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Immunogenicity of boiled compared with formalized leptospiral vaccines in rabbits, hamsters and humans

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SUMMARY

Leptospires (*Leptospira interrogans* serovar *pomona*) grown in chemically defined medium were immunogenic when given intradermally in humans if the leptospires were killed with formalin but not if they were boiled. Boiled leptospires were immunogenic for rabbits and hamsters and protected hamsters from challenge infection. On the other hand, boiled leptospires of the *biflexa* complex, serovar *patoc*, did retain some immunogenicity in humans, but the antisera did not protect hamsters against challenge with serovar *pomona*.

INTRODUCTION

Since Japanese workers first isolated the causal organism of Weil's disease in 1916, many leptospiral vaccines have been produced for animal and for human use (Alston & Broom, 1958). Early vaccines consisting of heat- or formalin-killed leptospires grown in media containing animal serum commonly caused severe clinical reactions (Wani, 1933; Borg-Petersen & Errebo-Knudsen, 1953). By preparing a whole leptospiral vaccine (Shen-Tor vaccine) from serovars *grippotyphosa* and *szwajizak* grown in chemically defined protein-free medium, Shenberg & Torten (1973) were able to reduce considerably the severity of adverse reactions to subcutaneous vaccination.

Takashima & Yanagawa (1975) reported reduced immunogenicity of heat-killed (60 °C) leptospires compared with unheated leptospires, but Painter & Ellinghausen (1976) found that leptospires heated to 98 °C were immunogenic.

The objectives of the present research were to investigate immunogenicity and usefulness of boiled or formalin-killed leptospires, particularly in humans, and to see whether the use of boiled and washed leptospires, injected intradermally rather than subcutaneously, would minimize and localize toxic reactions.

Since antibodies that cross-react in serological tests with non-pathogenic (*biflexa* complex) serovar *patoc* are produced by many patients infected with pathogenic leptospires (Turner, 1968), a second objective was to determine whether human antiserum to serovar *patoc* would cross-react with pathogenic serovars and protect from leptospirosis.

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MATERIALS AND METHODS

Leptospires and serological tests

The sources, culturing and enumeration of leptospires were described previously (Adler & Faine, 1977) as was the extraction of an erythrocyte-sensitizing F4 antigen and its use in haemagglutination (HA) tests (Faine, Adler & Palit, 1974). The microscopic agglutination test (MAT) and hamster protection test were performed as described previously, as was the method for fractionating sera on sucrose density gradients (Adler & Faine, 1978*a*).

Preparation of vaccines and human immunizations

Shen-Tor leptospiral grippotyphosa/szwajizak vaccine was kindly provided by Dr M. Torten, Israel Institute for Biological Research, Ness-Ziona, Israel. To prepare boiled leptospiral vaccine (BLV), the leptospires were centrifuged at 12000 *g*, washed 4 times in saline, held at 100 °C for 2 h, washed 4 times in saline, and then resuspended so that a 0·1 ml volume contained approximately 4×10^8 leptospires. Formalized leptospiral vaccine (FLV) was prepared by washing

Table 1. *The chemical composition of modified protein-free medium for growing leptospires for human vaccines*

(The pH was adjusted to 7·4–7·5 and the medium was sterilized by filtration through a 0·22 μm Millipore membrane filter.)

Component	Concentration (mg/l)
NA_2HPO_4	500
KH_2PO_4	150
$MgSO_4.7H_20$	150
$(NH_4)_2FeSO_4.6H_2O$	6
$CaCl_2.2H_2O$	14·7
EDTA	10
Tween 80*	50
Glycerol**	200
L-Asparagine	500
Thiamine	1
Cyanocobalamine	0·001
Sodium pyruvate	100
Sodium acetate	100

* Tween 80 passed through IRA-400 resin (see Materials & Methods).
† Glycerol was fat-extracted (see Materials & Methods).

