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LABORATORY INVESTIGATIONS

Synthetic Lipid A-Induced Uveitis and Endotoxin-Induced Uveitis—A Comparative Study

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Abstract: Endotoxin-induced uveitis (EIU) is an animal model of ocular inflammation induced by lipopolysaccharide (LPS). The lipid A (LA) region of the LPS chemical structure is believed to be responsible for virtually all the biological activities induced by LPS. The aim of this study was to perform a more detailed investigation of the potency of LA in reproducing EIU. Various doses of either LPS or LA were injected into the footpad of an inbred strain of Lewis rat and the inflammation patterns were compared by assessing the protein concentration, by cytological study, and by determining the inflammatory cell content in samples of aqueous humor obtained during 96-hour follow-up. Evaluation of the cell number and protein concentration ratio of both groups showed the LA-stimulated group presented a higher ratio than the LPS group (Welch's *t*-test, ($P < 0.00001$). It was noteworthy that even the injection of high doses of LPS could not reproduce the level of cellular infiltration induced by LA. Histological study confirmed the enhanced cellularity in the LA group, neutrophils being predominant in both the LPS- and the LA-stimulated groups. The divergent findings in these two models of uveitis may be valuable to further investigations of the process of inflammatory cell migration into the anterior chamber of the eye. **Jpn J Ophthalmol 1997; 41:355–361** © 1997 Japanese Ophthalmological Society

Key Words: Animal model, endotoxin-induced uveitis, lipid A, lipopolysaccharide, neutrophil, uveitis.

Introduction

Endotoxin-induced uveitis (EIU) is an animal model of uveitis induced by an injection of lipopolysaccharide (LPS).[1] It has been suggested that EIU could served as a model for human acute anterior uveitis associated with gram-negative infection. EIU can be produced by either intravitreal,[2,3] intracorneal,[4] intravenous,[1,5] intraperitoneal[6] or footpad[1,5,7] injection of LPS. LPS is capable of inducing uveitis in a dose-dependent[8] manner in a number of species, including rabbits, rats and mice.[9,10] EIU has been characterized by breakdown of the blood-aqueous barrier, leading subsequently to an extravasation of vascular fluid[6,11] protein and massive inflammatory cell infiltration into the anterior chamber (AC) of the eye, predominantly by neutrophils, a few monocytes/macrophages and T lymphocytes.[6,8]

LPS is an amphipathic constituent of the cell-envelope macromolecules of gram-negative bacteria.[12] Most of the biological activity of the endotoxin is ascribed to the lipid A[13] (LA) region of the LPS molecule.[14] The LA region of these strains of gram-negative bacteria differs in structure and in its effects. New insights in this field have made it possible to synthesize a large range of LA analogues,[15] from those with almost full biological activities[16–18] to LA analogues with very low biological activities.[19–22] Although it has already been reported that LA also induces ocular inflammation,[23] there have been no detailed investigations concerning its biological activity in the eye. Therefore, in this work, we carried out a comparative study between uveitis induced by *Escherichia coli* (*E. coli*) LPS or synthetic *E. coli* LA.

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Materials and Methods

Experiments were performed on female inbred strains of Lewis rats (Charles River Japan, Atsugi, Kanagawa) weighing 160–190 g. All rats were handled according to the ARVO Resolution on the Use of Animals in Research. We used the lyophilized LPS powder prepared by phenol extraction (*E. coli*, serotype 055:B5, lot 122H4125, Sigma, St. Louis, MO, USA) and the kit of synthetic LA [*E. coli*, LA-15-PP (506), molecular weight (MW) 2000.77, Dai-ichi Chemicals, Tokyo].

