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Analyses of Vaccination Protocols for *Leptospira interrogans* Serovar Autumnalis in Hamsters

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Abstract. Leptospirosis, caused by *Leptospira* spp., is a zoonotic disease found worldwide. Killed whole cell leptospiral vaccines have been used as effective vaccines to elicit specific antibodies for protection. However, the involvement of cytokine responses after vaccination is not well characterized. Hamsters were immunized with killed *L. interrogans* serovar Autumnalis before challenge to study cytokine mRNA expression levels (interferon [IFN]-γ, tumor necrosis factor [TNF]-α, interleukin [IL]-10, and IL-4). Vaccinated groups showed 92–100% survival rates, whereas control hamsters died within 6–10 days. However, live organisms were detected in vaccinated groups, and mild to moderate pathology was observed early in infection. IFN-γ and TNF-α mRNA expression levels correlated with the severity of infection and lung pathology, whereas IL-4 and IL-10 expression levels were significantly higher in vaccinated groups. In summary, commonly used vaccines changed the cytokine profiles and protected hamsters from death but failed to stimulate sterile immunity and were unable to prevent the occurrence of pathology.

INTRODUCTION

Leptospirosis is a zoonotic disease of global importance. Host infection by pathogenic *Leptospira* produces a diverse array of clinical manifestations ranging from subclinical to undifferentiated febrile illness to jaundice, renal failure, and potentially pulmonary hemorrhage. Pathogenesis mechanisms of *Leptospira* and the nature of protective immunity against leptospiral infection are poorly understood. The pathogenic mechanisms may be divided into direct effects by *Leptospira* and the host immune response to infection. Naturally acquired immunity that protects against re-infection by *Leptospira* does occur and has been assumed to be humorally mediated.[1] Protective immunity may be mediated by antibodies directed against serovar-specific leptospiral lipopolysaccharide, which stimulates the innate immune system through a Toll-like receptor-2–dependent mechanism, another potential mechanism of either protective immunity or immunopathology.[2] Previous studies have suggested that antibodies against *Leptospira* membrane–associated proteins may play a role in host defense.[3] Severe pulmonary hemorrhage is the most critical clinical presentation in patients infected with the organism.[4–6] The most remarkable is profuse lung hemorrhage, which is predominantly caused by capillary involvement and thrombocytopenia.[7,8] There is evidence that *Leptospira* may activate or damage capillary endothelial cells, leading to profuse leaking of plasma into the interstitium with consequent hypovolemia and generalized hemorrhage.[8] Toxic substances produced by the pathogen, such as endotoxins,[9] or by cytokine-producing activated host cells, for example, tumor necrosis factor α (TNF-α),[10] seem to be important in pathogenesis. Although protective serovar-specific vaccines against leptospirosis have been developed and used, the roles of cytokine responses in protective immunity have not been thoroughly studied. Therefore, we evaluated the mRNA expression levels of the pro-inflammatory cytokines, TNF-α and interferon γ (IFN-γ), anti-inflammatory cytokine, interleukin 10 (IL-10), and a cytokine related to antibody production, interleukin 4 (IL-4). We also evaluated antibody responses to leptospiral infection in hamsters immunized with either heat-killed or formalin killed *Leptospira*, which are generally used as animal vaccines. The study also observed the presence of *Leptospira* in some organs and the histopathologic findings in hamsters.

MATERIALS AND METHODS

***Leptospira* strain and cultivation.** Pathogenic *Leptospira interrogans* serovar Autumnalis no. RY21 was isolated from a patient with leptospirosis in Rayong province, Thailand. The isolate was subsequently cultured in Ellinghausen–McCullough–Johnson–Harris (EMJH) medium (BD Difco; Becton, Dickinson and Co., Sparks, MD) with 0.1% agar and enriched with 3% inactivated rabbit serum at 28–30°C. The LD_{50} of this isolate for hamsters is 158 cells. To maintain bacterial pathogenicity, aliquots of culture in EMJH medium containing 2.5% dimethyl sulfoxide were stored in liquid nitrogen until needed.

Preparation of vaccine for immunization. The 10% formalin-killed and heat-killed *Leptospira* vaccines used for immunization were prepared as described.[11,12] In brief, formalin-killed leptospires were prepared from 7- to 10-day-old cultures harvested by centrifugation at 12,000 × *g* for 10 minutes. The bacteria were washed four times in phosphate-buffered saline (PBS), pH 7.4, and resuspended in 10% neutral-buffered formalin for 60 minutes, thoroughly washed, and resuspended to a final concentration of 10^8 organisms/0.1 mL (vaccination dose) in PBS. The heat-killed vaccine was prepared by re-suspending the leptospires in PBS and boiling at 100°C for 10 minutes (final concentration, 10^8 organisms/0.1 mL). The sterility of vaccines was confirmed by absence of bacterial growth on blood agar plates and EMJH medium at 37°C and 30°C, respectively.

