Evidence for Endotoxin as a Causative Factor for Leptospiral Uveitis in Humans

Chidambaranathan Gowri Priya,1 Sivakumar R. Ratbinam,2 and Veerappan Muthukkaruppan1

PURPOSE. To understand the pathogenetic mechanism of leptospiral uveitis by determining the profile of infiltrating cells, the levels of cytokines, and the causative factor in aqueous humor (AH).

METHODS. AH and blood samples were collected from 22 patients with leptospiral uveitis that was confirmed by microscopic agglutination test (MAT). Nine patients with Behc¸et’s uveitis, 10 with phacolytic uveitis, and 13 with age-related cataract were included as control subjects. A cytomteric bead array was used to estimate human inflammatory and Th1/Th2 cytokines. The level of endotoxin in AH was estimated by limulus amebocyte lysate (LAL) test and by dot blot analysis using a leptospiral serovar lipopolysaccharide (LPS)–specific monoclonal antibody.

RESULTS. Except for one patient with leptospiral uveitis, AH from all other patients and control subjects was negative for Gram-negative bacterial endotoxin by LAL test. However, a significant level of serovar Copenhageni LPS was observed in AH of patients with leptospiral uveitis seropositive for the same serovar by MAT, in contrast to its absence in all control subjects. A selective infiltration of neutrophils as well as a significant increase in the levels of protein and cytokines IL-12p70, TNF, IL-6, IL-8, and IL-10 was observed in AH of patients with leptospiral uveitis. Phacolytic uveitis was associated with a high proportion of activated macrophages and increased levels of IL-6 and IL-8, whereas Behc¸et’s uveitis was associated with a predominant infiltration of neutrophils and increased levels of IFN-γ.

CONCLUSIONS. The results demonstrate the presence of serovar-specific LPS in AH, and thus it is likely that endotoxin is a causative factor in leptospiral uveitis. (Invest Ophthalmol Vis Sci 2008;49:5419–5424) DOI:10.1167/iovs.08-2174

Leptospirosis is an acute febrile illness caused by the spirochete of the genus Leptospira. It is a potentially epidemic disease, commonly found in tropical countries with a humid climate. It can cause both life- and vision-threatening complications. Uveitis develops as a late complication of the systemic illness in 40% of patients and has been reported even 1 year after acute illness.1 A major postmonsoon epidemic outbreak of leptospiral uveitis was reported from southern India in 1993. The patients had acute, anterior or pan, nongranulomatous uveitis with hypopyon.2 The etiology of leptospiral uveitis was confirmed by demonstrating the presence of specific anti-leptospiral lipopolysaccharide (LPS) antibodies in the serum of patients with leptospiral uveitis.3,4 However, the reason for the occurrence of acute ocular inflammation several weeks after recovery from acute systemic illness is not known.

Several animal models are available that aid in understanding the pathogenetic mechanism associated with the development of uveitis in humans. One such model is endotoxin-induced uveitis (EIU), in which systemic or intraocularly injected Salmonella typhi LPS migrates into the anterior chamber, possibly through the iris-ciliary body, resulting in a prominent infiltration of neutrophils and macrophages. The severity of uveitis is associated with elevated mRNA expression of TNF-α, IL-1β, IL-6, IFN-γ, MCP-1, and MCP-2 in the iris and ciliary body.5,6 Further, intraocular injection of TNF, IL-1, IL-2, IL-6, or IFN-γ was shown to induce ocular inflammation in experimental animals.7,8 However, in humans, the pathogenetic mechanism in uveitis associated with systemic infection and the causative factors are still undefined. The purpose of the present study was to determine the profile of infiltrating cells, the levels of the different cytokines, and the lipopolysaccharide (LPS) in AH of patients with leptospiral uveitis.