Induction of EIU

The LPS was dissolved in sterile saline 0.9% at concentrations of 62.5, 125, 250, 500, 1000, 1500, 2000 and 3000 μg/mL, and injected into a hind footpad of each animal with the precision microsyringe (Hamilton Co., Reno, NV, USA) in order to obtain a respective total dose per rat of 31.25, 62.5, 125, 250, 500, 750, 1000 and 1500 μg/kg. Control animals received an equal volume of saline. In a preliminary experiment, LPS dissolved in 0.025% triethylamine (TEA) failed to induce as much inflammation as obtained by LPS dissolved in saline (data not shown). Animals were sacrificed by CO_2 asphyxia 24 hours after LPS injection which has been reported to be the peak of ocular inflammation.[24]

Induction of Uveitis with LA

LA (1 mg) was dissolved in 0.025% TEA at concentrations of 25, 50, 100, 150, 200, 300, and 400 μg/mL in order to obtain a respective total dose of 12.5, 25, 50, 75, 100, 150 and 200 μg/kg when injected into the footpad. Assuming that the MW of LA is at least one-tenth of the MW of LPS, the initial dose of 100 μg/kg was selected (LPS dose response reached a plateau at 1000 μg/kg) to evaluate the time course response of LA. Animals were sacrificed by CO_2 asphyxia and samples of aqueous humor (AH) were obtained at 0, 6, 12, 18, 24, 30, 36, 48, 72 and 96 hours after the LA injection. For the dose-response analysis, all samples were collected at the peak time of inflammation.

AH Sampling

Approximately 30 μL of AH was obtained from each eyeball by inserting a 25-gauge scalp-vein needle (Terumo, Tokyo) into the AC of the eye under the operating microscope. The samples were transferred into a microfuge and mixed with EDTA (final-0.5% EDTA).

Assessment of Cells and Protein

Soon after the sampling procedure, the inflammatory cell content in AH was determined by means of the improved Neubauer hemocytometer under the microscope (Olympus, Tokyo). The remaining sample was centrifuged in order to precipitate the inflammatory cells, and the supernatant was collected and stored at −80°C until the protein assessment.

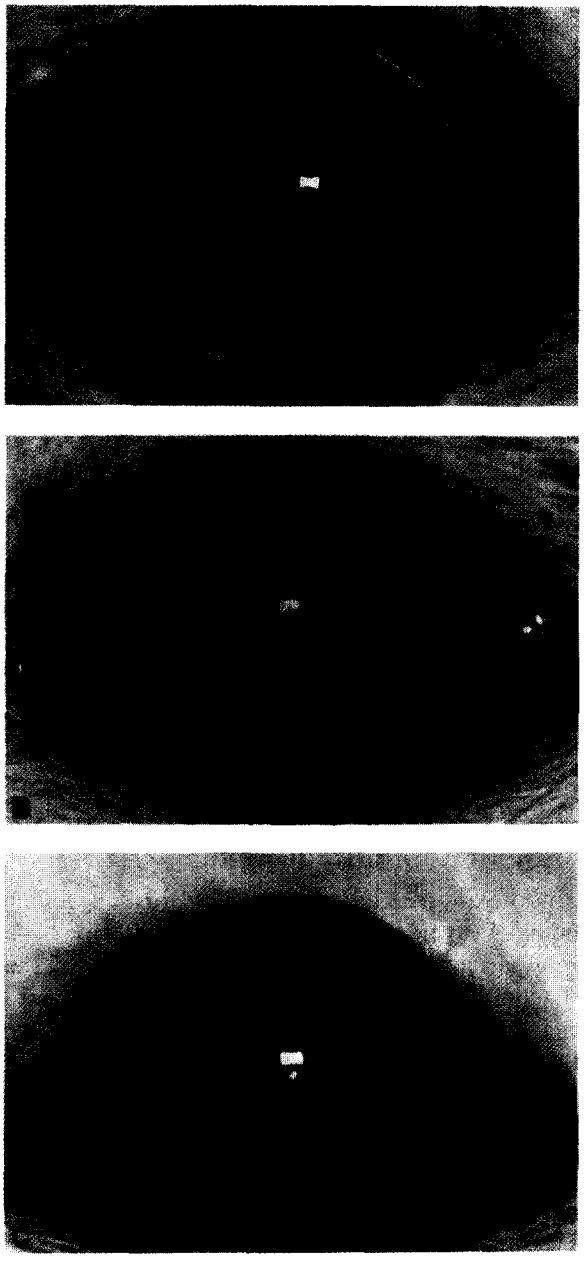

Figure 1. (**1A**) Normal rat eye (×18). (**1B**) Ocular findings in rat eye 24 hours after LPS injection (peak of inflammation). Iris vessels are conspicuously engorged. Note absence of fibrin clot in pupillary area (×18). (**1C**) Ocular findings in rat 30 hours after lipid A (LA) injection (peak of inflammation). Note engorgement of iris vessels, fibrin clot occluding pupil (arrowhead) and hypopyon (arrows) located between iris and crystalline lens (×18).