Experimental animals. Male 4- to 6-week-old Syrian golden hamsters (*Mesocricetus auratus*) were bred and housed at the Animal Laboratory Breeding Unit, Faculty of Medicine,

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Khon Kaen University. Hamsters were maintained and care under strictly hygienic conventional conditions under regulation of the National Laboratory Animal Center, Mahidol University, Thailand.

Immunization and challenge. Hamsters were divided into three groups (14/group). Group I was immunized with heat-killed *L. interrogans* (10^8 cells/0.1 mL/hamster), group II with formalin-killed *L. interrogans* (10^8 cells/0.1 mL/hamster), and group III with 0.1 mL PBS (control group). The immunizations were performed twice by subcutaneous injection at 45 and 30 days (D_{-45} and D_{-30}) before intraperitoneal challenge (D_0) with 100 μL of PBS containing 1.58×10^5 *L. interrogans* serovar Autumnalis (1,000 LD_{50}). Animals were observed daily for 30 days.

Blood collection for bacterial culture, reverse transcriptase-polymerase chain reaction, and microscopic agglutination test. The immunization protocol was repeated with 12 hamsters per group. Blood, kidneys, urine, liver, and lungs were taken for bacterial culture, and all tissues collected were subjected to histopathologic examination. Blood samples were also used for detection of antibodies and cytokine mRNA expression.

Blood samples were taken through the orbital plexus (300 μL) from all hamsters of each group 1 day before first immunization (D_{-46}) and on Days 2, 4, 7, 10, 21, and 30 after challenge using a sterile capillary tube containing 0.5 U of heparin. For the microscopic agglutination test (MAT), blood samples were taken through the orbital plexus 1 day before each immunization and challenge (D_{-46}, D_{-32}, and D_{-1}) and on Days 7, 14, 21, and 30 after challenge. Heparinized blood samples on Day 2 were also used for bacterial culture (50 μL of blood was inoculated into 5 mL EMJH), and all blood samples were centrifuged to obtain plasma for MAT and the pellet was used for RNA extraction within 1 hour after collection for reverse transcriptase-polymerase chain reaction (RT-PCR). The plasma was stored at −20°C until needed.

Histopathologic examination and bacterial culture. Three animals at a time from the immunized groups (a total of 12 hamsters from each group) were killed on Days 7, 14, 21, and 30 after challenge to collect blood and kidney, lung, liver, and urine samples for further experiments. All hamsters in the control group were killed on Day 7.

For histopathologic examination, lung, kidney, and liver tissues were prepared by fixing in 10% neutral-buffered formalin for 12 hours, dehydration by immersion in increasing concentrations of ethanol (70%, 95%, and then 100%) and then xylene before embedding in paraffin wax. The paraffin sections were stained with hematoxylin and eosin (H&E) for examination.

For bacterial culture, one lobe of lung and one kidney were homogenized in 1 mL EMJH medium and briefly centrifuged, and the supernatant was harvested. Ten-fold serial dilutions (10^{-1}, 10^{-2}, and 10^{-3}) of the supernatant were made in EMJH medium containing 3% rabbit serum; 100 μL of each dilution was inoculated into 5 mL of 0.1% semi-solid EMJH medium containing 200 μg/mL 5-fluorouracil. Ten-fold serial dilutions of urine from each hamster were also prepared and inoculated in the same way. The cultures were incubated at 30°C and observed weekly for 30 days.

MAT. Specific antibodies against *Leptospira* in serum samples from immunized hamsters were examined by MAT[13] using 10^8 cells/mL live *L. interrogans* serovar Autumnalis isolate no. RY21 cultured in EMJH medium as an antigen. Two-fold serial dilutions of serum samples starting from 1:50 were prepared in PBS in microtiter plates before the bacteria were added. The plate was incubated for 2 hours at room temperature in the dark and examined for agglutination by dark-field microscopy. The serum titer is the final dilution that shows 50% agglutination.