MATERIALS AND METHODS

Recruitment of Cases

Patients attending the Uvea Clinic, Aravind Eye Hospital, with a clinical diagnosis of leptospiral uveitis3,7,9 were recruited for the study. Demographic information on age, sex, place of residence, and socioeconomic status was collected on each patient. After a preliminary examination by a nonophthalmologist physician and a general ophthalmologist, all patients had a standard uveitis work-up. To identify the patients with a specific uveitis diagnosis, laboratory, and ancillary investigations were tailored for each patient, as determined by history and physical findings on presentation. Anatomic location of the inflammation was assigned based on International Uveitis Study Group criteria.10 Established diagnostic criteria13 were used to rule out other etiologic diagnoses, including HLA B27–related uveitis, Behc¸et’s syndrome, sarcoidosis, syphilis, tuberculosis, leprosy, acute retinal necrosis, VKH syndrome, and sympathetic ophthalmia. Cases were classified as idiopathic when a specific diagnosis was not known and were not included in the present study.

Twenty-two patients with diagnosis of leptospiral uveitis based on a specific combination of clinical features3 and a positive serology by MAT were included in the study.7 Ten patients with phacolytic uveitis with hypopyon, nine with acute Behc¸et’s uveitis, three with Fuch’s heterochromic cyclitis, and two with sarcoidosis were selected as the nonleptospiral uveitis control group. Thirteen patients with uncompli-
cated, age-related cataract who showed no symptoms of systemic or ocular infection were also included as control subjects. The study was approved by the Institutional Review Board of Aravind Eye Hospital and adhered to the tenets of the Declaration of Helsinki. Informed consent was obtained from all the patients before recruitment. Approximately 100 μL AH was collected from patients with leptospiral uveitis by anterior chamber paracentesis. Aqueous samples from patients with phacoalytic uveitis, Behçet’s uveitis, or cataract were collected at the time of surgery. Blood (5 mL) was obtained from all patients for serologic analysis and smear preparation. All sera were collected at the time of surgery. Blood (5 mL) was obtained from all patients with phacolytic uveitis, Behçet’s uveitis, or cataract were included as control subjects. The study was approved by the Institutional Review Board of Aravind Eye Hospital and adhered to the tenets of the Declaration of Helsinki. Informed consent was obtained from all the patients before recruitment.

Materials

Twenty leptospiral serovars and the monoclonal antibody F70 24-15 were obtained from Royal Tropical Institute, Amsterdam, The Netherlands. Cytometric bead array (CBA) kits for human inflammatory cytokines (IL-8, IL-1β, IL-6, IL-10, TNF and IL-12) and Th1/Th2 cytokines (IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ) were purchased from BD-Pharmingen (San Diego, CA); biotinylated goat anti-mouse Ig was stored at 4°C. Cytometric bead array (CBA) kits for human inflammatory cytokines (IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ) were purchased from BD-Pharmingen (San Diego, CA); biotinylated goat anti-mouse Ig was stored at 4°C. Streptavidin-HRP, 4-CN, BSA, Bradford reagent, and Folin Ciocalteau reagents were obtained from DakoCytomation A/S (Glostrup, Denmark); streptavidin-Pharmingen (San Diego, CA); biotinylated goat anti-mouse Ig was stored at 4°C. Chromogenic limulus amebocyte lysate (LAL; QCL-1000) was purchased from Cambrex BioScience, HRP, 4-CN, BSA, Bradford reagent, and Folin Ciocalteau reagents were obtained from DakoCytomation A/S (Glostrup, Denmark); streptavidin-Pharmingen (San Diego, CA); biotinylated goat anti-mouse Ig was stored at 4°C. The protein concentration was determined in AH and serum by the Bradford method of Bradford and Lowry, respectively, using bovine serum albumin as the standard.

Microscopic Agglutination Test (MAT)

All the serum samples were tested for the presence of anti-leptospiral antibodies by MAT. A panel of 20 serovars of Leptospira sp. was used as antigens and the end titer was defined as the highest dilution of serum having 50% agglutination. MAT was considered positive at 1:100 dilution of serum.11,12

Cytospin

Cells in AH were separated by centrifugation at 2000 rpm for 10 minutes at 4°C. The supernatant was transferred to a fresh tube and stored at −80°C for cytokine analysis. The cell pellet was resuspended in 200 μL of phosphate-buffered saline (PBS) and deposited onto glass slides by centrifuging at 400 rpm for 3 minutes (Shandon Cytospin 3; Thermo Fisher Scientific, Waltham, MA). After air drying, the blood smear and cytospin preparations were stained with Giemsa. The nature of infiltrating cells was then analyzed, and a minimum of 200 cells were counted for each sample.

