Lack of Demonstrable Memory T Cell Responses in Humans Who Have Spontaneously Recovered from Leptospirosis in the Peruvian Amazon

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Background. We tested the hypothesis that patients who have recovered from leptospirosis have peripheral blood memory T cells that are specific for *Leptospira* or *Leptospira* protein antigens.

Methods. Peripheral blood mononuclear cells (PBMCs) were obtained from patients who had recovered from leptospirosis, as well as from control individuals. PBMCs were assessed for in vitro proliferation, phenotyping, and cytokine production after stimulation with different strains of *Leptospira*, recombinant LipL32, or overlapping synthetic peptides of different outer membrane proteins.

Results. PBMCs from both control subjects and patients produced significant proliferative responses to all *Leptospira* strains. Proliferation from control PBMCs was significantly greater than responses produced by patient PBMCs. Select strains of *Leptospira* expanded both T cell receptor (TCR) αβ and TCRγδ T cells in both control subject and patient PBMCs. Patient and control subject PBMCs produced equivalent levels of tumor necrosis factor α and interferon γ, but patient PBMCs produced significantly less interleukin 10 than did control subject PBMCs after stimulation by different strains of *Leptospira*. PBMCs from patients failed to respond to recombinant LipL32 or to any of the *Leptospira* peptides.

Conclusion. *Leptospira* induced significant proliferative responses, TCRγδ T cell expansion, and cytokine production in both control subject and patient PBMCs. Patient PBMCs failed to recognize *Leptospira* protein antigens. Leptospirosis does not seem to generate memory T cells that can be activated by in vitro stimulation.

Leptospirosis is a zoonotic disease caused by spirochetes of the genus *Leptospira* [1]. This disease is found worldwide with a disproportionate impact in tropical climates [2]. Infection by *Leptospira* can lead to widely divergent clinical outcomes: symptomatic infection, which is common in regions where *Leptospira* are endemic [3]; an undifferentiated febrile illness or an aseptic meningitis syndrome with low morbidity [4]; or fulminant disease, which has diverse manifestations, including a septic shock–like syndrome, jaundice, renal failure, myocarditis or heart failure, hemorrhage, and death [5].

The role of cell-mediated immunity in host defense against *Leptospira* remains poorly understood in both animal models and human disease. One study has shown that cattle that receive a killed *Leptospira* vaccine develop protective immunity associated with CD4+ T cells and γδT cells that produce in vitro proliferative responses and interferon (IFN) γ secretion following stimulation with a *Leptospira* antigen preparation [6]. Naturally acquired immunity that protects against re-infection by *Leptospira* does occur, but this has been shown to be serovar-specific in animal models [7] and has been suggested to be mediated primarily by lipopolysaccharide (LPS)–specific antibody [7–10]. There is also evidence that antibodies specific to *Leptospira*...
membrane-associated proteins may play a role in host defense [7, 11] in animal models of vaccination.

Nonetheless, little is known about the human immune response to Leptospira. We have reported that in vitro stimulation of human peripheral blood mononuclear cells (PBMCs) obtained from leptospirosis-naive individuals with a pathogenic Leptospira isolate results in the expansion of not only T cell receptor (TCR) αβ T cells but also TCRγδ T cells and a high production of IFNγ and other Th1 cytokines [12]. In addition, an uncharacterized leptospiral glycolipoprotein has also been shown to induce in vitro production of tumor necrosis factor (TNF) α and interleukin (IL) 10 as well as up-regulate the expression of CD69 and human leukocyte antigen (HLA) DR on PBMCs [13]. Elevated levels of soluble IL-2R, IL-6, and TNFα have been demonstrated in serum samples obtained from patients treated for acute leptospirosis [14]. Heat-killed Leptospira have also been shown to induce IFNγ and IL-12 production from human whole-blood cultures [15]. Still, it is unknown whether people living in regions where leptospirosis is endemic develop naturally acquired protective immunity against severe disease or whether cellular immune responses develop following recovery from leptospirosis. A key question—whether Leptospira-infected patients who experience spontaneous recovery have protective immunological memory—is a fundamentally important question that has yet to be resolved. In this study, we tested the hypothesis that patients who have recovered from leptospirosis have peripheral blood memory T cells specific for Leptospira whose function could be demonstrated by in vitro recall responses to Leptospira or leptosporal proteins.

