The growth of *Leptospira australis* B in the kidneys of mice in the incipient experimental carrier state

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INTRODUCTION

The carrier state in leptospirosis has been known since 1915 (Ido, Hoki, Ito & Wani, 1916) and reviewed recently (Babudieri, 1958). The only facts about its pathogenesis are that (i) the carrier state may develop in unvaccinated or inadequately immunized animals 9–15 days after a generalized acute infection (Proehoeman, 1930; Dinger & Verschaffelt, 1930; Faine, 1962a) from which recovery may be either spontaneous, owing to antibody formation (Faine, 1957b) or assisted by antiserum or antibiotics; (ii) the leptospirae live in the convoluted tubules of the kidneys of the carrier usually without any tissue reaction to their presence (Kwee Tat Tjong, 1940); (iii) the leptospirae are excreted in the carrier's urine which may contain homologous antibody (van der Hoeden, 1936; Stuart, 1956). There is no explanation for the localizing of the leptospirae in the renal tubules, nor is there evidence that they multiply rather than survive there.

It has been shown (Faine, 1962a) that the carrier state is a result of a quantitative balance between host and leptospiral factors. This paper describes the growth of leptospirae in the host as it becomes a carrier.

METHODS

The strain, source, maintenance and weighing of mice, and the preparation of tissue suspensions were described previously (Faine, 1962a). *L. australis* B (Boyle), isolated in 1960 from man was kindly provided by Mr D. J. W. Smith of Brisbane. Details of its maintenance and virulence have been described (Faine, 1962a), as has been the direct counting method (Faine, 1957a). Leptospirae grown *in vivo* in mouse kidneys and found in them or in urine are referred to as 'renal' leptospirae.

Agglutination-lysis (AL) tests

These were performed using live antigens in suitable dilutions in 0·02 ml. volumes in haemagglutination trays. Results were read microscopically under dark-ground illumination following incubation for 2 hr. at room temperature or at 30° C. The temperature of incubation did not affect the result. The endpoint, taken as the highest dilution of antiserum showing agglutination, as described originally by Schüffner & Mochtar (1927), is more sensitive and in our hands more reproducible than a subjective assessment of the proportion of unagglutinated leptospirae in a suspension. Young cultures seeded with heavy inocula and

incubated 3–4 days at 30° C. provided suitable antigen suspensions without signs of autoagglutination. Urine for AL tests was serially diluted in twofold steps. Frequently non-specific killing of leptospirae was observed up to titres of 1/4. This was diluted out and could be easily distinguished from immune activity.

Antisera

Rabbits were immunized intravenously and mice intraperitoneally either with repeated injections of live cultured leptospirae, to provide antisera designated C, or with a single dose of renal leptospirae to provide antisera designated R.

Gel-diffusion precipitin tests

Several double-diffusion methods (Ouchterlony, 1958) or minor modifications were used which gave satisfactory results with known precipitating systems. Antigens were prepared by centrifuging cultures or suspensions of ground kidneys pooled from several mice at 10,000 g for 30 min. The sediments were resuspended to contain approximately 10^9 cultured leptospirae or 10^8 renal leptospirae/ml. Some suspensions were used intact, while others were sonically vibrated at 20 kc./sec. for 1 min.

Passive cutaneous anaphylaxis tests

Tests were performed according to the techniques of Ovary (1958). Guinea-pigs weighing 2–400 g. were sensitized intravenously with 0·4–0·5 ml. of a rabbit anti-leptospiral serum C or R of a titre 10^{-4}. Four hours later they were challenged intradermally with 0·1 ml. of antigen, 15 min. after an intravenous injection with 0·5–1·0 ml. of a 1 % saline solution of Evans Blue. Results were read by measuring the average diameter of blue areas in the intact animal after 30 min., and 36 hr. after injection of antigen.

Guinea-pigs were killed at the latter time and the diameter of the blue areas measured on the under-surface of the reflected skin. Suitable controls were included.

