mean of 3.51 ± 3.35 µg/mL of anti-PIB IgG (range, 0.30–13.1 µg/mL; median, 1.62 µg/mL) was also detected in these sera (figure 1). Uninfected control subjects averaged ≏16% levels of these specific IgGs, compared with the infected subjects (0.47 ± 0.11 µg/mL of anti-PIA IgG and 0.57 ± 0.27 µg/mL of anti-PIB IgG [figure 1]).

Stimulation of PBMC from N. gonorrhoeae–infected individuals with Por. We surmised that the anti-Por T cell response, which would be necessary for induction of antigen-specific B cell responses, would also be present in subjects with urogenital gonococcal infection. PBMC isolated from infected subjects were stimulated in vitro for 7 days with increasing concentrations of Por and then were examined for proliferative responses by use of a [3H]-thymidine incorporation assay. Figure 2 illustrates results from a single representative infected subject (figure 2A) and uninfected control subject (figure 2B), as well as composite results, expressed as SIs, from 30 infected individuals (figure 2C and 2D). A dose-dependent and antigen-specific proliferative response was observed when PBMC from infected subjects were stimulated with PIA at concentrations of 0.0001–100 µg/mL (0.003–3000 nM; figure 2C). PIA antigen–specific proliferative responses (SI ≥ 2.5) were observed in 80% (24/30) of the patients examined (figure 2C). Furthermore, in 67% (20/30) of the subjects examined, dose-dependent and antigen-specific proliferative responses (SI ≥ 2.5) were also measured after incubation with PIB at concentrations of 0.0001–100 µg/mL (0.003–3000 nM; figure 2D). No significant proliferative responses were measured when PBMC were incubated with GroES (SI, 1.3 ± 0.6) [40, 41] or GST (SI, 1.1 ± 0.6; figure 2A). No differences were observed between men and women.

These results indicate that a cellular immune response generated against Por could be detected in subjects infected with N. gonorrhoeae. To assess whether these cellular proliferative responses against gonococcal Por were antigen-specific and not due to cross-reactive responses to other proteins having porin homology [12], the proliferative responses of PBMC from normal volunteers to PIA and PIB were measured. No significant proliferative responses were seen in these normal volunteers when their PBMC were stimulated with PIA, PIB, or the control antigens (figure 2B). PBMC proliferative responses were ob-
Figure 2. Detection of cellular immune responses toward gonococcal porin (Por) in gonococcal-infected subjects. Peripheral blood mononuclear cells (PBMC; $1 \times 10^6$ cells/mL) isolated from 1 gonococcal-infected (A) and 1 gonococcal-uninfected (B) individual were incubated with medium alone (*) or with various concentrations of protein IA (PIA; ●), protein IB (PIB; ■), GroES (▲), or glutathion-S-transferase (GST; ×) for 7 days. Cells were pulsed with [³H]-thymidine 18 h prior to harvesting. Data are mean ± SD from 1 representative experiment. Stimulation indices mean [SI] of Por-stimulated PBMC from gonococcal-infected subjects after incubation with medium alone or with various concentrations of PIA (C) or PIB (D) for 7 days. Cells were pulsed with [³H]-thymidine 18 h prior to harvesting. The data are reported as a box/whiskers plot, where the upper and lower levels of the box represent the 75th and 25th percentiles and the whiskers represent the 90th and 10th percentiles. The open circles represent outliers. The solid line is the median value, and the dotted line is the mean. The maximal proliferative response of the PBMC when stimulated with a nonrelevant control antigen, GST, was an SI of 1.1 ± 0.6. All PBMC proliferated when stimulated with phytohemagglutinin at 10 $\mu$g/mL (SI, 98 ± 82). Experiments were performed for 30 patients and 5 normal volunteers. SI is the average cpm of wells with PBMC incubated with antigen divided by the average cpm of wells with PBMC incubated with medium alone for each individual patient.

Por stimulation of purified T lymphocytes in subjects infected with N. gonorrhoeae. Previous evidence indicates that antineisserial porins possess B cell mitogenic activity [42–45]. To determine whether the cellular proliferation after Por incubation with PBMC was due to antigen-specific T cell proliferation rather than nonspecific B cell (or other non-T cell) proliferation, experiments similar to the PBMC proliferation studies described above were performed, but by use of purified T lymphocytes and autologous irradiated PBMC as APCs. As shown in figure 3, antigen-specific proliferative responses were observed when purified T cells were stimulated with PIB in the presence of various numbers of irradiated syngeneic APCs. No proliferative responses were seen when T cells alone or the irradiated APCs were incubated with PIB. One representative experiment ($n = 5$) is displayed in figure 3.

