IMMUNISING POTENCY OF *LEPTOSPIRA INTERROGANS* SEROTYPE *CANICOLA* AFTER HEAT INACTIVATION AT DIFFERENT TEMPERATURES

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At present, only killed preparations are licensed as leptospiral vaccines in the United States. Their protective ability has been questioned for many years (Olitzki *et al.*, 1949; Hubbert and Miller, 1965; Stalheim, 1967; Glosser *et al.*, 1974), and certain difficulties, other than those associated with immunogenicity, have been described by Hanson (1973). Various inactivation methods have been used and some have been thought to affect immunising potency adversely (Smith, 1937; Brunner and Meyer, 1950; Hoag and Bell, 1955; Hubbert and Miller, 1965; Auran, Johnson and Ritzi, 1972; Shenberg and Torten, 1973).

The object of this investigation was to evaluate the immunising potency for hamsters of vaccines prepared from a virulent strain of *Leptospira interrogans* serotype *canicola* inactivated at various temperatures, and from an avirulent strain.

MATERIALS AND METHODS

Leptospires were cultured and counted, dry weights were determined and virulence was assayed as previously described (Ellinghausen, 1973; Ellinghausen and Painter, 1976).

Vaccines. The study was primarily concerned with heat-killed vaccines of a virulent strain of *Leptospira interrogans* serotype *canicola* (NADL A-13). Preservation in the laboratory has no effect on this strain's virulence for hamsters (Ellinghausen and Painter, 1976) and dogs can be infected with 2.6×10^4 leptospires. For purposes of comparison, the protective potency of a chemically-killed, commercial vaccine containing serotypes *icterohaemorrhagiae* and *canicola* was examined. In addition, a heat-killed vaccine prepared from the avirulent *canicola* strain (Hond Utrecht IV) was examined. Strains Hond Utrecht IV and NADL A-13 are serologically identical.

Inactivation took place in the glass containers used for culture, the leptospires being heated in a water bath or an autoclave. Exposure times in the water bath were measured from the moment of immersion. Before inactivation, late log-phase cultures in liquid polysorbate 80 (P-80) medium were diluted with bovine serum-albumin diluent (BSAD) (Ellinghausen, 1973) to contain 2.6×10^8 leptospires per ml. This number of cells corresponds to a nephelometer reading (NR) of 25 and a dry weight of 40 μ g per ml. BSAD was used for dilution to maintain the albumin concentration of vaccine suspensions at 1%.

The experiments described were carried out after preliminary tests had given an approximate indication of minimal lethal heat treatments. The leptospires were considered non-viable if no growth had occurred in 10 ml of P-80 semisolid medium inoculated with 1 ml of

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heated suspension after 30 days' incubation at 29°C. The effects on leptospire of temperatures ranging from -80°C to 121°C were examined.

For hamster-immunisation experiments, the leptospire were grown in aerated culture (Ellinghausen, 1966). Suspensions of *canicola* strain NADL A-13 were killed either by exposure to 50°C for 4 h, 98°C for 15 min. or 121°C for 15 min. Suspensions of *canicola* strain Hond Utrecht IV were killed by exposure to 50°C for 4 h. After inactivation, the vaccines were tested for freedom from contamination by culture in Trypticase Soy Broth. They were then dispensed in sterile glass bottles, sealed with aluminium-capped rubber stoppers and stored at -80°C until used. The vaccines were either used as prepared or diluted with BSAD so that the required dose was contained in a 1-ml volume.

Animal experiments. Immune response was evaluated in female Syrian hamsters weighing 40-50 g. Hamsters were vaccinated and challenged by intraperitoneal injection.

At death or slaughter, kidney, liver, lung, brain, gastro-intestinal and urogenital tissues were examined for macroscopic evidence of leptospirosis. Samples of the blood, kidney, liver, brain and urine were cultured on P-80 semisolid medium for 30 days at 29°C. Half the surviving hamsters were necropsied 15 days after challenge and the remainder 30 days after challenge.

Serology. Agglutinins were assayed by means of the microscopic agglutination (MA) test. Live suspensions of *canicola* strain Hond Utrecht IV diluted to a NR of 25 were used as antigens. Post-challenge titres were noted for all surviving hamsters in protection experiments. Post-vaccination titres were evaluated in a separate experiment on groups of five hamsters given doses of 80, 40, 25, 10, 5, or 2 µg of the strain NADL A-13 vaccine killed by exposure to 50°C for 4 h; serum was collected for the MA test 30 days after intraperitoneal vaccination. The serological data were summarised in terms of the geometric mean titres as used by Glosser *et al.* (1974).