RESULTS

Growth of leptospirae in the kidneys of mice

Urine from carrier mice contained 10^3–10^7 leptospirae/ml. Suspensions of the kidneys from individual carrier mice were prepared and the leptospirae counted. There was a range of 10^5–10^7 leptospirae per pair of mouse kidneys. Assuming a daily urinary volume of about 1 ml., carrier mice must excrete daily almost all the leptospirae in their kidneys, and leptospirae must grow there.

The growth of leptospirae in mouse kidneys was followed by injecting intra-peritoneally 31 mice (average weight 12·7 ± 0·2 g.) chosen at random from 41 mice (average weight 12·6 ± 0·2 g.) with $7·8 \times 10^3$ leptospirae in 1·0 ml. of a suspension of carrier mouse kidneys diluted 100-fold in buffered saline. All mice were weighed daily, the 10 uninfected mice (average weight 12·4 ± 0·5 g.) acting as weight controls. Pairs of infected mice selected at random before the experiment were killed at intervals. Serum and in one experiment urine were kept for AL tests and leptospirae were counted in suspensions of kidneys, urinary bladder and urine.

For the first 7 days suspensions of all the blood obtainable, the liver, spleen, heart, lungs and adrenals were also prepared and leptospirae counted. Four of the infected mice died of leptospirosis between the 3rd and 13th days. Their average weight was $11 \cdot 8 \pm 0 \cdot 5$ g. at infection. Elimination of the weights of these mice changed the average weight of the survivors insignificantly to $12 \cdot 8 \pm 0 \cdot 2$ g.

Leptospirae were never found in organs other than the kidneys. Counts of renal leptospirae and a summary of the findings, including gross changes seen at autopsy, are shown in Fig. 1. Two similar experiments using inocula of 1×10^3 and 5×10^3 leptospirae produced essentially similar results in all respects except that the onset of leptospiruria was delayed until later than 14 days with the lower dose. The spleens of all mice autopsied 6–12 days after infection were enlarged and pale showing gross enlarged grey follicles.

Fig. 1 shows that mice lose weight within 3 days of infection. In the acute phase detectable numbers of leptospirae are not found in the kidneys until the 6th day, rising to not more than 10^5 on the 7th day in all experiments. Numbers then fall abruptly so that leptospirae cannot be found until there is a steady regrowth from the 10th day to reach a high and relatively constant level by the 18th day. By this time all mice had leptospiruria. Although the data are not adequate for an accurate calculation of generation time, approximations are 7, 9 and 16 hr. for the three experiments.

AL titres against cultured *L. australis* B were measured in the supernatants of kidney suspensions. The results were negative until antibody appeared in the serum. After this time titres varied up to 1/32. The leptospirae contained in such suspensions survived apparently unaffected by antibody for more than 24 hr. at room temperature.

In similar experiments (Lane & Faine, to be published), the blood urea level rose almost immediately after infection from a normal value of 35 mg./100 ml. to a peak of 90 mg./100 ml. at 12 days. Its fall coincided with recovery of weight loss, the disappearance of lesions and of casts from the urine, as well as with the growth of leptospirae in the kidney.

Relative serum and urinary antibody levels

AL titres rose to 1/2–1/10 in mice by the 3rd–5th day after infection, rising rapidly to 10^{-4}–10^{-5} about the 10th day. Such high-titre sera often showed prozones up to 10^{-2}. Serum and urinary antibody from a typical experiment are shown in Fig. 1. Average urinary antibody titres reached 1/6–1/20 of the serum level at their highest levels, at about the time when re-growth of leptospirae was occurring in the kidneys.