Cytokine Analysis

The levels of human inflammatory cytokines (IL-8, IL-1β, IL-6, IL-10, TNF, and IL-12) and Th1/Th2 cytokines (IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ) in AH and serum were determined by flow cytometry using CBA as per the manufacturer’s instructions (BD Biosciences). Briefly, the six capture bead populations with distinct fluorescence intensities that were coated with cytokine-specific capture antibodies were mixed together in equal volumes. To 50 μL of the mixed-bead population, 50 μL of the recombinant standards (20–5000 pg/mL) or AH or serum samples and 50 μL of PE-conjugated detection antibodies were added and incubated for 3 hours at 25°C in the dark, to form sandwich complexes. The beads were then washed with the wash buffer, and the sample data were acquired using flow cytometry (FACSCalibur with BD CBA Software; BD Biosciences). The standard curve and sample results were generated in graphic and tabular format.

Protein Estimation

The protein concentration was determined in AH and serum by the method of Bradford and Lowry, respectively, using bovine serum albumin as the standard.

Chromogenic LAL Test

The level of Gram-negative bacterial endotoxin was quantified using the LAL test as per the manufacturer’s instructions (Cambrex BioScience, Inc.). Briefly, 50 μL standard or AH from patients was mixed with LAL supplied in the kit and incubated at 37°C for 10 minutes. The substrate solution was then added and incubated at 37°C for an additional 6 minutes. The reaction was stopped with 25% vol/vol glacial acetic acid in LAL reagent water. The absorbance was then read at 405 nm with the ELISA reader. A standard curve was constructed by using the standard in the range 0.1 to 1.0 EU/mL, and the concentration of endotoxin in each sample was determined. Since we found inhibitory factors in AH, when spiked with the standard, the observed inhibition was overcome by heating the AH at 70°C for 10 minutes, at a dilution of 1:20.

Leptospiral LPS Estimation

A Leptospira interrogans serovar Icterohaemorrhagiae serovar Copenhageni-specific monoclonal antibody (F70 24-15) was used to test for the presence of leptospiral LPS in AH. Antigen containing LPS from five leptospiral serogroups (Australia, Autumnalis, Icterohaemorrhagiae, Louisiana, and Patoc) were prepared according to the published protocol and used to test for the specificity of the monoclonal antibody. The leptospiral antigen and AH were transferred to the NC membrane by a vacuum filtration method with a dot-blot apparatus (Bio-Rad, Hercules, CA). After blocking with 5% skimmed milk powder in PBS for 2 hours at room temperature, the blots were incubated with monoclonal antibody F70 C24 (1:1000), followed by biotinylated anti-mouse Ig (1:3000) and streptavidin HRP (1:1000) in 1% BSA in PBS-Tween 20 (PBS-T) for 1 hour each. The blots were washed with PBS-T after each incubation and developed with 4-CN. The results were read by measuring the intensity per square millimeter of the dot in the gel documentation system (Bio-Rad). Using different concentrations of the antigen containing LPS from L. interrogans serogroup Icterohaemorrhagiae (15–125 ng/dot), a standard curve was constructed with intensity per square millimeter in the x-axis and concentration in the y-axis. A linear curve was observed between the range 31 to 125 ng/dot, from which the unknown concentrations were calculated (Quantity One software; Bio-Rad). Since the volume of AH collected from patients with uveitis varied, it was not possible to load 100 μL in each uveitis sample. However, the concentration of LPS in all the samples was determined using the standard curve and extrapolated for 100 μL.

Statistical Analysis

Analysis and graphic representation of the data was performed using commercial software (Stata, ver. 8.2 [Stata, College Station, TX] and Excel [Microsoft, Redmond, WA]). The data were tested using Mann-Whitney rank sum test with a significance level of 0.05.

Results

All 22 patients with leptospiral uveitis were serologically confirmed by MAT for leptospiral infection. They were positive for the pathogenic L. interrogans serovars Copenhageni (n = 8), Icterohaemorrhagiae (n = 2), Autumnalis (n = 3), Australis (n = 2), Andamana (n = 1), Hardjo (n = 1), and Louisiana (n = 1) and to the saprophytic Leptospira biflexa serovar Patoc (n = 4). All the nonleptospiral uveitis and cataract patients recruited for the study were negative for leptospiral antibodies.