Preparation of hamster RNA from blood. Two hundred microliters of heparinized blood from each hamster was treated with ice-cold erythrocyte lysis solution (140 mmol/L NH_4Cl and 17 mmol/L Tris, pH 7.2). White blood cells were collected by centrifugation at 2,000 × *g* for 10 minutes, washed with PBS, pH 7.2, and homogenized in 1 mL Trizol reagent (Invitrogen, Life Technologies, Carlsbad, CA). Each sample was incubated at room temperature for 5 minutes and centrifuged at 11,000 × *g* for 10 minutes at 4°C. The supernatant was transferred to a new tube, and RNA was extracted with an equal volume of chloroform and then with 500 μL of isopropyl alcohol. The RNA pellet was precipitated with 1 mL absolute ethanol, air-dried, and dissolved in 50 μL of RNase-free water. Contaminating DNA was digested with RNase-free DNase (Promega, Madison, WI), and the quality and quantity of RNA were studied by electrophoresis and spectrophotometry. The DNase-treated RNA was stored at −70°C until needed.

Determination of cytokine gene expression. *cRNA synthesis for standard real-time PCR.* Spleen cells from normal hamsters at a concentration of 1×10^6 cells/mL were stimulated with 10 μg/mL of concanavalin A (Con A; Sigma, St. Louis, MO) in Roswell Park Memorial Institute (RPMI) containing penicillin (100 U/mL) and streptomycin (100 μg/mL). After 24 hours of stimulation, the cell suspension was harvested, washed in PBS, and subjected to total RNA extraction using Trizol reagent (Invitrogen). The standard cRNA of each cytokine (TNF-α, IFN-γ, IL-10, and IL-4) and a housekeeping gene, hypoxanthine phosphoribosyl transferase (HPRT), used as a control were prepared from this total RNA as described previously.[14,15]

The cRNA construction was carried out by converting the total RNA to single-stranded cDNA, and the T7 promoter sequence (TAATACGACTCACTATAGGGA) was added to the 5′ end of the forward primers and Oligo-d $(T)_{15}$ to the 5′ end of the reverse primers (Table 1) by PCR amplification. The PCR reaction contained 50–100 ng of cDNA, 10× PCR buffer, 1 unit of *Taq* DNA polymerase, 0.2 mmol/L dNTP, 1.5 mmol/L $MgCl_2$, and RNase-DNase-free water to a final volume of 25 μL. The amplification conditions included pre-amplification for one cycle at 94°C for 2 minutes and amplification for 35 cycles as listed in Table 1 with a final extension at 72°C for 4 minutes for one cycle. To obtain cRNA, the purified PCR products containing the T7 promoter were used as templates for *in vitro* transcription with MEGAscript (Ambion, Austin, TX). Two microliters of reaction buffer, 2 μL of each dNTP (75 mmol/L ATP, CTP, GTP, and UTP), 2 μL enzyme, and 1 μg of PCR product in a reaction volume of 20 μL were incubated at 37°C overnight. The cDNA was removed by RNase-free DNase I (Ambion). The cRNA was precipitated by adding 30 μL nuclease-free water and 25 μL 7.5 mol/L lithium chloride and incubated at −20°C for at least 30 minutes before centrifugation at 12,000 × *g* for 15 minutes. The cRNA pellet was washed with 70% ethanol, resuspended

TABLE 1
Sequences of the forward and reverse primers and PCR conditions used for real-time PCR or cRNA *in vitro* transcription

Gene*	Purpose	Primer sequence	PCR condition (denature—anneal—extend)	Size (bp)
HPRT	Real-time PCR	F: 5′GCGATGTCATGGTAGAGA R: 5′GGGAGTGGATCTATCACA	94°C/10 s—60°C/30 s—72°C/30 s	128
	cRNA	F: 5′TAATACGACTCACTATAGGGAGCGATGTCATGGTAGAGA R: 5′TTTTTTTTTTTTTTTTGGGAGTGGATCTA TCACA	94°C/15 s—64°C/30 s—72°C/60 s	164
IFN-γ	Real-time PCR	F: 5′CCAGAGGAGCATAGACACCA R: 5′ACAGCACCGACTTCTTTTCC	94°C/10 s—60°C/30 s—72°C/30 s	208
	cRNA	F:5′TAATACGACTCACTATAGGGACCAGAGGAGCATAGACACCA R: 5′TTTTTTTTTTTTTTTTACAGCACCGAC TTCTTTTCC	94°C/10 s—65°C/30 s—72°C/60 s	244
TNF-α	Real-time PCR	F: 5′TGCCTATGCCTCAGCCTCTT R: 5′TGGAGCCGATGATAGGGTTG	94°C/10 s—60°C/30 s—72°C/30 s	127
	cRNA	F: 5′TAATACGACTCACTATAGGGATGCCTATGCCTCAGCCTCTT R: 5′TTTTTTTTTTTTTTTTGGAGCCGATGATAGGGTTG	94°C 15 s—64°C/30 s—72°C/60 s	163
IL-4	Real-time PCR	F: 5′TGAACCAGGTCACAGA R: 5′CGTGGACTCATTCACA	94°C/10 s—50°C/30 s—72°C/30 s	251
	cRNA	F: 5′TAATACGACTCACTATAGGGATGAACCAGGTCACAGA R: 5′TTTTTTTTTTTTTTTTCGTGGACTCATTCACA	94°C/15 s—60°C/30 s—72°C/60 s	287
IL-10	Real-time PCR	F: 5′CATGCTCCGAGAGCTGA R: 5′CTGCAGTTGCCTCCTGA	94°C/10 s—60°C/30 s—72°C/30 s	256
	cRNA	F: 5′TAATACGACTCACTATAGGGACATGCTCCGAGAGCTGA R: 5′TTTTTTTTTTTTTTTTCTGCAGTTGCCTCCTGA	94°C/15 s—65°C/30 s—72°C/60 s	292