Cytokine production of T lymphocytes from gonococcal-infected individuals after stimulation with Por. Having demonstrated that porin-specific T cells were present in subjects infected with N. gonorrhoeae, we next examined the cytokine...
profiles of the porin-stimulated T cells to determine whether the responses generated were characteristic of Th1 or Th2 cells. Peripheral blood mononuclear cells (PBMC) or purified T cells and irradiated antigen-presenting cells (APCs) isolated from infected subjects were incubated with medium alone or with various concentrations of protein IB (PIB) for 7 days. Purified T cells (1 × 10⁶/mL) were incubated with Por in the presence of 2 × 10⁶/mL (●) or 1 × 10⁶/mL (■) irradiated syngeneic APCs or no additional APCs (○). PBMC (▲) or irradiated APCs alone (×) were also incubated with Por or medium as controls. Cells were pulsed with [³H]-thymidine 18 h prior to harvesting. Data are stimulation indices defined as the fold increase in cellular proliferation from 1 representative experiment (n = 5; see text and figure 2 legend).

In a subset of patients (n = 3), intracellular cytokine analysis was performed at earlier time points (at days 2 and 4 in addition to day 7) of incubation of the patients’ PBMC with Por or medium. A similar pattern of cytokine production was seen. Only IL-4 production was detected (but to a lesser degree compared with day-7 results), whereas no production of the other tested cytokines (IL-2, IL-10, IFN-γ, or TNF-α) was noted at these earlier time points. Proliferation of infected patients’ PBMC when the cells were incubated with Por, as determined by [³H]-thymidine incorporation, was also measured at these earlier time points, and, although greater than baseline, the SIs were less than the SI at day 7 (data not shown).

Cytokine production of VLAα/β⁺-positive T lymphocytes from gonococcal-infected subjects after stimulation with Por. Mucosal lymphocytes preferentially home to their site of origin or traffic to other mucosal sites via the mucosal homing receptor VLAα/β⁺, which binds to the mucosal addressin MAdCAM-1 [46, 47]. Interestingly, antigen-specific VLAα/β⁺-positive lymphocytes have been detected in the systemic circulation after mucosal antigenic challenge [48–51]. Moreover, recent work has demonstrated that women with gonococcal disease have higher numbers of CD4⁺ T cells in vaginal washings compared with uninfected individuals, indicating that CD4⁺ T cells can circulate to the vaginal mucosa [52]. We have shown that circulating T lymphocytes from subjects infected with N. gonorrhoeae generate IL-4 when incubated with Por. Because gonorrhea is a mucosal disease, the ability of VLAα/β⁺-positive T cells from infected subjects to produce IL-4 after Por incubation was evaluated. PBMC from infected subjects were stimulated with PIB (10 µg/mL) for 7 days in vitro, and VLAα/β⁺-positive CD4⁺ or CD8⁺ T cells were examined for cytokine production by use of intracellular staining and FACS analysis. Antigenic stimulation with PIB significantly increased the percentage of IL-4⁺ VLAα/β⁺-positive CD4⁺ T cells (51.2% ± 17.1%, P < .0001) and CD8⁺ T cells (54.4% ± 16.4%, P < .003), compared with incubation with medium alone (24.4% ± 23.5% and 26.4% ± 26.0%, respectively; figure 5B).