**MATERIALS AND METHODS**

**Reagents.** Purified protein derivative (PPD) was obtained from Mycos Research. Fluorescein isothiocyanate (FITC)/phycocerythrin (PE)—conjugated anti-human TCRαβ and TCRγδ was obtained from Caltag Laboratories. Overlapping peptides for in vitro stimulation of human PBMCs were from the following Leptospira species: (1) Leptospira interrogans L1130 LipL32, AAS69953.1, (2) L. interrogans L1130 LipL21, AAS68648.1, (3) L. interrogans L1130 OmpL1 AY461983, and (4) L. interrogans L1130 LipL41, AE017298. All peptides were 15 aa in length, with 10-aa overlaps between sequential peptides. The overlapping peptides covered the full length of each protein: (1) 53 peptides for LipL32, (2) 36 peptides for LipL21, (3) 62 peptides for OmpL1, and (4) 69 peptides for LipL41. Pools containing 10–12 peptides were prepared and used for in vitro stimulation. His6-LipL32 fusion protein was purified by affinity chromatography and dialyzed against phosphate buffered saline (PBS) containing 10% glycerol, 0.3% Triton X-100 [16]. The purity of the His6-LipL32 fusion protein was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis and shown to be free of LPS using an limulus amebocyte lysate test.

**Patients and control subjects.** There were 23 patients and 30 control subjects enrolled in the study. Isolates were not obtained from all patients. Diagnosis was made on the basis of microscopic agglutination. Although serological testing does not necessarily identify an infecting serovar [17] and there is broad cross-reactivity for different serovars (hence, the percentages add to >100%), the frequency of serovar identification was as follows: Icterohaemorrhagiae (30%), Copenhageni (26%), Bratislava (26%), Var10 (65%), Australis (17%), Autumnalis (8.7%), Canicola (13.0%), and Cynopteri (4.3%). In patients who had recovered from leptospirosis, the blood sample was collected 0.5–2.8 years after diagnosis by macroscopic agglutination testing. Control subjects (n = 30) were healthy volunteers with no history of Leptospira exposure (subjects lived in Lima, Peru, and were seronegative for Leptospira). For cell proliferation, phenotyping, and cytokine production, PBMCs were stimulated with different Leptospira species. Var10, RGA, and HAI188 were assessed for inducing cell proliferation, phenotyping, and/or cytokine production in PBMCs from 13 patients and 13 control subjects. Seven other different control subjects were stimulated with varying numbers of live versus formalin-fixed Leptospira (Var10 and RGA), and 6 of these control subjects were also assessed for proliferation following stimulation with HAI024, HAI864, and HRL280 strains. Finally, PBMCs from 10 patients and 10 control subjects were stimulated with recombinant proteins and synthetic peptides and tested for cell proliferation. The study was approved by the Committee of Ethics of the Universidad Peruana Cayetano Heredia and the University of California San Diego Human Subjects Protections Program; informed consent was provided by all subjects.

**Bacteria.** For in vitro stimulation of PBMCs, Leptospira strains were grown in modified Ellinghausen-McCullough-Johnson-Harris liquid to log phase, counted using a Petroff-Hauser counting chamber with a dark field microscope, and then washed once and adjusted to the correct density. In some experiments, the bacteria were formalin-fixed, 0.5% by 2 h then washed 4 times and adjusted to the correct number in sterile PBS. The following isolates were used in this study: RGA was obtained from the American Type Culture Collection and is a high-passage strain (ie, is a high-passage strain). Fresh isolates included Leptospira icterohae-morrhagiae and has been continuously cultured for decades (ie, is a high-passage strain). Fresh isolates included Leptospira icterohae-morrhagiae and has been continuously cultured for decades (ie, is a high-passage strain). Fresh isolates included Leptospira icterohae-morrhagiae and has been continuously cultured for decades (ie, is a high-passage strain). Fresh isolates included Leptospira icterohae-morrhagiae and has been continuously cultured for decades (ie, is a high-passage strain).
interrogans serovar Icterohaemorrhagiae), and (4) HRL280 (L. santarosai serovar unique as determined by pulsed-field gel electrophoresis [PFGE]). These strains were isolated from symptomatic patients in the Iquitos region of Peru and were identified by sequencing of the 16S ribosomal gene and PFGE [20].