Resistance of renal leptospirae to antibody

Leptospirae excreted in the urine of carrier mice survived there for more than 36 hr. although urinary antibody titres varied up to 1/32. Renal leptospirae were not agglutinated or lysed by any of the following preparations containing antibody up to final titres of 10^{-3}: (i) the carrier's own serum added either to urine or to the kidney suspension; (ii) mouse or rabbit antisera C or R against the strain of
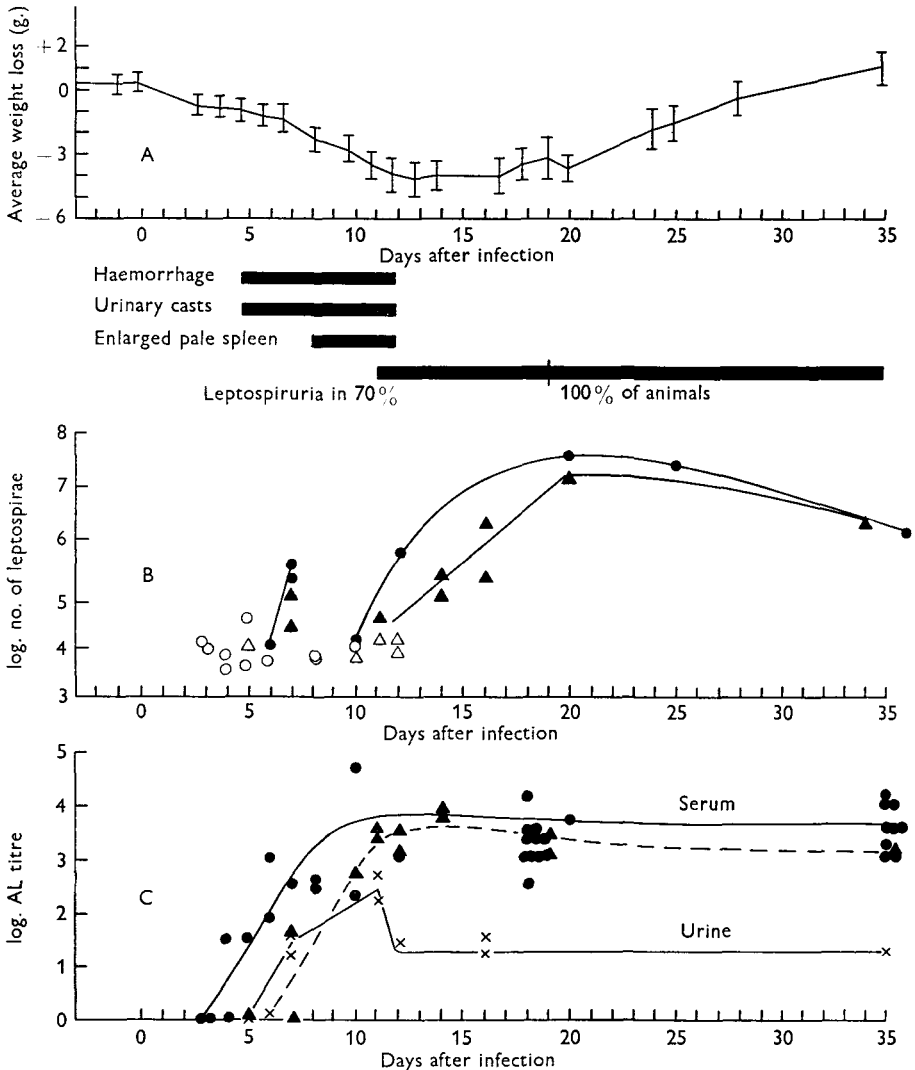
Fig. 1. (A) Loss of weight, (B) growth of leptospirae in the kidneys and (C) serum and urinary antibody titres in 2 experiments where mice were infected intra-peritoneally with renal leptospirae in mouse kidney suspension and killed at intervals in a pre-arranged order. A, Weight loss, shown for experiment 1 only, is the difference ($\pm$ S.E.) between the average weights of 10 control mice and test mice. Thirty test mice (average weight $12 \cdot 6 \pm 0 \cdot 2$ g.) commenced the experiment, reducing in number to 9 at day 35. Weight losses over $1 \cdot 8$ g., from days 8–20, were significant ($P < 0 \cdot 001$). B, Log number of leptospirae in suspensions of the kidneys of mice inoculated with $3 \cdot 8 \times 10^3$ leptospirae (experiment 1, ●—●) or $3 \cdot 0 \times 10^3$ leptospirae (experiment 2, ▲—▲). The symbols ○ or △ indicate for experiments 1 and 2 respectively the largest number of leptospirae which could have passed undetected because of the lower limit of the counting method. C, AL titres of serum from mice during experiment 1 (●—●) and experiment 2 (▲- - -▲), and from urine in experiment 2 (× — ×). (Data for urine titres in experiment 1 are not available.)