leptospires 4 times in saline and then resuspending them for 10 min in 10% neutral-buffered formalin. The leptospires were then washed 8 times in saline and resuspended to a density of 4×10^8 leptospires/0·1 ml dose. The sterility of vaccine preparations was checked by the Bacteriology Department, Alfred Hospital. Vaccines to be used in humans were grown in modified Shenberg's (1967) protein-free medium (Table 1) because unmodified Shenberg's medium was unsuitable for the growth of serovar *pomona* or *patoc*. Tween 80 was passed through Amberlite IRA-400 resin as described by Staneck, Henneberry & Cox (1973). Glycerol was fat-extracted by shaking with an equal volume of ether. The process was repeated 4 times and residual ether was evaporated from the glycerol at 56 °C. The final

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protein-free medium was dispensed in 50 ml volumes in flat 200 ml bottles which were inoculated with 1 ml of leptospiral culture and incubated on their side to provide a large surface area. Leptospires used for vaccine preparation were subcultured successively at least 30 times in the protein-free medium.

Human immunizations were performed within the guidelines on human experimentation set down by the National Health and Medical Research Council, Canberra, Australia. Volunteers were injected intradermally (ID) in the flexor aspect of the left forearm with a 0·1 ml dose of vaccine. Biochemical and haematological tests were performed by the diagnostic laboratories of Alfred Hospital on the day of vaccination and again 2 and 9 days later. Blood was taken by venipuncture at intervals after immunization, for serological tests. Volunteers were not known to have had leptospiral infections and were seronegative before immunization.

Injection of animals

New Zealand White rabbits were immunized with boiled or unheated leptospires intramuscularly (IM) with incomplete Freund's adjuvant as described previously (Adler & Faine, 1978*b*). Rabbits to be vaccinated intradermally (ID) were given a single injection of 0·1 ml of the appropriate preparation into the shaved flank. They were bled from the marginal ear vein. Hamsters were infected with leptospires of serovar *pomona* as described previously (Adler & Faine, 1978*a*).

Dermal toxicity tests in guinea-pigs

Adult albino guinea-pigs were injected ID in their shaved backs with 0·1 ml of the test material. After 30 min, 0·5 ml of 1 % Evans Blue (plus 0·3 ml of antiserum if appropriate) were injected intravenously. The diameter of the blue zone was measured after 1 h and after 24 h. This method was used so that the antigen, dose and route of injection were the same as that used in humans.

RESULTS

Immunogenicity of boiled leptospires in rabbits

The antibody response of rabbits to boiled leptospires of serovar *pomona* was compared with that to unheated leptospires (Fig. 1). It is apparent that boiling did not reduce the immunogenicity of leptospires injected with incomplete Freund's adjuvant with respect to antibodies detectable by either the MAT or HA with F4 antigen.

The antibody response to BLV and FLV injected ID without adjuvant is shown in Fig. 2. Thus, when injected in a manner which might be suitable for human immunization, boiled leptospires (BLV) were as immunogenic as unheated leptospires in the form of FLV. Booster injections given 57 or 64 days after primary immunization stimulated a further rise in antibody level (Fig. 2).

Protective capacity of BLV and FLV in hamsters

The immunogenicity of BLV and FLV of serovar *pomona* in hamsters is shown in Table 2. The minimum immunogenic and protective dose of either vaccine was 0·1 ml of a $1/10^3$ dilution, corresponding to 4×10^5 leptospires.