Protein Exudation in AH

A significant (P < 0.01) increase in the level of protein was observed in AH of patients with leptospiral (median [minimum, maximum]: 5.3 mg/mL [0.95, 53]), phacoalytic (10 mg/mL [3, 46.4]), or Behçet’s uveitis (13.94 mg/mL [2.0, 33.4]) compared with those with cataract (0.5 mg/mL [0.1, 0.8])
However, the serum protein levels were the same in all four groups (Fig. 1).

### Selective Infiltration of Neutrophils

A significantly higher proportion ($P < 0.001$) of neutrophils was observed in the AH of patients with leptospiral or Behc¸et’s uveitis than in those phacolytic uveitis (Table 1). The latter group was distinctly different from others on the basis of macrophages as predominant infiltrating cells. Comparison of the cellular profile in AH and corresponding blood samples indicated a selective infiltration of neutrophils in the AH of leptospiral and Behc¸et’s patients and macrophages in patients with phacolytic uveitis. No cells were observed in the AH of the cataract control subjects (Table 1).

### Cytokines in AH and Serum

Fifty microliters of undiluted AH or serum samples were analyzed with CBA for human inflammatory and Th1/Th2 cytokines. A significantly higher concentration of IL-6, IL-8, IL-12p70, TNF, and IL-10 was observed in AH of patients with leptospiral uveitis than in control subjects with cataract (Fig. 2). Further, leptospiral uveitis was significantly different from other types in IL-12p70, TNF, and IL-10. IL-6 levels were similar in all types of uveitis.

As shown in Figure 3, we did not observe increased levels of Th1/Th2 cytokines in patients with leptospiral uveitis. Of interest, the signatory Th1 cytokine IFN-$\gamma$ was significantly higher in Behc¸et’s uveitis than in control subjects with cataract. The levels of the inflammatory/Th1/Th2 cytokines in serum were below the detection limit in patients with leptospiral uveitis and control subjects.

### Level of Endotoxin in AH

**LAL Estimation.** AH samples from five patients with leptospiral uveitis seropositive by MAT, three with Behc¸et’s uveitis and three with cataract were tested for the presence of endotoxin by LAL assay. Except for one AH (0.656 EU/mL) from a patient with leptospiral uveitis, all were negative for Gram-negative bacterial endotoxin.

**Dot Blot analysis.** Antigenic preparations containing LPS from different serogroups were used to test for the specificity of the monoclonal antibody for \textit{L. interrogans} serogroup Icterohaemorrhagiae serovar Copenhageni. A positive reaction after Giemsa staining, a minimum of 200 cells were counted for each patient.

### Table 1. Profile of Infiltrating Cells in AH and Blood of Leptospiral Uveitis Patients and Control Subjects

<table>
<thead>
<tr>
<th>Cellular Profile</th>
<th>Median (Minimum, Maximum)</th>
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<tbody>
<tr>
<td><strong>Aqueous humor</strong></td>
<td></td>
</tr>
<tr>
<td>Neutrophils ($n = 10$)</td>
<td>86 (56, 99)*†</td>
</tr>
<tr>
<td>Lymphocytes ($n = 10$)</td>
<td>12 (1, 34)*†</td>
</tr>
<tr>
<td>Monocytes ($n = 6$)</td>
<td>85 (48, 91)*†</td>
</tr>
<tr>
<td>Macrophages ($n = 10$)</td>
<td>0</td>
</tr>
<tr>
<td>Eosinophils ($n = 10$)</td>
<td>0</td>
</tr>
<tr>
<td><strong>Blood</strong></td>
<td></td>
</tr>
<tr>
<td>Neutrophils ($n = 10$)</td>
<td>64 (50, 76)</td>
</tr>
<tr>
<td>Lymphocytes ($n = 10$)</td>
<td>62 (52, 81)</td>
</tr>
<tr>
<td>Monocytes ($n = 6$)</td>
<td>73 (63, 89)</td>
</tr>
<tr>
<td>Macrophages ($n = 10$)</td>
<td>69 (59, 76)</td>
</tr>
</tbody>
</table>