The bars between graphs A and B show the times when principal changes were seen macroscopically at autopsy during the infection, on the same time scale as the graphs. Leptospiruria was seen in 70 % of the mice from day 12, and in 100 % of mice from day 19 onwards.

L. australis B used in infections; (iii) mouse antisera prepared by immunizing with *L. australis* B grown in homologous rabbit antiserum C or R; (iv) rabbit anti-mouse globulin either alone or with any of the above antisera (i)–(iii). Incubation at room temperature, 30° or 37° C., with or without guinea-pig complement did not affect the results. These observations have been confirmed in a mouse of the Walter and Eliza Hall Institute coloured strain, inoculated in North Queensland with blood from a patient. This mouse, kindly supplied by Miss M. L. Emanuel, was a heavy excretor of *L. australis* A. Renal leptospirae excreted by the mouse were unaffected by its serum which showed an AL titre of 10^{-3} against a cultured strain of *L. australis* A.

Evidence for antigenic differences between renal and cultured leptospirae was sought using cross-absorption, passive cutaneous anaphylaxis (PCA) and gel diffusion precipitin tests as well as ability to grow in antisera. Volumes of 0·1 ml. of rabbit antisera C or R adjusted to a final titre of 10^{-3} were each absorbed twice with equal volumes of a concentrated suspension either of cultured or of renal leptospirae. The leptospirae were centrifuged and each supernatant tested against cultured leptospirae. There was complete cross-absorption. PCA tests were performed in guinea-pigs and the results read as described on p. 436. Guinea-pigs sensitized with rabbit R or C antisera were challenged with intradermal injections of 0·1 ml. of concentrated suspensions either of cultured or of renal leptospirae. Control injections of 0·1 ml. of culture medium, and of mouse kidney suspension were interspersed among the test injections. Either set of antigens gave a positive reaction of 7–8 mm. average diameter either 5 or 36 hr. after challenge when the dose contained not less than about 10^8 leptospirae. Controls gave reactions up to 3–4 mm. in diameter.

Gel diffusion precipitin tests were set up using antigens from cultures or from renal leptospirae, in each case either sonically disrupted or untreated. No precipitation was observed with tenfold dilutions up to 10^{-3} of antigen with any of a range of similar dilutions of rabbit or mouse antisera C or R, each with AL titre of 10^{-4}.

Survival and agglutinability of leptospirae grown in antisera

Three batches of media were prepared each containing antisera to final AL titres of 10^{-2}. One contained rabbit antiserum C, one contained rabbit antiserum R and one contained antiserum from mice immunized with leptospirae grown in medium to which rabbit antiserum C had been added. Two tubes of each medium with antiserum and two of ordinary medium were inoculated either with a stock cultured *L. australis* B or with freshly isolated renal *L. australis* B in their first subculture. Duplicates were incubated at 30° or 37° C. No differences were seen between the two strains, or between cultures at 30° and 37° C. In all the media the leptospirae grew in irregular flakes up to 2–3 mm. diameter at the bottom of the container. Microscopically the flakes were seen to be tangles of live, vigorously motile leptospirae. Numerous small clumps and individual leptospirae were also present. On subculture in similar media an increasing number of leptospirae remained unagglutinated, giving rise to characteristic turbidity. The individual survivors did not appear to be affected by further addition of fresh antisera either

of type C or R, or by the addition of sheep anti-rabbit globulin. The leptospirae from all cultures grew typically and luxuriantly when transferred to ordinary medium, with concomitant dilution of antiserum.