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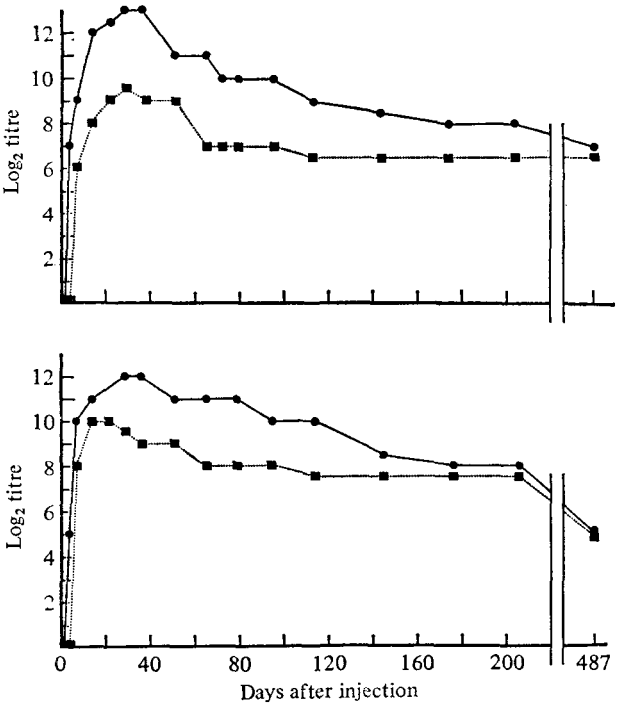

Fig. 1. The typical antibody responses in rabbits immunized IM with adjuvant, with boiled leptospires (upper graph) or with unheated leptospires (lower graph) of serovar *pomona*. The titres of three individual rabbits did not differ by more than one doubling dilution. ●——●, MAT titre; ■.....■, F4 antibody titre.

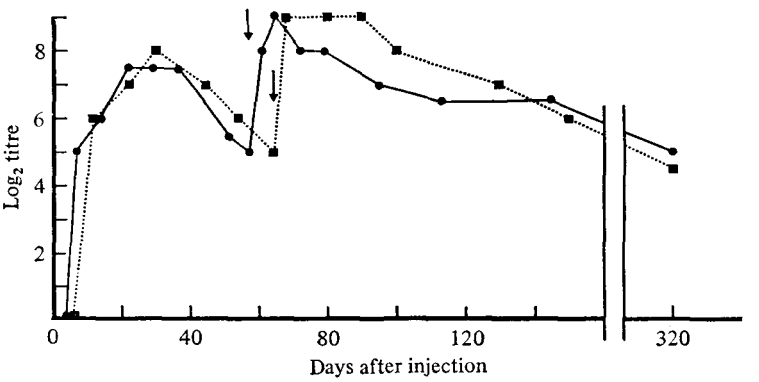

Fig. 2. The typical rabbit MAT antibody response following immunization ID with 0·1 ml of serovar *pomona* BLV or FLV. A booster injection was given on days 57 and 64 respectively (arrows). The titres of three individual rabbits did not differ by more than one doubling dilution. ●——●, Immunized with BLV. ■.....■, Immunized with FLV.

Reactogenicity of vaccines in guinea-pigs

BLV and FLV prepared from leptospires grown in modified protein-free medium were tested for dermal toxicity by injecting 0·1 ml volumes ID into the shaved backs of guinea-pigs followed by an IV injection of Evans Blue (Materials and

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Methods). Both BLV and FLV produced a blue zone of 3–4 mm diameter visible after 1 h and lasting 48 h. The reaction was less than that to 0·1 ml of a commercial typhi/paratyphi A/paratyphi B vaccine (5 mm).

Table 2. *Protection of hamsters from serovar* pomona *infection by vaccination with homologous BLV or FLV*

Immunized with*	No. of leptospires in vaccine dose	MAT titre†	No. of deaths. no. tested
BLV	4×10^8	256	0/12
	4×10^7	256	0/3
	4×10^6	32	0/3
	4×10^5	8	0/3
	4×10^4	0	3/3
	4×10^3	0	2/3
FLV	4×10^8	512	0/8
	4×10^7	256	0/3
	4×10^6	32	0/3
	4×10^5	4	0/3
	4×10^4	0	3/3
	4×10^3	0	3/3
Not immunized		0	20/20

* Immunized ID in the flank with a volume of 0·1 ml 3 weeks before challenge with at least 2×10^8 leptospires of serovar *pomona*.

† Pooled serum MAT titre of each group in serum taken on the day of challenge.