* Gene and GenBank accession numbers: *HPRT* (AF047041), *IFN*-γ (AF034482), *TNF*-α (AF046215), *IL-4* (AF046213), and *IL-10* (AF046210).

in 20 μL diethyl pyrocarbonate-dH_2O (DEPC-H_2O), and stored at −80°C.

cDNA synthesis for real-time PCR. The standard cRNA of each cytokine was serially diluted 10-fold to obtain 10^1–10^8 copies, and cDNA from each dilution was synthesized using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen) according to the manufacturer's instructions. cDNA of each cytokine from each sample was also synthesized from total RNA in parallel with the standards. All cDNA samples were kept at −20°C until needed for real-time PCR.

Real-time PCR. Real-time PCR was performed using the LightCycler machine (Roche Applied Science, Indianapolis, IN). Primers to amplify each hamster's cytokine and *HPRT* genes were designed from sequences available in GenBank (Table 1). Ten microliters of 2× Platinum SYBR Green qPCR superMix-UDG (Invitrogen) was mixed with 1 μL of bovine serum albumin (Sigma; 1 mg/mL), 10 μmol/L of each forward and reverse primer, 2 μL of either cDNA from the sample or cDNA generated from standard cRNA, and nuclease-free water to a final volume of 20 μL. Amplification was carried out for 45 cycles as indicated in Table 1. Melting curve analysis was performed for 1 cycle at 95°C/0 seconds, 55°C/15 seconds, and 95°C/0 seconds (slope = 0.1°C/second) for denaturation, annealing, and melting, respectively. The copy number of each cytokine or *HPRT* gene was quantified from the standard curve. The result was expressed as the ratio of copy number of the cytokine genes over the copy number of HPRT.

Statistical analysis. For the cytokine assay, changes within groups over time were analyzed with the paired *t* test. The differences between vaccinated and control animals were analyzed using a one-way ANOVA test. The software used for the statistical analysis was SPSS 11.0 for Windows (SPSS, Inc., Chicago, IL).

RESULTS

Survival and organ sterility. All infected control hamsters died within 6–10 days of challenge. The hamsters vaccinated with heat-killed and formalin-killed *Leptospira* showed 100% (14/14) and 92% (13/14) survival, respectively (Figure 1). When the immunization experiment was repeated with 12 hamsters per group, 2 hamsters in the control group died on Day 6, and the rest were moribund by Day 7 after challenge

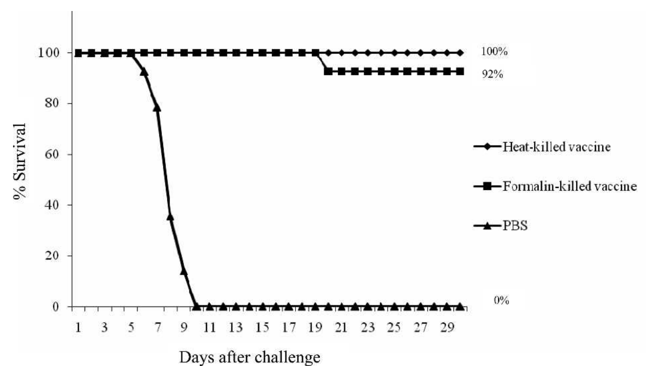

FIGURE 1. Survival of hamsters immunized with heat-killed (♦) or formalin-killed (■) *L. interrogans* serovar Autumnalis and control hamsters (▲) after challenge with *L. interrogans* serovar Autumnalis and observed for 30 days.

and therefore were killed and their organs were collected for H&E staining and bacterial culture. Urine samples were also collected for bacterial culture. The control group was positive for *Leptospira* in all kidney samples (100%), 7 of 10 (70%) urine samples, and 1 of 10 (10%) lung samples (Table 2). In contrast, leptospires were not seen by Day 21 in any of the cultures from vaccinated groups. However, one third of kidney samples from the formalin-killed vaccine group and one third of kidney and one third of urine samples of the heat-killed vaccine group were culture positive by Day 30.