The protein concentration in AH was determined according to the method of protein assay described by Bradford et al[25] (Bio-Rad Labs, Richmond, CA, USA). Bovine serum albumin was used as the internal standard and the absorbance was read by microplate spectrophotometer (595 nm, MTP-22, Corona Electric, Tokyo).

Histology

Histological study of the eyeball was performed for the LPS- and LA-stimulated groups. Animals were sacrificed at the peak time of inflammation (24 and 30 hours, respectively), the eyeballs enucleated and submitted to hematoxylin and eosin (H&E) stain.

Cytological Study of Inflammatory Cells in AH

Smears of AH (3 μL) obtained at peak time of inflammation from either the LPS- or LA-stimulated group were prepared on silane-coated glass slides (Muto Pure Chemicals, Tokyo) and then dried at room temperature (RT) for 30 minutes. The specimens were fixed with 0.1 mol/L PBS (9.25% formalin and 45% acetone, pH = 6.6) for 30 seconds. They were briefly rinsed 4 times in order to avoid the detachment of the cells from the glass slide and dried at RT. Specimens were then stained with Diff-Quick I (International Reagents, Kobe) for 30 seconds and Diff-Quick II (International Reagents) for 5 seconds. After rinsing, they were dried at RT and covered with microcover glass using an immersion oil (Olympus).

White Blood Cell (WBC) Count in Peripheral Blood Specimens

Rats were anesthetized by IM injection of a solution of chlorpromazine and ketamine chloride at 2:1 ratio (Wintamin, Shionogi Pharmaceutical, Osaka; and Ketalar, Sankyo, Tokyo, respectively) at 0, 6, 12, 18, 24 or 30 hours after the stimulation with LPS or LA. Blood samples were drawn from the portal vein and mixed with EDTA (final–0.5% EDTA) for the determination of the number of peripheral WBC. After performing hemolysis, the number of WBC was assessed by an automated call counter (sensibility 6, threshold 5, model MEK-5158, Nihon-Kohden, Tokyo).

Statistical Analysis

Data are presented as the mean ± *SD*. Differences were analyzed for significance by the Student's two-tailed *t*-test or Welch's two-tailed *t*-test for independent means ($P \leq 0.05$).

Results

Clinical Findings

A normal rat eye is shown in Figure 1A. Figure 1B shows a rat eye 24 hours after stimulation with 1 mg/kg of LPS. Conspicuous engorgement of the radially stretched iris vessels is observed. Figure 1C shows a rat eye 30 hours after stimulation with 100 μg/kg of LA. In this group, the presence of high cellular concentration in the AC can be clinically visualized by the presence of the hypopyon between the crystalline lens and the iris. Fibrin deposition could also be noted in the pupillary area.

Cell and Protein Concentration in the AC

Ishiguro et al[24] have reported that ocular inflammation reached a peak at 24 hours after stimulation with LPS. Accordingly, we investigated the dose re-