Immune human or rabbit sera did not induce a heterologous cutaneous anaphylaxis, nor did previously infected immune guinea-pigs show a greater immediate or delayed reaction than normal guinea-pigs. This lack of reactogenicity was unusual but may be due to rapid local neutralization and clearance of the leptospires. The reaction was similar to the reaction to Shen-Tor leptospiral vaccine which has been tested and approved for human immunization by the Israel Ministry of Health.

Following these tests showing minimal dermal reactions, human volunteers were immunized. Three separate trials were conducted.

Immunogenicity of vaccines in humans

Trial 1. Two subjects were immunized with *pomona* BLV and 2 with *patoc* BLV. All biochemical and haematological tests were normal. The BLV caused erythema over a region of approximately 10–15 mm with a 5–10 mm central induration. The reaction began 1–2 h after immunization and subsided by 48–72 h. There was some localized tenderness but no pain or itchiness. No systemic reactions or discomfort were observed. The reaction to *patoc* BLV was greater than that to *pomona BLV*. The *pomona* vaccinees did not produce any detectable antibody up to 2 months after immunization, while the two who received serovar *patoc* BLV produced low titres of agglutinins and also antibodies to erythrocyte-sensitizing

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F4 antigen of serovar *patoc* (Fig. 3) (Adler & Faine, 1978*a*). Fractionation of their sera on sucrose density gradients showed that the reacting antibody in both tests was detected solely in the IgM-containing fractions.

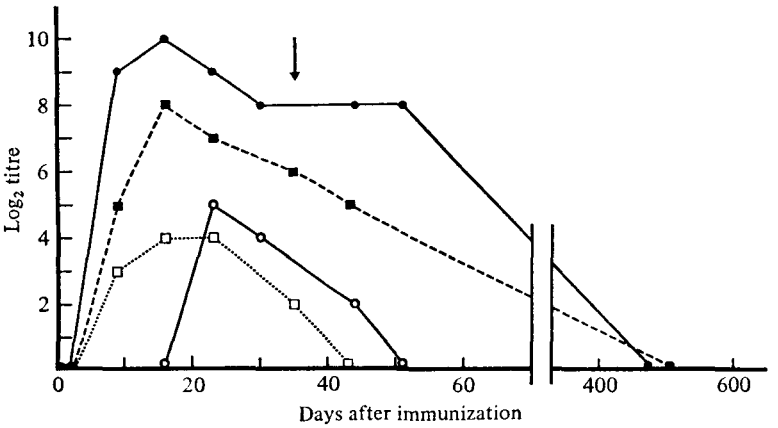

Fig. 3. The antibody response of two human subjects following immunization with *patoc* BLV. A booster injection was given on day 35 (arrow). ●——●, Subject 1, F4 antibody titre; ○——○, Subject 1, MAT titre; ■.....■, Subject 2, F4 antibody titre; □.....□, Subject 2, MAT titre.

The sera of these *patoc* immunized subjects did not react with F4 from serovar *pomona*, in contrast to the sera from patients with naturally acquired *pomona* infections, which cross-reacted to high titres with *patoc* F4 (Adler & Faine, 1978*a*). Neither did the sera agglutinate leptospires of serovar *pomona* or representative serovars of other serogroups. The sera from subjects in Trial 1 taken at weekly intervals from 2 days to 2 months after immunization failed to protect hamsters from infection with serovar *pomona* when the sera were injected in volumes of up to 0·8 ml. All subjects were given a second injection of the same antigen preparation 5 weeks after their first injection. The dermal reactions were similar to those after the initial injection. No hypersensitivity reaction was evident. No subsequent antibody or change in antibody titre was observed (Fig. 3).

Trial 2. Three subjects were immunized with *pomona* BLV. No antibody was detected in sera taken up to 6 weeks after immunization. The subjects had 5–8 mm erythematous zones at the site of injection, which subsided after 48 h.