DISCUSSION

In mice becoming leptospiral carriers the course of infection was essentially similar to that in guinea-pigs inoculated with attenuated cultures or with small numbers of virulent leptospirae (Faine, 1957b). Compared with acute fatal infection the onset of lesions is delayed while virulent individual leptospirae grow *in vivo* from small numbers in the inoculum. The generation-time of leptospirae in mice estimated in these experiments was similar to that reported in guinea-pigs (Faine, 1957b; Larson, Treick, Edwards & Cox, 1959). However, infection in mice was established within 3 days, measured by weight loss and nitrogen retention. Unless the animal becomes a carrier, growth of leptospirae stops on the 7th–8th day together with and presumably on account of an increase in antibody in man (Ido *et al.* 1916), guinea-pigs (Faine, 1957b) and mice (Fig. 1). The carrier state is characterized by a second wave of growth of leptospirae confined to the kidney. These leptospirae are almost certainly exposed to antibody during growth in renal tubules. It has been shown that antibody can pass from the bloodstream through renal tubules into urine in normal mice (Faine, 1962b) and in man (Remington & Finland, 1961). Urinary antibody in leptospirosis is well known (van der Hoeden, 1936; Stuart, 1956). Thus leptospirae are exposed to antibody in the tubules of intact kidneys, and will not be affected by tissue and circulating antibody solely as a result of the artefact made by crushing kidneys into a suspension.

Schüffner & Bohlander (1943) speculated that renal leptospirae might be relatively resistant to antibody if they survived in its presence in renal tubules. Antibody in urine, kidney suspensions or serum was active against cultured leptospirae, and protective against the acute phase of leptospirosis (Stuart, 1956; Faine, 1962a) although inactive against renal leptospirae. Urinary antibody is therefore similar to serum antibody in its action on cultured leptospirae. Furthermore, renal leptospirae revert to a susceptible form *in vivo* equally well as in culture. Support for the contention that renal leptospirae are resistant to antibody is given by the observations of Schüffner & Bohlander (1942), and Broom (1944). In their diagnostic experience leptospirae freshly cultured from animals often failed to be agglutinated at all, or typically, or to full titre until subcultured a few times. Presumably, such strains are slow to revert from the 'renal' to the 'cultured' state.

Antigenic differences were not detected between cultured and renal leptospirae, but precipitin reactions were not observed with several antigen preparations using high AL titre rabbit or mouse sera, although other precipitating systems worked satisfactorily under identical conditions. Antiglobulin failed to alter the effect of antiserum or to prevent the emergence of persisters, which occurred at dilutions greatly exceeding observed prozones. Hence it is unlikely that renal leptospirae resist antibody because they are coated with blocking antibody or globulin.

Although a stable antigenic change was not found to follow the growth of leptospirae in homologous antiserum in these experiments or those of Schüffner & Mochtar (1927) it has been reported that several repeated cultures in antibody may produce a stable antigenic variant (Pike & Schulze, 1958). If such changes can result from growth under appropriate conditions in the presence of antibody in the kidney, the nature of the carrier state itself can provide all the factors necessary for the emergence of a new serotype. Further exhaustive studies of these problems are required, in particular of the antigenic homogeneity of apparently pure cultures and of whether the change to the 'renal' or antibody-resistant form represents a selection or a mutation. Genetic studies to investigate these points are in progress.

The presence of antibody does not prevent leptospirae from growing in renal tubules. Thus there are two essential conditions permitting the carrier state: (i) pathogenic mechanisms which allow initial acute infection, and (ii) growth of leptospirae *in vivo*, including absence of phagocytosis in the kidney.

SUMMARY

The growth of *L. australis* B in the kidneys of young mice which become carriers was followed after experimental intraperitoneal infection. There was a primary growth corresponding to generalized acute infection and terminated at the time of appearance of antibody. A secondary growth of leptospirae followed in the kidneys alone about 7–10 days after infection, coinciding with the onset of leptospiruria and recovery from infection. Subsequently mice carried about 10^6–10^7 leptospirae in their kidneys permanently.

The leptospirae in the urine or in the kidneys of carriers were resistant to the action of antibody in the serum or urine of the host animal, or in rabbit antisera prepared against mouse-renal leptospirae or against cultured leptospirae of the infecting strain. No antigenic differences were detected between renal and cultured leptospirae. An analogous situation is the growth *in vitro* of leptospirae in homologous antiserum. The mechanism permitting growth of leptospirae in homologous antiserum *in vivo* or *in vitro* is unknown.