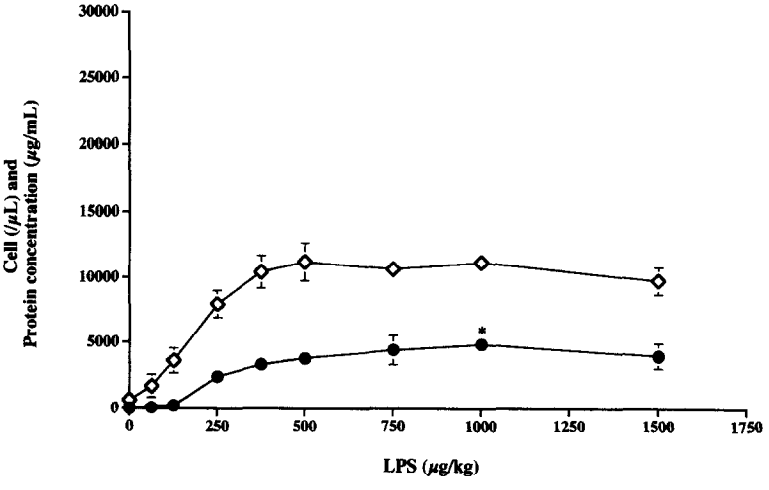

Figure 2. Dose-response evaluation of uveitis induced by LPS. Sample of AH was collected 24 hours after stimulation. Highest protein concentration (11.2 + 1.4 mg/mL) was obtained at dose of 500 μg/kg and peak of cell concentration (*4866 + 670 cell/μL) was obtained at dose of 1000 μg/mL. Values are means ± *SD* (n = 10). ◇ Protein (μg/mL); ● Cell (/μL).

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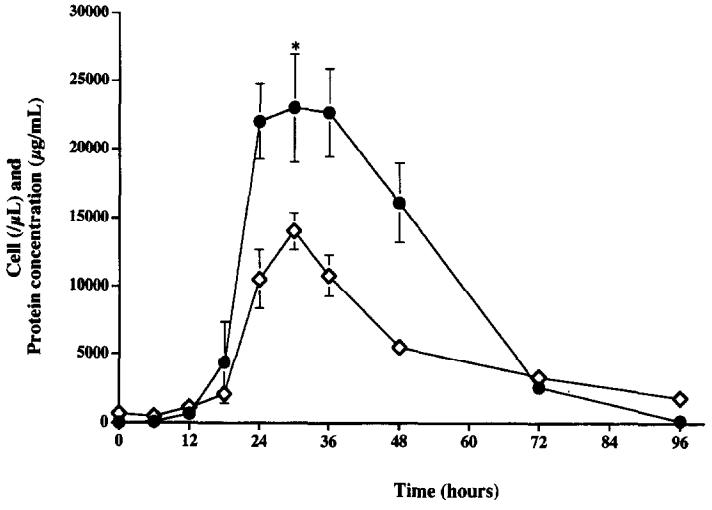

Figure 3. Time course evaluation of uveitis induced by LA (100 μg/kg). Peak of inflammation shown by both protein (14.5 ± 3.6 mg/mL) and cell (*22875 ± 3724 cell/μL) parameters was seen 30 hours after LA stimulation. Values are means ± *SD* (n = 8). ◇ protein (μg/mL); ● Cell (/μL).

sponse in samples collected at this time point. In the dose response of the LPS group, the cell number peaked at the dose of 1000 μg/kg (*4,866 ± 670 cells/μL) (n = 10) and the protein concentration peaked at dose 500 μg/kg (11.2 ± 1.4 mg/mL) (n = 10) (Figure 2). In the time course evaluation in the LA group (100 μg/kg), the peak of the inflammation was detected at 30 hours after footpad stimulation in both cell and protein parameters (Figure 3). The dose response in the LA group showed that both protein concentration and cell number peaked at dose 100 μg/kg (14.5 ± 3.6 mg/mL; *22875 ± 3724 cells/μL, respectively) (n = 8) (Figure 4). The evaluation of the cell number/protein concentration ratio at all peak times with every dose of both compounds showed that the LA group presented a higher ratio compared with the LPS group. This indicates that cells in the AC were more numerous in the LA group than in the LPS group at any given level of protein concentration. The number of cells in the AH of the LPS-stimulated group (1 mg/kg) and in the LA-stimulated group (100 μg/kg) at their respective peak time of inflammation were statistically significant. ($P < 0.00001$, *Welch's t-test) (Figure 5).

Histology

Histological examination performed with H&E stain confirmed the clinical findings observed in both the LPS and the LA groups. In the LPS group (Figure 6A) a mild cell infiltration in the ciliary body and AC was observed. In the LA group, the cellular migration into the AC was more evident, showing a pool of inflammatory cells between the crystalline lens and the iris (Figure 6B).