Histologic examinations. Gross pathology of lungs in all control hamsters (non-vaccinated) at 7 days after infection showed multiple foci of hemorrhage visible on the surface (Figure 2C1, arrow). Lungs of vaccinated hamsters showed a few focal areas of hemorrhage on the surface on Days 7 and 14 after infection (Figure 2, A1, A3, B1, and B3), but all samples taken on Days 21 and 30 (Figure 2, A5, A7, B5, and B7) appeared normal. Livers and kidneys from either vaccinated or control groups appeared normal (data not shown).

Light microscopy observations of the lungs were consistent with the gross examinations. In the control group, hemorrhage in lungs involved an estimated 30–60% of total lung parenchyma. The alveolar hemorrhages appeared as large foci with interstitial congestion (Figure 2, C2 and C3), and mild to moderate inflammatory infiltrates and foci of pulmonary edema (Figure 2, C4) were observed in all control hamsters. In lungs from hamsters vaccinated with heat-killed *Leptospira*, minimal pulmonary hemorrhage was found in 1–10% of total lung parenchyma (Figure 2, A4). Only one third of the hamsters showed interstitial congestion on Days 7 and 14 after infection (Figure 2, A2 and A4), but samples taken from Day 21 onward appeared normal (Figure 2, A6 and A8). Two of three hamsters vaccinated with formalin-killed *Leptospira* showed minimal pulmonary hemorrhage in an estimated 5–10% of total lung parenchyma on Day 7 (Figure 2, B2), and one third of the hamsters showed interstitial congestion on Days 7, 14, and 21 (Figure 2, B2, B4, and B6). Normal histology was seen by Day 30 (Figure 2, B8). The remaining hamsters showed normal histology (data not shown).

Light microscopic findings of the kidneys and livers from vaccinated and control hamsters did not show any differences. Most of the kidneys showed only mild congestion, and glomeruli and tubular interstitial areas appeared normal. No interstitial nephritis was observed (data not shown). Liver lobules showed varying pathology from normal to mild congestion and mild steatosis in both vaccinated and control groups. Portal tracts appeared normal. No abnormality of bile ducts and blood vessels was noted (data not shown).

TABLE 2

Bacterial culture results of blood, kidney, lung, and urine from vaccinated and control hamsters after challenge with *L. interrogans* serovar Autumnalis

Hamster groups	Days after challenge	No. positive for *L. interrogans*/ no. of samples tested			
		Blood	Kidney	Urine	Lung
Heat-killed vaccine	2	12/12	ND	ND	ND
	7	ND	0/3	0/3	0/3
	14	ND	0/3	0/3	0/3
	21	ND	0/3	0/3	0/3
	30	ND	1/3	1/3	0/3
Formalin-killed vaccine	2	12/12	ND	ND	ND
	7	ND	0/3	0/3	0/3
	14	ND	0/3	0/3	0/3
	21	ND	0/3	0/3	0/3
	30	ND	1/3	0/3	0/3
Control	2	12/12	ND	ND	ND
	7	ND	10/10*	7/10*	1/10*

* All remaining animals on Day 7 were killed, and samples were collected.
ND = not determined.

Serologic examinations. All vaccinated hamsters developed antibodies against *L. interrogans* serovar Autumnalis before challenge (Table 3). In hamsters vaccinated with heat-killed *Leptospira*, detectable levels of antibodies were observed 2 weeks after the first immunization, and the MAT titers increased and remained at high levels after challenge until the end of the experiment (geometrical mean MAT titer = 459 on Day 30 after challenge). Hamsters vaccinated with formalin-killed *Leptospira* sero-converted later, and the highest geometrical mean MAT titer was 213 at 14 days after challenge before decreasing substantially.

TNF-α, IFN-γ, IL-4, and IL-10 responses in vaccinated and control hamsters. The mRNA expression level of all cytokines in all groups, except IL-4 in the control, was significantly increased after challenge ($P < 0.05$; Figure 3). The levels of IL-4 and IL-10 expression in vaccinated hamsters were significantly higher on Days 2, 4, and 7 than the infected control group ($P < 0.05$) and remained constant until Day 30 after challenge.