**PBMC culture.** PBMCs were isolated using Ficoll-Paque Plus (Pharmacia). Cells were set up in 96-well plates at 2 × 10^5 cells/well (cell proliferation and cytokines) or in 24-well plates at 1 × 10^6 cells/well (cell phenotyping) with one of the following: (1) medium only, (2) varying numbers of live *Leptospira* species, (3) varying quantities of LipL32 recombinant, (4) 10 µg/mL from each peptide pool, with each pool containing no more than 12 peptides, or (5) PPD *Mycobacterium tuberculosis* (5 µg/mL). PPD was used as a positive control for measuring an in vitro memory response, because a majority of the population had been vaccinated with BCG. Cell cultures were incubated in Roswell Park Memorial Institute 1640 medium (Gibco) containing 5% heat-inactivated human serum and penicillin-streptomycin.

**Cell proliferation assay.** Cell proliferation was measured from 96-well plate cultures after varying times in culture. Each well was pulsed with 1 µCi of [³H] thymidine (Sigma) and cultured for 5 h. The total incorporated counts per min (CPM) of [³H] thymidine were determined by a 1205 Liquid Scintillation Counter (Betaplate). The mean CPM of triplicate cultures was calculated. Results are expressed as CPM or proliferation index, which is the ratio of the CPM of cultures exposed to antigen to CPM from control cultures treated with medium alone. A proliferation index ≥2.0 was considered to be positive.

**Flow cytometry analysis.** The phenotype of different T cell populations present in PBMCs stimulated by different antigens was determined using flow cytometry. PBMCs were cultured in 24-well plates (1 × 10^6 cells/mL) with different antigens and then assessed at 6 days for the percentages of T cells bearing TCRαβ versus TCRγδ. The PBMCs were incubated with a panel of monoclonal FITC- or PE-conjugated monoclonal antibodies specific for T cells expressing TCRαβ versus TCRγδ. In parallel, additional cells were also incubated with the corresponding isotype control monoclonals. After washing, cells were analyzed using a FACSCalibur (Becton Dickinson). The percentages of positive cells were obtained using the software Cellquest (Becton Dickinson).

**Cytokine measurements.** PBMC cultures, as described above, were incubated for 6 days. At the end of culture, supernatants were collected. TNFα, IFNγ, and IL-10 were measured in culture supernatants by enzyme-linked immunosorbent assay (ELISA) (BD OptEIA kit; BD Bioscience)

**Statistical analysis.** Data are expressed as mean values (± standard deviation). The proliferation index, cell surface phenotypes (TCRαβ vs TCRγδ), and cytokine profiles in the in vitro response to *Leptospira* antigens between PBMCs from patients who have recovered from leptospirosis versus control subjects were compared by means of the Mann-Whitney U test.

To compare treatments with different antigens (live or formalin-fixed bacteria), the Wilcoxon rank-sum test was used. *P* values <.05 indicate a significant statistical difference. Stata software, version 9.0 (Stata) was used for statistical analysis.

**RESULTS**

*Leptospira* induce proliferative responses in PBMCs from healthy humans. We have previously shown that PBMCs from normal healthy volunteers with no previous infection due to *Leptospira* gave high in vitro proliferative responses upon exposure to *L. interrogans* (strain M20) [12]. These vigorous proliferative responses were associated with the production of high levels of Th1 cytokines and expansion of both TCRαβ and TCRγδ T cells. As an essential first step towards determining whether PBMCs from patients who had recovered from leptospirosis could give an in vitro memory response, we initiated a series of experiments to investigate whether PBMCs from normal humans responded in a similar fashion to other *Leptospira*. PBMCs were assessed for proliferation following in vitro exposure to Var10 versus RGA. Data presented in Figure 1 show the results from a representative experiment in which we determined the best dose of bacteria for inducing a proliferative response and comparing fixed versus live *Leptospira*. Optimal proliferative responses were found using high numbers of *Leptospira* (10^7 cells/mL) and were maximal at day 6 of

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Figure 2. Different Leptospira strains induce proliferation of control peripheral blood mononuclear cells (PBMCs). PBMCs were cultured in microtiter wells at $2 \times 10^6$ cells/well with one of the following: (1) medium only, (2) purified protein derivative (PPD) (5 $\mu$g/mL), or (3) different strains of Leptospira ($1 \times 10^5$ cells). Cell proliferation was assessed at 6 days by measuring the amount of [3H] thymidine incorporation in counts per min (CPM). Results are from 1 experiment that was representative of 6 independent experiments (6 different controls).