Significant increases (P < .001) in the percentage of IL-4⁺ VLAα/β⁻-negative CD4⁺ T cells (from 22.7% ± 20.7% [medium] to 43.4% ± 16.1% [Por]) and CD8⁺ T cells (from 26.4% ± 26.9% [medium] to 43.7% ± 16.1% [Por]) were also observed after stimulation with Por (figure 5C). No significant production of IL-2, IL-10, IFN-γ, or TNF-α was observed in either PIB-incubated or medium-incubated VLAα/β⁻-positive
Figure 4. T cells from gonococcal-infected subjects generate interleukin (IL)-4 after stimulation with gonococcal porin (Por). Peripheral blood mononuclear cells (PBMC; $1 \times 10^6$/mL) from infected subjects were incubated with medium alone or protein IB (PIB; 10 µg/mL) for 7 days. By use of flow cytometric analysis, cytokine production was determined by labeling cells extracellularly with anti-CD4-fluorescein isothiocyanate (FITC) or anti-CD8-FITC and anti-CD3-CyChromeC and intracellularly with anti-IL-2, anti-IL-4, anti-IL-10, anti-interferon (IFN)-γ, anti-tumor necrosis factor (TNF)-α-phycoerythrin (PE; black line), or rat IgG-PE (solid gray). A total of $1 \times 10^5$ CD3+ cells were analyzed per sample. (A) Cytokine staining in CD4+ T cells; (B) Cytokine staining in CD8+ T cells. Histograms are from 1 representative experiment ($n = 10$).
Table 1. Detection of interleukin-4 in culture supernatants of peripheral blood mononuclear cells (PBMC) stimulated with porin from subjects with gonorrhea.

<table>
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<tr>
<th>Subject group</th>
<th>Medium&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Protein IB&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>Uninfected</td>
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<tr>
<td>Infected</td>
<td>Not detectable&lt;sup&gt;d&lt;/sup&gt;</td>
<td>48.0±4.4 pg/mL</td>
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<sup>a</sup> PBMC used at a concentration of 1×10<sup>6</sup> cells/mL were cultured in medium for 7 days.
<sup>b</sup> PBMC used at a concentration of 1×10<sup>6</sup> cells/mL were incubated with protein IB (10 μg/mL) for 7 days.
<sup>c</sup> Tissue culture supernatants were assayed from 5 uninfected subjects.
<sup>d</sup> Level of sensitivity for quantitative ELISA was <20.0 pg/mL.
<sup>e</sup> Tissue culture supernatants were assayed from 10 infected patients.

Discussion

The specific immune responses generated against gonococcal antigens in patients infected with this mucosal pathogen are largely unknown [13, 18, 20]. The significance of systemic versus mucosal immune responses in the susceptibility to and protection from gonococcal infection remains undefined. Most studies that have examined human antigenonococcal immune responses have focused predominantly on humoral immune responses [13, 18, 20]. As antibody responses against proteins, such as Por, must be T cell dependent, Por-specific T cells must also be present in subjects infected with N. gonorrhoeae. In humans, there have been minimal investigations examining the T cell responses to N. gonorrhoeae. In this study, T cell responses to Por in patients with gonorrhea were characterized to evaluate, as a first step, whether T cell antigenonococcal reactivity might be associated with immune protection from this disease.

Anti-Por IgG antibodies have been detected in the serum of patients with gonorrhea [14]. In this study, a broad range of serum anti-PIA and anti-PIB IgG concentrations was observed, and the levels were significantly higher than those in normal volunteers with no history of gonorrhea. These results coincide with previous reports demonstrating that serum antigenonococcal OMP antibodies can be present infected individuals [13, 15, 18].

The development of an antibody response against a pathogen entails a series of coordinated interactions between antigen-specific B cells and T cells involving the exchange of costimulator signals and cytokines necessary for cellular growth,
Figure 5. VLAα4/β7-positive T cells from gonococcal-infected subjects generate interleukin (IL)-4 after stimulation with gonococcal porin (Por). Peripheral blood mononuclear cells (PBMC; 1 x 10⁶ cells/mL) from infected subjects were incubated with medium alone or protein IB (PIB; 10 μg/mL) for 7 days. By use of flow cytometry analysis, IL-4 production was determined by labeling cells extracellularly with anti–CD4-fluorescein isothiocyanate (FITC) or anti–CD8–FITC and anti–VLAα4/β7–biotin/streptavidin–PerCP and intracellularly with anti–IL-4–phycoerythrin (PE) or rat IgG-PE. A total of 1 x 10⁶ CD4⁺ or CD8⁺ cells were analyzed per sample. (A) Cytokine staining in CD4⁺ and CD8⁺ VLAα4/β7-positive T cells after incubation with medium or PIB (10 μg/mL) for 7 days. Less than 5% of cells stained positively for the isotype control. Dot plots are from 1 representative experiment (n = 8 patients analyzed). Increase in IL-4–producing VLAα4/β7-positive (B) and VLAα4/β7-negative (C) T cells after incubation with medium or PIB (10 μg/mL) for 7 days. Less than 5% of cells stained positively for the isotype control. Data are the percentage of IL-4–positive T cells (n = 8 patients analyzed). The data are reported as a box/whiskers plot, where the upper and lower levels of the box represent the 75th and 25th percentiles and the whiskers represent the 90th and 10th percentiles. The open circles represent outliers. The solid line is the median value, and the dotted line is the mean.
proliferation, and immunoglobulin production [27, 53, 54]. Because anti-Por antibodies can be detected in patients with gonorrhea, it is logical to expect the presence of Por-specific T cells in them as well. Por-specific PBMC proliferation was observed in the majority of subjects with gonorrhea. In addition, Por-specific T cell proliferation was observed when purified T cells from subjects with gonorrhea were stimulated with Por-pulsed autologous APCs. Moreover, Por failed to stimulate T cell proliferation in the absence of APCs, demonstrating a lack of a direct mitogenic effect by porin on human T cells [42–45]. These observations demonstrate that individuals infected with N. gonorrhoeae generate an antigen-specific T cell response against Por.