In comparison, IFN-γ expression levels in vaccinated hamsters were increased but were significantly lower than the infected controls on Days 2, 4, and 7 after challenge. Moreover, IFN-γ levels in vaccinated groups decreased after Day 4 and returned to almost basal levels on Day 10 after challenge, whereas the IFN-γ level in infected controls remained high at Day 7. The TNF-α mRNA expression levels in all groups were not significantly different on Day 2, but vaccinated groups showed more decline in TNF-α expression by Day 7. However, only the heat-killed vaccine group differed significantly from the infected control group on Days 4 and 7. The infected control group was either dead or moribund by 7 days after challenge, whereas heat-killed or formalin-killed vaccine groups survived (100% and 92% respectively; Table 2).

These results indicate that expression levels of the inflammatory cytokines (TNF-α and IFN-γ) in vaccinated groups were inversely correlated with survival, whereas expressions of the Th2 cytokine (IL-4) and anti-inflammatory cytokine (IL-10) were increased and positively correlated with the hamster survival after challenge (Figure 3; Table 2).

DISCUSSION

The pathogenic mechanisms of leptospirosis are a combination of direct effects by *Leptospira* and the host immune response to the infection. For example, inflammation in many organs caused by deposition of immune complexes contributes to the severity of the symptoms.[16] In the last decade, it has been shown that a pivotal point in the immune response is whether a Th1-type response (intracellular microbe killing) is induced, characterized by T-cell production of IFN-γ and a bias of the antibody response toward IgG2, or induction of a Th2 response (extracellular microbe killing), characterized by production of IL-4 by T cells and antibodies of the IgE, IgG1, and IgA isotypes.[17] The Th1 and Th2 profile elicited by leptospires is of interest for the success of vaccine development.

It is well established that the effectiveness of the killed vaccine is serovar-specific because of antibody-mediated pro-