Trial 3. Six subjects took part in Trial 3. Three of them received standard 0·1 ml doses of *pomona* BLV. The results were similar to those in Trial 2, with no detectable antibody response. The other 3 received 0·1, 0·05 or 0·02 ml doses of *pomona* FLV. Their MAT antibody responses to *pomona* are shown in Fig. 4. Subjects 1 and 2 (given FLV) also produced F4 antibody, which paralleled the rise and fall of the agglutinins and peaked at a titre of 16 on day 17 after immunization. Tests performed with fractions of sera from subjects 1 and 2 failed to detect antibodies other than IgM.

Volumes of 0·2 ml of serum from subjects in trial 3 were used in hamster protection tests. Sera taken 17, 31 and 67 days after immunization with BLV did not protect hamsters from serovar *pomona* infection. Sera taken at similar times after

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subjects 2 and 3 were immunized with FLV also failed to protect hamsters. However, serum of subject 1 from day 17 had a hamster protective titre of 2 and serum from day 31 had a hamster protective titre of 1. These protective titres are consistent with the MAT titres of the sera, in view of the results obtained in passive protection experiments with human sera (Adler & Faine, 1978*a*). Thus the failure of sera of subjects 2 and 3 to protect was due to low protective titres correlated with their low MAT titres. This was confirmed by the finding that a volume of 0·8 ml of serum, from days 17 and 30, from subject 2 protected 2/2 hamsters. A volume of 0·4 ml of serum did not protect.

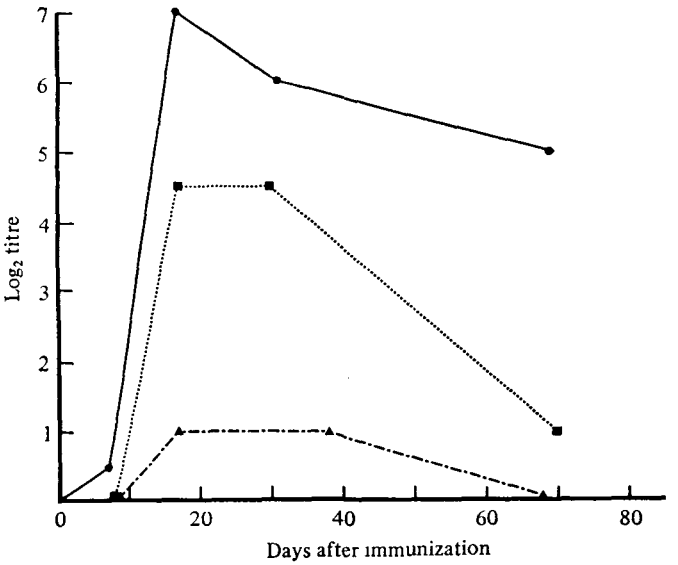

Fig. 4. The MAT antibody responses of three human subjects following immunization with serovar *pomona* FLV. ●, Subject 1; ■, Subject 2; ▲ Subject 3.

DISCUSSION

Serovar *pomona* BLV failed to stimulate antibodies detectable by MAT or HA in humans. However, the two subjects immunized with serovar *patoc* BLV produced antibodies which reacted to F4 in HA tests, and also low titres of agglutinins. Both types of antibody were specific to *patoc* since they did not react with heterologous strains of leptospires and did not protect hamsters from serovar *pomona* infection. Agglutinins were found only in the IgM class, as would be expected from a typical first response (Pike, 1967). A possible criticism may be that IgG antibodies were not detected by agglutination tests because IgG is not as good an agglutinator as IgM. However, in an analogous situation IgG agglutinins were readily detectable in humans with naturally acquired leptospiral infection (Adler & Faine, 1978*a*).

A similar agglutinin specificity was observed by Rottini *et al.* (1972) in the sera of children immunized with a serovar *patoc* vaccine although they found that antibodies were mainly IgG. Rottini *et al.* (1972) did not perform specific antileptospiral tests with IgG fractions, but used the less specific and less sensitive method

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of measuring by radial immunodiffusion the reduction in total serum IgG concentration after absorption of serum with leptospires. Shishkina *et al.* (1976) found that the agglutinins produced in man in response to a hexavalent vaccine were IgM antibodies. They found no increase in total serum IgG, and IgG agglutinins were detected (all at MAT titres $\leqslant$ 20) in only 3 out of 190 vaccinees.