Cytology

Cytological study of the AH samples showed that the inflammatory cells present in the AC consisted mainly of neutrophils in both models. Mononuclear

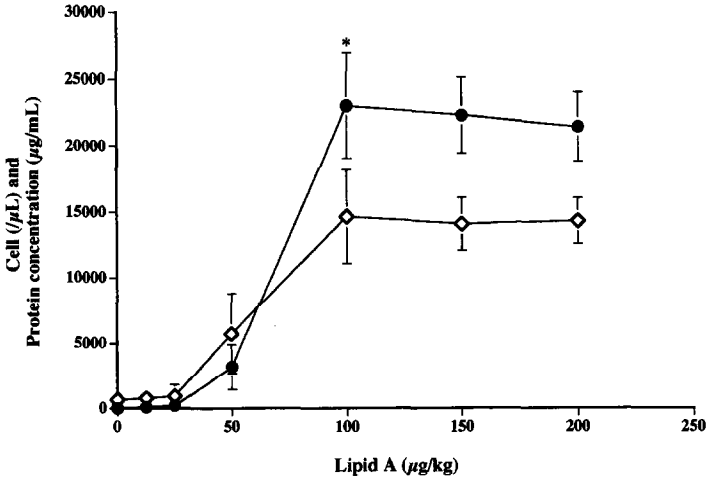

Figure 4. Dose-response evaluation of uveitis induced by LA. Sample of AH was collected 30 hours after stimulation. Highest protein concentration (14.5 ± 3.6 mg/mL) and peak of cell concentration (*22875 ± 3724 cell/μL) were obtained at dose of 100 μg/mL. Values are means ± *SD* (n = 8). ◇ Protein (μg/mL); ● Cell (/μL).

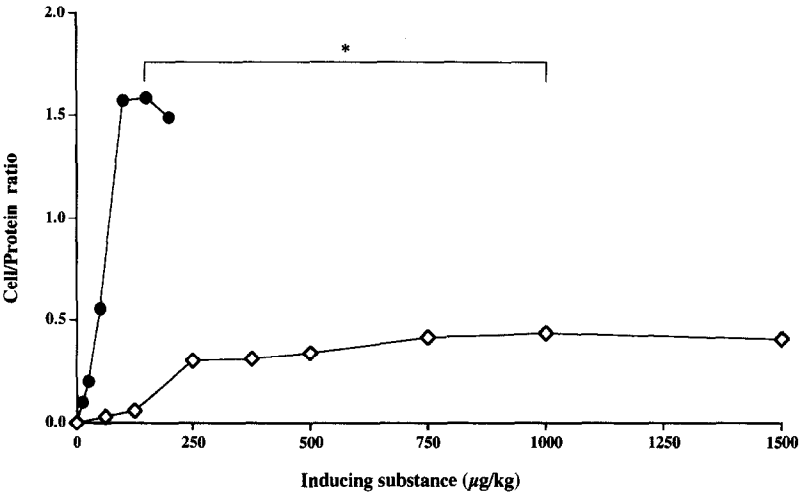

Figure 5. Cell/protein ratio indicates number of cells present in AC at given level of protein concentration. LA group presented higher ratio than LPS group. Difference between number of cells in the LA group (100 μg/kg; n = 8) and LPS group (1 mg/kg, n = 10) at their respective plateau level of cellular infiltration was statistically significant. *$P < 0.00001$, Welch's *t*-test. ◇ LPS; ● LA.

cells, macrophages and a few lymphocytes were also present (LPS group—Figure 7A, LA group—Figure 7B).