RESULTS

The effect of temperature on canicola suspensions

The influence of intermediate and high temperatures on the viability, optical density and cell count of *canicola* suspensions is shown in table I. At 98°C, only 2 min. were required to inactivate completely a suspension containing 2.6×10^8 cells per ml and the cell count was reduced by 87% after exposure for 15 min. At 50°C, a period of 4 h was required to ensure inactivation and this treatment reduced cell counts by approximately 60%. When a suspension containing 2.6×10^8 cells per ml was frozen for 24 h at -80°C and thawed, 9.7% of the cells were motile and a 1-ml inoculum containing 2.6×10^3 or more of these cells was required for the demonstration of viability by subculture.

Challenge of hamsters at intervals after vaccination with different doses of heat-killed strain NADL A-13 vaccine and with commercial vaccine

The strain NADL A-13 vaccine was inactivated by heating at 50°C for 4 h. Hamsters in three groups of 30 received the NADL A-13 vaccine in doses of 40 µg, 25 µg and 10 µg. Thirty hamsters in a fourth group each received 1 ml of a 1 in 4 dilution of commercial vaccine. A fifth group of 30 remained unvaccinated as controls. Each of the five groups was divided into sub-groups of 10 to allow for challenge 14, 30 and 120 days after vaccination. The challenge dose was 26×10^6 cells of strain NADL A-13.

TABLE I

Influence of exposure to different temperatures on the viability and optical density of canicola suspensions

Temperature (°C)	Time of exposure (h)	Viability	Nephelometer reading	Percentage light transmission (400 nm)	Countable cells per ml (millions)
25	0	+	25	74	260
50	4	—	18	80	100
98*	0.25	—†	17	75	32
—80	24	+‡	13	80	155

Before exposure to the various temperatures, all suspensions contained 2.6×10^8 cells per ml.

* From 25°C, temperature increased to 74°C in 45 s; to 85°C in 60 s; to 93°C in 90 s; and to 98°C in 120 s.

† Suspension was non-viable after 2 min.

‡ Inoculum of 2.6×10^3 cells required to demonstrate viability.

All unvaccinated hamsters died from leptospirosis after challenge, but all vaccinated hamsters survived except two that died when challenged 30 days after receiving 10 µg of strain NADL A-13 vaccine and a single animal that had been vaccinated with the commercial vaccine.

Of the 88 animals that were protected from death by the strain NADL A-13 vaccine, only four (killed 15 days after challenge) gave positive cultures from kidney and urine; these four hamsters received either 10 µg or 25 µg of vaccine and were challenged either 14 or 30 days later. Blood, liver and brain cultures were negative.

Of the 29 animals that were protected from death by the commercial vaccine, only two gave positive cultures from kidney and urine; blood, liver and brain gave negative results on culture.

Because both vaccination and challenge injections were made by the intraperitoneal route, it was necessary to exclude any complication raised by the possibility of non-specific protection. Accordingly, the above experiment included an additional 20 hamsters inoculated with 1 ml of either P-80 medium or BSAD and challenged 14 days later. Like the untreated controls, all these hamsters died within 5 days of challenge and consistently yielded isolates of strain NADL A-13 from blood, liver, brain, kidney and urine. Macroscopic evidence of leptospiral infection consisted of enlarged and engorged kidneys and liver, and extensive haemorrhages in the lungs and gastro-intestinal and urogenital tracts.

The geometric means of the MA titres measured 15 days after challenge, within the nine sub-groups of hamsters that received strain NADL A-13 vaccine, varied from 190 to 260.

Vaccination with virulent and avirulent canicola strains, and the effect of exposure to heat on protective potency

Table II shows that the avirulent strain Hond Utrecht IV, like the virulent strain NADL A-13, gave complete protection when used as a vaccine killed by heating at 50°C for 4 h. Strain NADL A-13 vaccine killed by heating at 98°C for 15 min. also gave complete protection, but heat inactivation at 121°C for 15 min. gave only partial protection as judged by deaths and by cultures made from the survivors. The MA titres measured 15 days after challenge were lower in hamsters that had received vaccine heated at 121°C than in hamsters treated with other vaccines.