In the trial of immunogenicity of *pomona* BLV in humans, 8/8 subjects failed to respond, 2 of whom received booster injections. An unheated formalin-killed vaccine (FLV), which was immunogenic in rabbits and immunogenic and protective in hamsters, was injected into 3 volunteers in whom it evoked the production of antisera which protected hamsters from acute infection, and agglutinins which were detected only as IgM. In the 3 subjects the titre of agglutinins produced depended on the immunizing dose but remained low in comparison with titres found in infected humans. Although definite conclusions cannot be drawn from such a small number of subjects, it appears that an intradermal injection of 0·1 ml of vaccine containing 4×10^4 leptospires would be suitable to stimulate agglutinating and hamster protective antibodies in humans. The use of ID rather than subcutaneous injections kept the reactions localized at the injection site.

As early as 1937, Esseveld reported that leptospires killed by boiling stimulated antibodies in rabbits and that *L. icterohaemorrhagiae* heated to 70 °C could actively immunize humans (cited by Alston & Broom, 1958). Painter & Ellinghausen (1976) found that killing leptospires by heating to 98 °C for 15 min did not decrease immunogenicity or protection for hamsters of a *canicola* vaccine in a 40 μg dose although leptospires heated at 121 °C for 15 min were less immunogenic and protective, and did not prevent renal infection. The retention of immunizing capacity for both rabbits and hamsters after 'boiling' of the leptospires was confirmed in the present study.

In contrast, Takashima & Yanagawa (1975) found that 40 μg of leptospires of serovar *icterohaemorrhagiae* heated at 60 °C for 1 h, washed in water and lyophilized, failed to protect guinea-pigs challenged with the same strain, although larger doses of 200 and 1000 μg protected. A control unheated preparation protected in a dose of 40 μg.

In the present research, boiled leptospires of serovar *pomona* retained their immunogenicity for rabbits and hamsters but not for humans. This cannot be explained on the basis of dosage because similar doses of approximately $5{\cdot}7 \times 10^6$/kg for man and 8×10^6/kg for hamsters were used, although the minimum immunogenic dose of antigen is not directly related to the size of the animal. The immunogenicity of an intradermal dose of FLV containing the same number of organisms confirmed this. Similarly, Torten *et al.* (1973) found that 2×10^8 formalin-killed (approx. $2{\cdot}8 \times 10^6$/kg) leptospires were immunogenic in 29 of 51 (57 %) human volunteers.

The antibody response following vaccination of animals gives rise to both IgM and IgG agglutinins (Negi, Myers & Segre, 1971; Tripathy, Smith & Hanson, 1975). Humans differ, by producing only IgM antibody, resembling the response following natural infection in which they respond with mainly IgM antibody (Adler & Faine, 1978*a*), while animals produce both IgM and IgG antibodies (Graves &

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Faine, 1970; Morris & Hussaini, 1974; Crawford, 1972). Although the capacity of serum to protect hamsters depended on the titre of agglutinins and both classes of antibody protected, IgG antibodies persist for longer than IgM and thus stimulation of IgG antibodies would be desirable in vaccination. This was not achieved in the present research. It is possible that multiple vaccinations may evoke IgG antibodies and future experiments should investigate this possibility.

Growth of leptospires in protein-free medium may reduce their immunogenicity by altering their antigenic structure. Graves (1972) observed that leptospires of serovar *holland* (Waz) grown in Tween 80 albumin medium, contained less of one of the trypsin extractable antigens than did leptospires of the same serovar grown in serum-containing medium. Kida *et al.* (1977) showed that a vaccine prepared from leptospires grown in protein-free medium was as protective in guinea-pigs as a preparation grown in rabbit serum medium. However, these authors did not determine minimum protective doses of vaccines, so they would not have detected quantitative differences in immunogenicity.

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