PBMCs from patients who had recovered from leptospirosis fail to give enhanced in vitro proliferation to Leptospira. A series of experiments were then performed using PBMCs from patients who had been identified as having recovered from leptospirosis. The goal was to directly test the hypothesis that such patients would have peripheral blood memory T cells specific for Leptospira, whose function could be demonstrated by enhanced in vitro proliferative responses to Leptospira. We predicted that PBMCs from these patients would give a significantly higher proliferative response to Leptospira, compared with that of PBMCs from control individuals who had never been exposed to or infected with Leptospira. We then compared the in vitro proliferative responses of PBMCs from 13 different patients versus the responses of PBMCs from 13 different control volunteers to 3 different strains of Leptospira: Var10, RGA, and HAI188 (Figure 3). Surprisingly, when stimulated by Var10 and RGA, PBMCs from patients produced significantly lower responses, compared with responses by control PBMCs. Control PBMCs produced significantly higher responses to these Leptospira, and this result was reproducible. However, HAI188 induced lower (but identical) proliferative responses from both patient and control PBMCs. This finding was also observed at 3 days after stimulation and with varying numbers of either live or fixed Leptospira (data not shown). Even though PBMCs from patients gave a lower response to Leptospira, they produced higher responses to the recall antigen PPD, when compared with PBMCs from control subjects. Thus, patients who have recovered from leptospirosis seem to have a diminished or suppressed in vitro proliferative response to Leptospira.

Leptospira-induced expansion of TCR\(\alpha\beta\) versus TCR\(\gamma\delta\) T cells. To further investigate the TCR phenotypes associated with proliferative responses induced by different Leptospira strains, we performed a series of experiments to characterize the T cells present at 6 days of culture after exposure to 3 different Leptospira strains (Var10, RGA, and HAI188). Figure 4 shows the percentages of TCR\(\alpha\beta\) versus TCR\(\gamma\delta\) T cells present in cultures at 6 days after stimulation with the different Leptospira strains. TCR\(\alpha\beta\) T cells were the majority of T cells present in all cultures. The only significant difference between patients and control subjects for these T cells (TCR\(\alpha\beta\)) were cultures stimulated with RGA. In these cultures, control PBMCs contained slightly more TCR\(\alpha\beta\) T cells than did the patient PBMCs. However, there was a statistically significant difference in the percentage of TCR\(\gamma\delta\) T cells in Leptospira cultures when
Figure 4. Preferential expansion of T cell receptor (TCR) αβ+ (A) and TCRγδ+ (B) T cells. Patient and control peripheral blood mononuclear cells (PBMCs) were set up in 24-well culture wells (1 x 10^6 cells/well) and stimulated with Var10 versus RGA versus HA188 (1 x 10^6 cells). Cultures were terminated on day 6, and cells obtained from each culture were phenotyped by flow cytometry. Data shown are the mean of 13 experiments (13 different patients and 13 different control subjects). *P < .05 for the difference between responses of patient PBMCs versus control PBMCs.

Comparing the different strains and when comparing patients versus controls, Var10 consistently induced high percentages of TCRγδ T cells in control cultures. Surprisingly, this was not observed in PBMCs from patient cultures. In contrast, PBMCs from patients yielded a high percentage of TCRγδ T cells in cultures stimulated with RGA, compared with control PBMCs, which yielded significantly fewer TCRγδ T cells at 6 days of culture. The significance of these differences is unclear, but they seem to indicate that there is preferential expansion of TCRγδ T cells in response to different serovars.