Systemic Evaluation

Animals receiving LPS showed leukopenia at 6 hours after the stimulation (57 ± 34%) ($n = 3$), returned to the baseline at 12 hours and showed a slight leukocytosis (180 ± 34%) ($n = 3$) at 30 hours. In the LA-stimulated group, the WBC count was near the baseline at 6 hours (112 ± 12%) ($n = 3$), elevated at 12 hours (167 ± 18%) ($n = 3$) and returned to the initial level at 18 hours (134 ± 8.1%) ($n = 3$), remaining stable until 30 hours (126 ± 3%) ($n = 3$) (Figure 8). At 24 hours, there was no statistically significant difference between the two models, LPS (129 ± 57%) ($n = 3$) and LA (136 ± 3%) ($n = 3$). At 30 hours the difference was statistically significant ($P < 0.001$), but paradoxically, the WBC count was higher in the LPS group (180 ± 3%) ($n = 3$) than in the LA group (126 ± 3%) ($n = 3$) ($P < 0.001$).

Discussion

In this study, we could confirm that the LA region of LPS does play an important role in inducing uveitis in the Lewis rat. At the beginning, we expected that the synthetic LA would induce ocular inflammation similar to that induced by LPS because there were previous reports ascribing to LA nearly all of the biological activity induced by LPS. However, this comparative investigation showed that LA induced increased inflammatory cell migration into the AC compared to the LPS model.

The analysis of the dose response curve of the LPS- and LA-stimulated models showed that the number of cells present at the plateau level differed

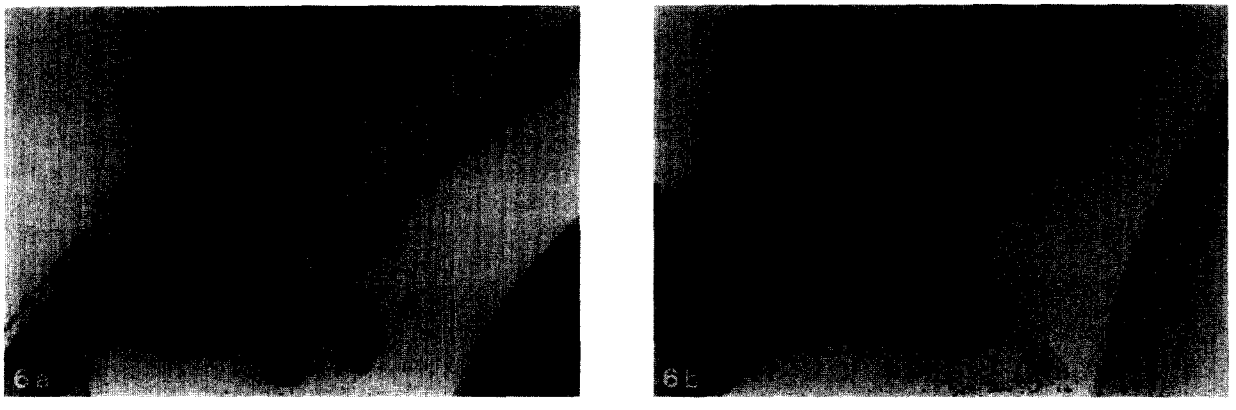

Figure 6. (**6a**) Section of eye from LPS-stimulated group. Note infiltration of inflammatory cells in ciliary body and in posterior chamber (H&E, ×25). (**6b**) Section of eye from LA group. Intense infiltration of ciliary body by inflammatory cells can be seen. Inflammatory cells are also present in posterior chamber, sedimented just behind iris (arrow) (H&E stain, ×25).

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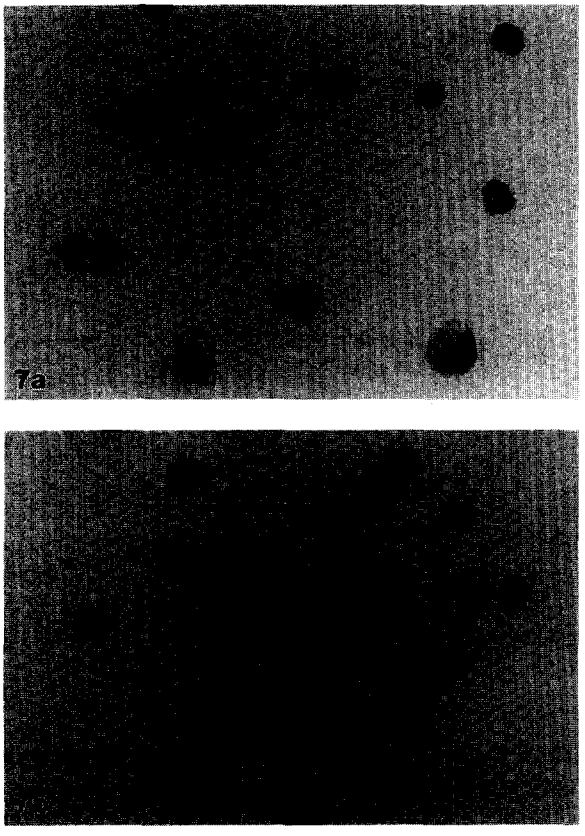