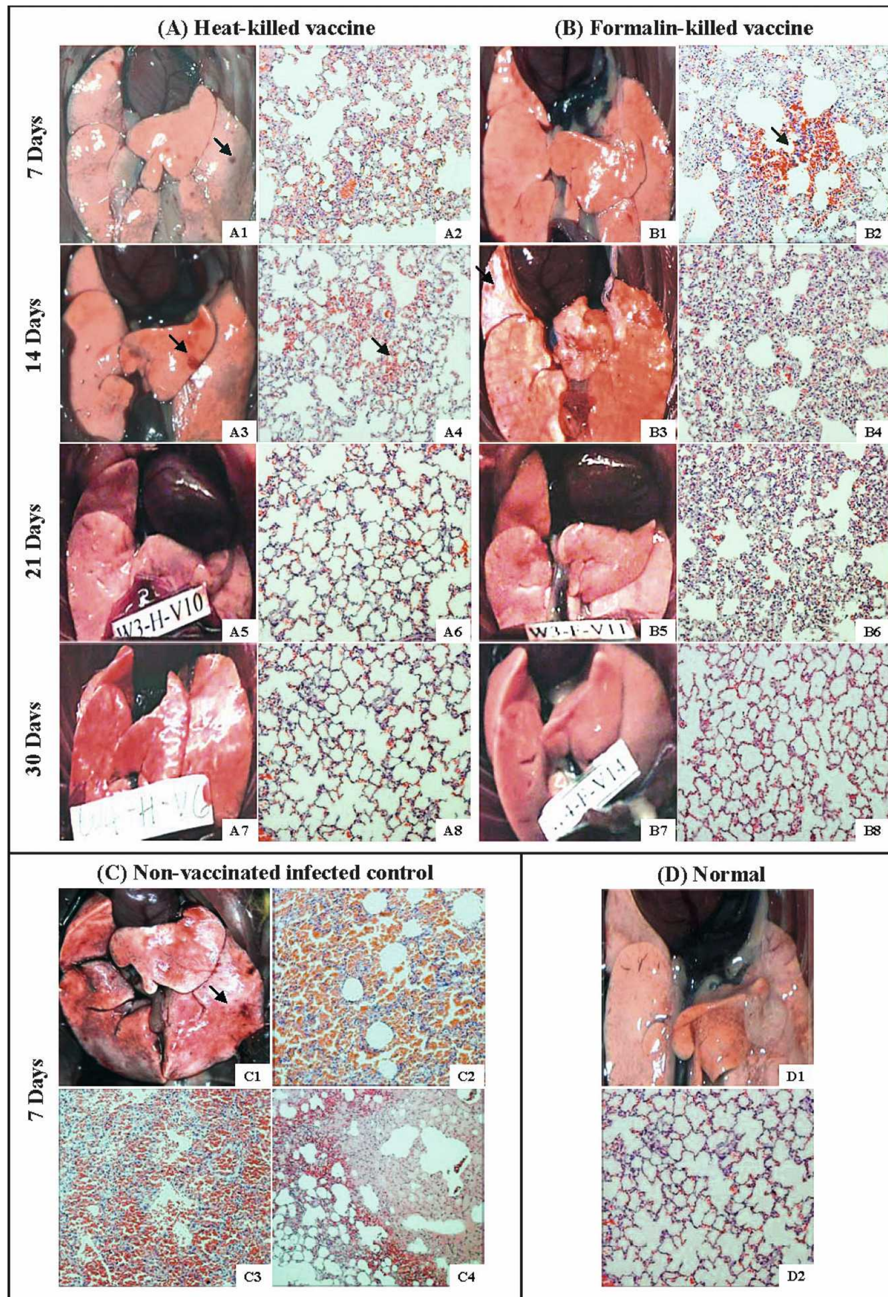

FIGURE 2. Gross pathology (H&E stain; magnification, ×200) of lungs from hamsters vaccinated with heat-killed (**A**) or formalin-killed (**B**) leptospires, control hamsters (non-vaccinated) (**C**), and normal hamsters (**D**). The vaccinated hamsters were killed for blood and organ collection on Days 7, 14, 21, and 30 after challenge with live leptospires, whereas the control group was killed on Day 7. The gross pathology of lungs on Day 7 of vaccinated hamsters showed a few focal areas of hemorrhage visible on the surface (**A1** and **B1**) compared with many foci on most of the lung surface in control hamsters (**C1**). The H&E staining from lungs of normal hamsters showed thin alveolar septae (**D2**), whereas all control hamsters showed large foci of hemorrhage (**C2** and **C3**) and thickening of alveolar septae caused by infiltrates of mononuclear inflammatory cells (**C3**, top left) and foci of pulmonary edema (**C4**). Lung samples from one third of vaccinated hamsters on Days 7 (**B2**) and 14 (**A4**) showed small foci of hemorrhage and thickening of alveolar septae (**A2**, **B2**, and **B4**) caused by infiltrates of mononuclear inflammatory cells. Lung samples from one third of vaccinated hamsters on Day 21 (**B6**) show thickening of alveolar septae. The pulmonary hemorrhage is indicated by the arrows. This figure appears in color at www.ajtmh.org.

tection. For example, the Japanese leptospiral vaccine, which consists of formalin-killed leptospires of serovars Australis, Autumnalis, Hebdomadis, and Copenhageni, can provide 60–100% protection only for the serovars included in the vaccine.[18,19] This is also seen with leptospiral vaccines used in the United States.[20]

In our study, hamsters immunized with heat-killed or formalin-killed *Leptospira* showed 100% and 92% survival rates, respectively, after challenge with the homologous serovar. However, hamsters in vaccinated groups showed sublethal but severe pathology at the early stage (7 days) and took at least 3 weeks to recover to normal. Moreover, the vaccination was shown here to be insufficient for preventing colonization of the kidney by leptospires.

TABLE 3
Geometric mean plasma MAT titers from hamsters immunized with heat-killed or formalin-killed *L. interrogans* serovar Autumnalis

Day	MAT titers (geometric mean) Heat-killed	Formalin-killed	Control
−46	< 50	< 50	< 50
−32	55	< 50	< 50
−1	181	126	< 50
7	344	172	< 50
14	375	213	ND*
21	436	168	ND*
30	459	141	ND*

The immunization was done twice by subcutaneous injection at 45 days and then 30 days before challenge (D_{-45} and D_{-30}). The animals were challenged by intraperitoneal injection on Day 0.
* Not determined because hamsters in the control group were killed on Day 7.

Histopathologic examination of the lungs showed a good correlation with antibody titer in that mostly mild pathology was seen in immunized groups, whereas severe lung hemorrhages were observed in the control group. The role of antibody may not only clear the bacteria but also remove or neutralize components released from the organism, such as lipopolysaccharide and glycolipoprotein, which have been reported to be a cause of tissue pathology.[21,22] As seen in humans, in addition to specific tissue lesions (e.g., interstitial nephritis and hepatic central-lobular necrosis), a generalized vasculitis is accompanied by hemorrhagic phenomena in all tissues.[8,23] However, severe pathology was not observed in any liver and kidney samples, even from control hamsters. Their glomeruli and tubular interstitial areas appeared normal even though leptospiral cultures were positive for all kidney samples. Because only a few spirochetes were observed in the damaged tissue, some factors released from spirochetes may be responsible for the acute systemic inflammation that is clinically found in the most severe cases such as in sepsis or the systemic inflammatory response syndrome of infectious origin.[9,10]