Cytokine production by PBMCs from patients versus from control subjects. To explore whether antigen-induced cytokine production could be used to assess in vitro memory T cell responses, we compared cytokine production by PBMCs from patients versus cytokine production by PBMCs from control subjects after in vitro stimulation with Var10, RGA, or HA188. PBMCs were established as described above for measuring proliferation, except culture supernatants were harvested at 48 h and at 6 days of culture. Levels of IFNγ, TNFα, and IL10 were assessed in supernatants by ELISA (Figure 5), which used supernatants from cultures at 6 days. There was no difference in the levels of IFNγ or TNFα produced by PBMCs from patients versus controls when stimulated with any of the 3 different Leptospira strains. However, PBMCs from patients always produced significantly less IL10 than did PBMCs from control subjects, regardless of which Leptospira strain was used for stimulation; this finding held true after PPD stimulation, as well. Identical results were also obtained from supernatants obtained from cultures at 48 h after stimulation (data not shown). The significance of lower levels of IL10 being produced by PBMCs from patients is unclear. However, the absence of elevated levels of cytokine production by PBMCs from patients is compatible with the proliferation data, which also showed no differences between patient and control PBMCs.

Stimulation of PBMC by Leptospira proteins and peptides. Whole Leptospira, either fixed or live, induced proliferation in PBMCs from healthy control individuals (Figures 1 and 2). Because this phenomenon could complicate or mask memory T cell responses from patient PBMCs, we next investigated whether purified Leptospira proteins or synthetic peptides derived from previously recognized protein antigens could preferentially induce a high proliferative response in PBMCs from patients. Thus, PBMCs from patients and control subjects were assessed for proliferation at 6 days after incubation with one of the following: (1) medium, (2) PPD (positive control for recall responses), (3) recombinant LipL32, or (4) overlapping peptides from LipL32 (Figure 6A), Lip21, OmpL1, or LipL41 (Figure 6B). Although PBMCs from both patients and control subjects gave strong proliferative responses to the recall antigen PPD, no increase in proliferation was observed from either control or patient PBMCs when exposed to any of the peptides or LipL32. These results suggest that no T cell memory to these antigens developed during infection and recovery from leptospirosis.

DISCUSSION

The role of T cells in protection and recovery from leptospirosis remains poorly understood in humans. In the present study, we demonstrated that PBMCs from humans who spontaneously recovered from leptospirosis in the hyperendemic setting of the Peruvian Amazon do not mount an antigen-specific T cell proliferative response to leptospiral antigens. The lack of PBMC proliferation was in response to a variety of leptospiral
antigens, including intact whole *Leptospira* cells as well as recombinant and synthetic peptide antigens, which have previously been shown to be recognized by antibodies from human and animal model infections. We also observed that whole leptospiral cells stimulated PBMCs from both previously infected and *Leptospira*-naive humans. This observation could reflect a superantigen-like property associated with *Leptospira*, and it appeared to be more pronounced with long-term in vitro cultivated *Leptospira* than with fresh leptospiral isolates.

A major goal of this study was to compare PBMCs from patients who had recovered from leptospirosis with PBMCs from *Leptospira*-naive individuals. Surprisingly, we found that PBMCs from patients who had recovered from leptospirosis gave a significantly lower proliferative response to Var10 and RGA, compared with the response given by PBMCs from control subjects. However, TCRγδ-positive T cells expanded from patient PBMCs following in vitro exposure to the long-term in vitro cultivated *L. interrogans* serovar Icterohaemorrhagiae RGA strain, which was not the case for PBMCs from control subjects. The significance of this difference is unclear, but it might suggest that RGA is a common strain associated with this particular area of Peru and that the expansion of TCRγδ-positive T cells reflected a novel type of memory response in this T cell lineage. We also compared cytokine production by PBMCs from patients versus naive control subjects after in vitro stimulation by the 3 different *Leptospira* strains, Var10, RGA, and HAI188. PBMCs from control subjects and patients produced equivalent levels of TNFα and IFNγ after in vitro exposure to the 3 *Leptospira* strains tested. We cannot rule out the possibility that patient PBMCs might have developed memory but only to the infecting *Leptospira* strain. However, we believe this to be unlikely, because patient PBMCs failed to respond to any of the proteins or peptides. Interestingly, patient PBMCs always produced less IL10 than did PBMCs from control individuals. The significance of this difference is not clear but could reflect a difference in the cytokine profiles produced by PBMCs from patients versus by PBMCs from control subjects and will be further investigated.