Figure 7. (**7a**) Smear of AH from LPS-stimulated group collected 24 hours after stimulation. Neutrophils are predominant but monocytes and lymphocytes are also present. (Diff Quick stain, ×200). (**7b**) Smear of AH from LA-stimulated group collected 30 hours after stimulation. Neutrophils are predominant, monocytes and lymphocytes can also be noted. (Diff Quick stain, ×200).

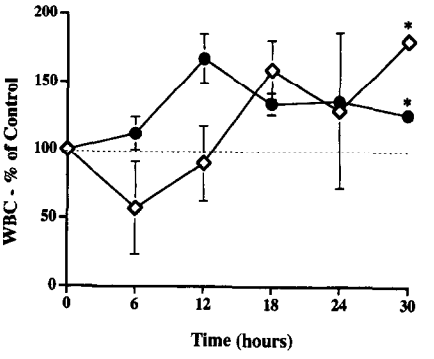

Figure 8. In LPS group, WBC number decreased at 6 hours after stimulation (57 ± 34%) if compared to control group (baseline). At 24 hours (peak time of inflammation in LPS group) WBC number was not significantly different between LPS and LA groups. At 30 hours (peak time of inflammation in LA group), LPS group showed higher WBC count than LA group. Values are means ± *SD* (n = 3). *$P < 0.001$, Student's t-test. ◇ LPS; ● LA.

significantly between the two models, that is, 4866 ± 670 cells/μL in the LPS group and 22875 ± 3724 cells/μL in the LA-stimulated group (Welch's t-test, $P < 0.00001$). It was noteworthy that even the injection of high doses of LPS could not reproduce the level of cellular infiltration induced by LA. Although the cytological findings did not differ in the type of inflammatory cell, the histology of ocular specimens showed a marked deposition of cells in the AC of the LA-stimulated group. If the cause of this discrepancy were due only to differences between the MW of LPS or LA, albeit at different concentrations, the dose-response curve of both substances should have reached a plateau at an equivalent level.

This fact led us to consider the presence of some other mechanism in the regulation of inflammatory cell migration induced in the LPS- or the LA-model of uveitis. These differences may be attributable to the presence of some active structure other than the LA region in the LPS molecule or some active substance existing in the LPS as an impurity, either acting directly or indirectly as an inhibitor of cell migration into the AC.

Manthey et al[26] have reported that commercially available phenol-extracted LPS still includes traces of endotoxin proteins.[27,28] They have also demonstrated in murine macrophages that the endotoxin proteins are positive-acting regulatory proteins for TNF-alpha and IL-1-beta gene expressions. Ishii et al[29] have also demonstrated the diverse activity of commercially available phenol-extracted LPS (containing 3% endotoxin proteins) and synthetic LA, in regard to the expression of the murine macrophage platelet-activating factor receptor gene. These cytokines and receptors are also known to be involved in the mechanism of EIU but their exact role is still not well understood.[30]

Furthermore, our systemic study revealed that the LPS model showed a decrease in the number of peripheral WBC in the first 6 hours followed by a significant leukocytosis 30 hours after stimulation. These findings could not be noted in the LA model, suggesting that LPS and LA produce different effects at the systemic level also.

Our ongoing detailed comparative study, focusing on the conspicuous difference in the amount of inflammatory cell migration into the anterior chamber of the LPS and LA models, may provide a new insight into the modulation of inflammatory cell migration in uveitis.

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