We observed a broad variation in the concentration of serum IgG in infected subjects. Similarly, anti-Por PIA- and PIB-specific T lymphocyte responses were observed in 80% and 67% of the study patients, respectively. Similar responses to each of the Por antigens by individuals infected with strains containing differing Por serotypes may be explained by the overall similarity of the gonococcal porins. The porins used in this study were purified from two highly characterized laboratory strains of N. gonorrhoeae, PIA from UU1 and PIB from Pgh 3-2 [30, 35, 55]. Por is an allelic protein consisting of two main isoforms, PIA and PIB [7], and there is moderate antigenic variability between them [9, 12, 56]. It is possible that Por from UU1 and Pgh 3-2 differ structurally and antigenically from Por expressed by strains infecting some patients. Therefore, the inability to detect anti-Por immune responses in some infected subjects may be explained by a lack of specific T or B cell epitopes on Por derived from laboratory gonococcal strains that were present on Por of the infecting strains. However, neisserial-porin T cell epitopes are derived from sequences of the porin that contain significant homology and are in the putative membrane-spanning regions of this protein [57–59]. Therefore, the difference in T cell epitopes of the two well-characterized Por antigens versus those in Por derived from patients’ own infecting strains should be minimal. To stimulate the patients’ PBMC, which were obtained during the acute phase of gonococcal infection, with the Por from their infecting strains would be logistically difficult, because the Por could not be obtained at the time of the primary PBMC isolation. Therefore, we believe that measuring anti-Por T cell reactivity in patients with gonorrhea by use of Por from these two laboratory strains is a valid approach and permits the acquisition of meaningful data.

Cytokines perform an essential role in regulating immune responses. They are intricately involved in directing the type of immune response generated (Th1 or Th2) and can either enhance the clearance of a pathogen or influence its virulence [60, 61]. The cytokine profile of Por-stimulated T cells from infected individuals was examined by intracellular staining and FACS analysis. Significant increases in the percentage of IL-4-producing CD8+ T cells were seen after antigenic stimulation with Por, but no production of other cytokines (IL-2, IL-10, IFN-γ, or TNF-α) was observed, indicating that infected individuals mounted a Th2-type T cell response against Por.

Interestingly, significant increases in the percentage of IL-4-producing CD8+ T cells after Por stimulation were also seen. Previous investigations have likewise reported the presence of these cells in humans, also known as Tc2 cells [62–65]. Tc2 cells have also been generated in vitro via T cell receptor activation of CD8+ T cells in the presence of high concentrations of exogenous IL-4 (≥50 ng/mL), along with anti–IFN-γ or anti–IL-12 antibodies [62, 66, 67]. Tc2 cells observed in this study were unlikely to be experimental artifacts, because no exogenous IL-4 or anti-cytokine antibodies were added to the tissue culture. It is also improbable that these Tc2 cells arose from a nonspecific bystander effect, because the levels of exogenous IL-4 in the accompanying culture supernatants containing the Tc2 cells were too low for the bystander effect to occur. The detection of Tc2 cells in this setting is a novel finding, because gonorrhea is an acute mucosal disease, and Tc2 cells have been previously detected only in subjects with chronic diseases, such as AIDS or atopic dermatitis [62, 63, 65].