* Significantly different from AH of phacolytic uveitis patients ($P < 0.001$).
† Significantly different from the corresponding blood sample ($P < 0.005$).
was observed only with the antigen from serovar Copenhageni (Icter-Cop) and not with other serogroups, indicating the specificity of the antibody (Fig. 4A). Further, the positive reaction remained after the antigen was treated with proteinase K (Fig. 4B); but was totally lost after periodate treatment (Fig. 4C) confirming that LPS is the antigen detected by this monoclonal antibody. High levels of serovar-specific LPS was observed in AH of patients with leptospiral uveitis (Figs. 5, 6) and interestingly, the sera of these patients were MAT positive for the same serovar Copenhageni.

DISCUSSION

Though there are animal models that aid in understanding the pathogenic mechanism associated with the development of uveitis, only sparse information is available on humans. Most of these reports are based on analysis of the cytokine levels in a heterogenous group of patients with uveitis, only a few have analyzed the levels in the defined entity of uveitis. The present study is the first of its kind in selecting a homogenous group of patients with uveitis of confirmed leptospiral etiology to identify the profile of cytokines in AH and to identify the associated causative factor. The etiology in all the recruited leptospiral uveitis cases was confirmed by MAT, the gold-standard test for leptospirosis, even in a geographic region like India where it is more common. We have demonstrated that MAT positivity is due to IgM antibodies toward leptospiral LPS.

Analysis of AH and corresponding blood samples from patients with leptospiral uveitis revealed: (1) protein exudation, the first sign in the breakdown of the blood–aqueous barrier, (2) a selective infiltration of neutrophils into AH in contrast to...
that observed in autoimmune uveitis, mediated by T-cells\textsuperscript{20} and (3) a higher level of the inflammatory cytokines IL-12p70, IFN-γ, TNF, IL-6, IL-8, and the regulatory cytokine IL-10 in AH compared with their serum samples, indicating local production. Thus, the specific profile of cytokines in AH of patients with leptospiral uveitis indicates that it is mediated by inflammatory cytokines, since the signatory cytokines of Th1/Th2 were not observed. Further, in vitro studies have shown that heat-killed leptospires were able to induce production of IL-12p40, TNF-α, IL-6, IL-8, and IL-10 in AH, synthesized by the iris and ciliary body.\textsuperscript{5,27} Therefore, it is possible on the basis of these findings that leptospiral LPS may be the initiating factor for the development of acute anterior uveitis, several weeks to months after systemic infection. There are reports to suggest that LPS from the original infecting organism. Further, the control subjects with Behçet’s uveitis and cataract were negative for Gram-negative endotoxin in their AH. Therefore, demonstration of a significant concentration of infecting serovar specific LPS in AH suggests that leptospiral uveitis is endotoxin mediated. However, the source and pathologic effect of leptospiral LPS in the AH must be elucidated.

Behçet’s disease is a chronic inflammatory disorder characterized by recurrent attacks of different clinical manifestations, including oral ulcers, genital ulcers, uveitis, skin lesions, arthritis, venous thrombosis, arterial aneurysms, and lesions in the central nervous and gastrointestinal systems.\textsuperscript{33} The etiology of Behçet’s disease is unknown but is considered to be chronic, autoimmune uveitis. In the present study, a predominant infiltration of neutrophils and a higher concentration of IFN-γ were observed in AH of Behçet’s uveitis, confirming the earlier reports of Shimada et al.\textsuperscript{34} and Lacomba et al.\textsuperscript{17}

Phacolytic uveitis, caused by the leakage of the lens proteins was included in the present study as the noninfectious uveitis control. Accordingly, leptospiral uveitis could be distinguished from phacolytic uveitis on the basis of the pattern of infiltrating cells\textsuperscript{35} and cytokines.

This study provided evidence that distinguishes leptospiral uveitis from phacolytic uveitis and Behçet’s uveitis (Table 2). The specific combination of clinical features in leptospiral uveitis\textsuperscript{9} along with laboratory confirmation of leptospiral etiology, the nature of infiltrating cells, and the profile of cytokines collectively indicate that leptospiral uveitis is a distinct entity, different from phacolytic, Behçet’s, and possibly other forms of uveitis.

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References