The carrier condition results from the ability of virulent leptospirae to (i) grow in the host and produce lesions in the primary, acute generalized infection, (ii) grow in renal tubules in the presence of antibody.

REFERENCES

BABUDIERI, B. (1958). Animal reservoirs of leptospires. *Ann. N.Y. Acad. Sci.* **70**, 393.

BROOM, J. C. (1944). Footnote to abstract. *Bull. Hyg.* **19**, 396.

DINGER, J. E. & VERSCHAFFELT, F. (1930). Recherches expérimentales sur quelques souches de leptospires. *Ann. Inst. Pasteur*, **45**, 396.

FAINE, S. (1957a). Virulence in leptospira. I. Reactions of guinea-pigs to experimental infection with *Leptospira icterohaemorrhagiae*. *Brit. J. exp. Path.* **38**, 1.

FAINE, S. (1957b). Virulence in leptospira. II. The growth *in vivo* of virulent *Leptospira icterohaemorrhagiae*. *Brit. J. exp. Path.* **38**, 8.

FAINE, S. (1962a). Factors affecting the development of the carrier state in leptospirosis. *J. Hyg., Camb.*, **60**, 427.

FAINE, S. (1962b). Antibody in renal tubules of mice. *Aust. J. exp. Biol.* (In the Press.)

Ido, Y., Hoki, R., Ito, H. & Wani, H. (1916). The prophylaxis of Weil's Disease. *J. exp. Med.* **24**, 471.

Kwee Tat Tjong (1940). Over de positie der leptospiren in de nier bij chronische uitscheiders. Thesis, Batavia. Quoted by Van Thiel (1948), p. 51.

Larson, A. D., Treick, R. W., Edwards, C. L. & Cox, C. D. (1959). Growth studies and plate counting of leptospires. *J. Bact.* **77**, 361.

Ouchterlony, Ö. (1958). Diffusion-in-gel methods for immunological analysis. *Progr. Allergy*, **5**, 1.

Ovary, Z. (1958). Immediate reaction in the skin of experimental animals provoked by antibody–antigen interactions. *Progr. Allergy*, **5**, 459.

Pike, R. M. & Schulze, M. L. (1958). Serologic variants of leptospira types resulting from growth in immune serum. *J. Immunol.* **81**, 172.

Proehoeman, S. (1930). Studies over de epidemiologie van de ziekte van Weil, over haren verwekker en de daaraan verwante organismen. Thesis, Amsterdam. Quoted by Van Thiel (1948), p. 117.

Remington, J. S. & Finland, M. (1961). Precipitating antibody in normal human urine. *Proc. Soc. exp. Biol., N.Y.*, **107**, 765.

Schüffner, W. & Bohlander, H. (1942). Klinische und bakteriologische Beobachtung einer Laboratoriumsinfektion mit Schlammfieber. Anhaltende Leptospirurie. *Zbl. Bakt.* (1 Abt. *Orig.*), **149**, 193.

Schüffner, W. & Bohlander, H. (1943). Ueber den verschiedenen Verlauf der durch Leptospiren hervorgerufenen Nierenprozesses bei Feldmaus und Ratte. *Z. ImmunForsch.* **104**, 237.

Schüffner, W. & Mochtar, A. (1927). Versuche zur Aufteilung von Leptospirenstammen mit einleitenden Bemerkungen uber den Verlauf von Agglutination und Lysis. *Zbl. Bakt.* (1 Abt. *Orig.*), **101**, 405.

Stuart, R. D. (1956). The importance of urinary antibodies in the diagnosis of leptospirosis. *Canad. J. Microbiol.* **2**, 288.

Van der Hoeden, J. (1936). Anticorps spécifiques de la maladie de Weil dans l'urine. *Ann. Inst. Pasteur*, **56**, 206.

Van Thiel, P. H. (1948). The Leptospiroses. Leiden: Universitaire Pers.