Although FACS data indicated that T cells incubated with either medium alone or Por produced IL-4, IL-4 was detected only in supernatants of Por-stimulated PBMC obtained from subjects infected with N. gonorrhoeae (table 1). IL-4 was possibly present in culture supernatants of both medium- and Por-stimulated PBMC from noninfected subjects and in the supernatants of medium-cultured PBMC from infected subjects, but, since the sensitivity of the quantitative ELISA was ≤20 pg/mL, it is possible that the concentration of IL-4 in the supernatants was below this level.

The FACS data indicated that after Por stimulation, T cells generated only IL-4. IL-2, IFN-γ, and TNF-α production normally occurs in T cells within the first 24–48 h of antigenic stimulation [68]. In this study, it is likely that the production of these cytokines either did not occur at all or was not sustained during Por stimulation. IL-4 appears to be the primary cytokine induced by Por stimulation of antigen-specific T cells, which would result in suppression of Th1-type cytokine production (e.g., IL-2, IFN-γ, and TNF-α), as would be expected to occur during a Th2-type T cell response [69]. Moreover, in the 3 patients in whom intracellular cytokine detection was performed on PBMC incubated with PIB or medium alone at days 2 and 4 in addition to day 7, only production of IL-4 was detected at these earlier time points, whereas no production of IL-2, IL-10, IFN-γ, or TNF-α was noted, consistent with our hypothesis that Por is stimulating a Th2-type response in patients with gonococcal disease.

Circulating lymphocytes traffic to mucosal sites via the mucosal homing receptor VLAα/β2 [46, 47, 70], and antigen-specific VLAα/β2-positive lymphocytes have been detected in the systemic circulation after mucosal antigenic challenge [47–49, 51, 71]. Because gonorrhea is a mucosal disease, we examined the level of IL-4 production in VLAα/β2-positive T cells on
incubation with Por. We observed significant increases in IL-4-producing VLAα/β7-positive CD4+ and VLAα/β7-positive CD8+ T cells after Por incubation. This demonstrates that circulating Por-specific T cells (producing IL-4 on Por incubation) could potentially traffic to mucosal sites that are infected. However, we also observed simultaneous increases in IL-4–producing VLAα/β7-negative CD4+ and VLAα/β7-negative CD8+ T cells after Por stimulation. There is little evidence that the urogenital immune system harbors aggregated lymphoid follicles [47, 72, 73]. Therefore, once antigenically stimulated, the dendritic and Langerhans’ cells located in the urogenital epithelium must traffic to nearby draining lymph nodes to activate T cells [47, 74, 75]. Antigen-stimulated dendritic cells can traffic to a variety of lymph nodes [76–78]; therefore, the observed PI-specific VLAα/β7-negative T cells were probably derived from systemic lymph nodes, where they had been activated by urogenital-derived dendritic cells that had processed and presented Por antigen.

Two recent publications offer a second hypothesis, at least in women. Yeaman et al. [79] have demonstrated that lymphoid aggregates can be detected in endometrial tissues of normal menstruating women and that the size of these aggregates varies during the menstrual cycle (secretory phase > proliferative phase > postmenstrual phase). In addition, Levine et al. [52] have demonstrated that the number of CD4+ T cells in vaginal washings from women with acute gonococcal disease is greater than that of normal uninfected women. Therefore, it is possible that antigen presentation of gonococcal proteins, such as Por antigen, and resultant T cell stimulation can be occurring locally and therefore can account for the presence of VLAα/β7-positive T cells specific for Por in the circulating lymphocyte pool.

This study demonstrates that individuals with gonorrhea generate a Th2-type immune response against Por. These findings correlate with previously published data indicating that individuals with mucosal *N. gonorrhoeae* infection develop antibodies directed against the major gonococcal outer membrane components, particularly the porins [14–16, 18, 20]. Most significantly, the results reported here illustrate that antigenococcal Por-specific T cells can be detected in the peripheral blood of individuals with gonorrhea. Experimental approaches that assay the peripheral blood of infected individuals for T cell reactivity directed against mucosal pathogens are also supported by this work, because we demonstrated that Por-specific T cells, locally stimulated, can be detected in the peripheral blood of individuals with gonorrhea. These cells could potentially traffic to mucosal sites. These observations will aid studies that examine T cell antigenococcal reactivity and its association with immune protection from this disease.

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