The cell-mediated immune response has also been shown *in vitro* to play a role during leptospiral infection.[24,25] Glycolipoprotein extracted from the pathogenic *L. interrogans* serovar Copenhageni can induce peripheral blood mononuclear cell activation as measured by the secretion of TNF-α and IL-10 and the increase in the expression of CD69 and HLA-DR, which are markers of cellular activation.[26] The induction of *in vitro* production of Th1 cytokines by Peripheral Blood Mononuclear Cells (PBMCs) and cell proliferation in αβT cells and γδT cells was also shown by Klimpel and others.[27] In immunized hamsters, besides antibody formation, vaccination induced significant changes in patterns of inflammatory cytokine profiles. The expression levels of pro-inflammatory cytokines in the vaccinated hamster were correlated with pathogenesis because they were significantly lower than the control

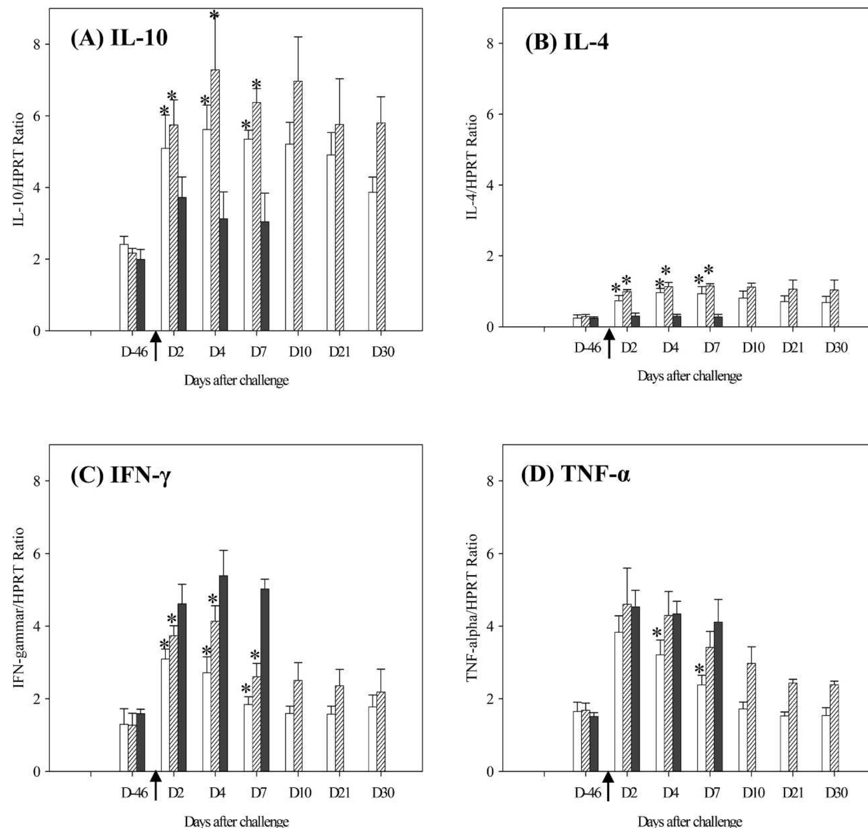

FIGURE 3. Expression levels represented as the ratio of cytokine copy number to HPRT copy number as determined by reverse transcription real-time PCR analysis of blood samples from control and vaccinated hamsters. IL-10 (**A**), IL-4 (**B**), IFN-γ (**C**), and TNF-α (**D**) expression in hamsters immunized with heat-killed (□) or formalin-killed (▨) *L. interrogans* serovar Autumnalis and control hamsters (■) before and after challenge with *L. interrogans* serovar Autumnalis. The result is expressed as cytokine/HPRT ratio. All animals were challenged on Day 0 (**arrow**). The asterisks (*) indicate statistical difference ($P < 0.05$) between control and immunized groups on the same day. Each bar represents the mean ± SD.

group. This corresponded with the report of Estavoyer and others[28] that a significant increase of TNF-α in patients infected with leptospires is associated with severity of the disease. The level and persistence period of pro-inflammatory cytokines may also correlate with the virulence of leptospires compared with the study of Vernel-Pauillac and Merien[29] that used a less virulent leptospiral isolate for infection and obtained only a transient period of cytokine response.

Taken together, our findings suggest that vaccination does change cytokine profiles. The IFN-γ and TNF-α mRNA expression levels were correlated with the severity of infection and lung pathology. Moreover, this study showed that the current commercially available killed vaccines may not efficiently prevent pathology in vaccinated populations and may not completely prevent the pathogen from colonizing the kidney and subsequent shedding in urine. Therefore, the pathogen may still persist and cause re-infection from time to time even in a vaccinated herd. Ideally, future leptospiral vaccines would induce both humoral and cell-mediated responses, whereby manipulation of the cell-mediated response could foreseeable provide a broad species-wide protection.

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