Serological response to heat-killed leptospirae

The post-vaccination and post-challenge MA titres of individual hamsters given heat-killed vaccines varied widely, but the geometric mean of titres was useful as an index of group response. The geometric means of the titres of vaccinated, unchallenged hamsters increased with dose. By the 30th day after vaccination, doses of 2, 5, 10, 25, 40 and 80 µg had produced geometric mean titres respectively of 5, 10, 20, 100, 160 and 200.

DISCUSSION

The unvaccinated hamsters were completely susceptible to the challenge given. In contrast, hamsters immunised with heat-killed *canicola* vaccines prepared from the homologous strain and from an avirulent strain were, under optimal conditions, fully protected as judged by survival and by cultural methods. As *canicola* suspensions killed at 98°C and at 50°C were both capable of conferring complete protection on hamsters, it seems unlikely that essential immunogens are lost as a result of inactivation at temperatures of 98°C or below.

Viable leptospirae were present in the kidney and urine of all hamsters that survived challenge after vaccination with *canicola* suspensions killed at 121°C. It would seem that the immunity against residual renal infection elicited by *canicola* suspensions killed at lower temperatures is antibody mediated. None of the survivors given vaccine killed at 121°C had culturable leptospirae in the liver, brain or blood. The explanation may be either that the kidney provides a more favourable environment than the other three tissues, that leptospirae in renal tissue are protected to some degree, or that the immunogens eliciting protection against leptospiuria may be distinct from those that protect against systemic infection. If the latter possibility were correct, differences in the distribution of these immunogens among vaccine strains or serotypes might account for the well-known inconsistencies in protective performance of leptospiral vaccines.

Previous reports indicating that the doses of leptospiral vaccines required to prevent leptospiuria are in excess of those required to prevent death are

TABLE II

Protection of hamsters by canicola vaccines inactivated by different heat treatments*

Canicola vaccine strain	Vaccine killed by exposure to	Deaths in groups challenged 14 days after vaccination†	Number of survivors that, at slaughter, gave positive cultures from		Geometric mean of MA titres of survivors 15 days after challenge
			blood, liver and brain	kidney and urine	
NADL A-13	98°C for 15 min.	0/10	0	0	175
NADL A-13	121°C for 15 min.	6/15	0	9	<100
NADL A-13	50°C for 4 h	0/10	0	0	170
Hond Utrecht IV	50°C for 4 h	0/10	0	0	165

* Vaccine dose = 40 µg.

† Challenge dose of strain NADL A-13 = 26×10^6 cells. Ten unvaccinated hamsters all died after challenge and cultures from all sites were consistently positive.

supported by this investigation, but the degree of excess appeared not to be unduly large. In our tests, a dose of 25 µg prevented death and infection of the blood, liver and brain in all of the experimental hamsters, and 40 µg prevented the development of leptospiuria. No hamster vaccinated with 10 or 25 µg and slaughtered as late as 30 days after challenge was culturally positive in any tissue examined. Thus, the leptospiuria found in challenged hamsters vaccinated with the heat-killed vaccines appeared to be of a temporary nature. It would be of interest to examine the virulence of isolates from the urine of vaccinated hamsters that have been challenged. In the present study, limitation of leptospiuria was a useful indication of the immunising capacity of vaccines.

The data suggest that the dosage required to protect hamsters was greater when the animals were challenged 30 days or less after vaccination than when they were challenged later. This accords with the conclusions of previous investigators who identified leptospiral protective antibodies with the slowly developing immunoglobulins of the IgG class.

There were no local or systemic reactions in any hamster given intraperitoneal injections of any of the vaccines or suspending fluids. The literature cited by Alston and Broom (1958) indicates that heat-killed vaccines injected subcutaneously into a variety of animal species do not cause serious reactions.

The results of this study indicate that some strains of *Leptospira interrogans* serotype *canicola* contain potent immunogens that are highly resistant to heat. Information is needed on the possible correlation of dose response in the hamster with that in other species, especially the dog. Because they are easy and convenient to prepare, heat-killed vaccines may provide a useful tool for studies of immunity to leptospirosis.

SUMMARY

The immunogenicity of *Leptospira interrogans* serotype *canicola* suspensions inactivated by various degrees of heat exposure was examined in hamsters.

No differences between leptospirae killed at 50°C and at 98°C were shown. After exposure to 121°C, suspensions retained their ability to protect against lethal infections but lost their ability to prevent leptospiruria. Tests with vaccines inactivated at or below 98°C showed that the doses required for complete protection varied with the interval between vaccination and challenge. Larger doses were required to prevent the development of leptospiruria than to prevent death.

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