In a final attempt to detect memory T cells after recovery from leptospirosis, PBMCs from patients versus PBMCs from naive control subjects were tested for in vitro proliferation after exposure to recombinant and synthetic peptide *Leptospira* antigens. This strategy would hopefully address the possibility that in vitro stimulation of PBMCs by whole *Leptospira* might be complicated by nonspecific or superantigen-like activity of *Leptospira*, which was capable of inducing high proliferation in control PBMCs. However, PBMCs from patients failed to give any proliferative response to either recombinant, purified, LPS-free LipL32, or to any of the overlapping peptides from 4 *Leptospira* outer membrane proteins.

We do not know whether people living in leptospirosis hy-

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**Figure 5.** Cytokine profile produced by peripheral blood mononuclear cells (PBMCs) obtained from control subjects versus patients. PBMCs were stimulated with one of the following: (1) medium only, (2) purified protein derivative (PPD) (5 μg/mL), or (3) *Leptospira* (1 × 10⁸ cells): Var10 versus RGA versus HAI188. Culture supernatants were collected at 6 days of culture and assessed for different cytokine levels. 

A, tumor necrosis factor (TNF) α; B, interferon (IFN) γ; and C, interleukin (IL) 10. Data presented are the mean of 13 experiments (13 different patients and 13 different controls). *P* < .05 for the difference between patients and control subjects.
Figure 6. Peripheral blood mononuclear cells (PBMCs) from patients fail to respond to *Leptospira* antigens. PBMCs from patients who have recovered from leptospirosis and from control subjects were cultured with one of the following: (1) medium only, (2) purified protein derivative (PPD), (3) RGA (1 × 10^6 cells), (4) LipL32 recombinant protein, or (5) overlapping peptides to LipL32, LipL21, OmpL1, and LipL41. Data presented is the proliferation index at day 6 of culture. A, LipL32 recombinant and synthetic peptides; B, LipL21, OmpL1 and LipL41 synthetic peptides. Recombinant LipL32 were used at the indicated μg/200 μL. Data presented are from 1 experiment that was representative of 10 different experiments (10 different patients and 10 different control subjects). *P < .05 for patient versus control PBMCs.

perendemic regions, such as Iquitos, Peru, develop naturally acquired protective immunity against leptospirosis. If immunity develops in these patients, then one potential way to assess this immunity would be to show in vitro recall responses to either *Leptospira* or leptospiral antigens by PBMCs from immune patients who have recovered from infection. In vitro recall responses have been demonstrated using PBMCs from individuals immune to many different pathogens or antigens. Of direct relevance to the present study, *Burkholderia pseudomallei* is able to stimulate in vitro memory T cell–mediated cytokine and proliferative responses [21], in contrast to our findings here. Thus, we reasoned that, if immunity to leptospirosis develops in patients who recover from infection, then PBMCs from these patients should give enhanced in vitro proliferative responses, when compared with the responses from PBMCs from control individuals. Our previous studies showed that naive PBMCs gave high proliferative responses and produced high levels of Th1 cytokines in response to in vitro exposure to *L. interrogans* serovar Copenhageni, strain M20 [12]. This strain induced the expansion of TCRαβ CD4+ T cells and a 10–50-fold expansion of TCRγδ T cells. This observation was surprising and suggested that this strain might contain superantigen-like traits. Thus, a major goal
of this study was to investigate other strains of *Leptospira* for their ability to induce PBMC proliferative responses and cytokine production.

This study is, to our knowledge, the first to investigate the ability of PBMCs from patients who experienced spontaneous recovery from leptospirosis to give in vitro recall responses to *Leptospira* and individual leptospiral antigens. We show that PBMCs from healthy volunteers respond vigorously to in vitro exposure to whole *Leptospira* and that some *Leptospira* strains can induce expansion of both TCRαβ CD4+ and TCRγδ+ T cells. More importantly, we present evidence that suggests that leptospirosis does not lead to the generation of memory T cells specific for *Leptospira* that can be demonstrated by in vitro stimulation.

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