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VIRULENCE IN LEPTOSPIRA. I: REACTIONS OF GUINEA-PIGS TO EXPERIMENTAL INFECTION WITH *LEPTOSPIRA ICTEROHAEMORRHAGIAE*

S. FAINE*

From the Sir William Dunn School of Pathology, University of Oxford†

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IT is well known that pathogenic leptospira lose virulence when grown in culture. There have been no studies of the mechanism of virulence, although Dinger (1932) and Chang (1947) claimed, without confirmation, that they could preserve virulence *in vitro* by various means. The aim of this study was to compare the fate of virulent and avirulent *Leptospira icterohaemorrhagiae* in young guinea-pigs and to investigate some host reactions which might determine the outcome of infection.

MATERIALS AND METHODS

Two strains, " Field " and " Jackson ", of the " complete biotype " of *L. icterohaemorrhagiae* were kindly supplied by Dr. J. C. Broom from the collection of the Leptospira Reference Laboratory.

Virulent spirochaetes.—The " Field " strain was virulent for guinea-pigs. It was maintained at maximum virulence by continual passage through successive guinea-pigs of infected blood or liver obtained at death of the infected animal. The leptospira were cultivated periodically from the blood of moribund guinea-pigs and maintained in culture for use in experiments *in vitro*. The number of " culture passages " referred to in the text is the number of subcultures since isolation from the guinea-pig reservoir. The virulence of organisms obtained directly from the animals was of the order of LD_{50} = one organism.

Avirulent spirochaetes.—The " Jackson " strain has been avirulent for young guinea-pigs for many years. A dose of 10^{10} leptospira produced no ill effects, and lesions could not be found in a 300 g. guinea-pig on histological examination of the organs one week after the intraperitoneal injection of 10^9 leptospira.

Culture

The organisms were cultivated in a modified Korthof medium containing salts, 0·08 per cent " Neopeptone " and 10 per cent rabbit serum. Except where stated they were grown at 30°. Cultures were made isotonic when required by adding 1/10th vol. of 8 per cent NaCl.

* Nuffield Dominions Demonstrator.

† Present address: Department of Microbiology, Otago University Medical School, Dunedin, New Zealand.

Counting

A direct counting method was used. An " Agla " micrometer syringe half filled with liquid paraffin was mounted pointing downwards at an angle of about 45° on a retort stand. The ferrule of a cut-off No. 19 needle attached to the syringe was surrounded with plasticine. An expendable capillary tube 5–10 mm. long, approx. 2–3 mm. in diameter at the lower end, was fitted over the needle and embedded in the plasticine. With the micrometer, 0·01 ml. of the fluid to be counted was drawn into the capillary tube, expelled on a clean slide and covered carefully with a square $\frac{3}{4}$ in. (1·9 cm.) No. 0 coverslip. The used capillary was then discarded. The spirochaetes were counted directly under dark-ground using a 4 mm. or 8 mm. objective and a 15 × eyepiece containing a graticule ruling, of which all but ten central squares were obscured, and observing the usual conventions regarding organisms lying on a line. Five, ten or a larger convenient number of squares were counted, distributed in the four corners and the centre or otherwise evenly and systematically over the coverslip area. The number of spirochaetes per ml. of fluid was obtained by multiplying the average number of organisms per eyepiece square by the number of squares in the coverslip area, for each objective, and by 100 (the reciprocal of the volume of the drop).

Error of this method.—Results were repeatedly reproducible within 10 per cent in duplicates. Motility of the spirochaetes did not introduce difficulties at the magnifications used, but care was necessary to avoid drying or drifting movements. The method may be adapted to counting small numbers of organisms by searching systematically a large number of fields. Counts down to an order of 10^3 or even 10^2 organisms per ml. may be made. Very densely grown cultures may require dilution to a suitable density before counting.

Counting under aseptic conditions.—It was found best to remove aseptically with a sterile Pasteur pipette small aliquots of fluids required to be kept uncontaminated. The aliquot delivered into a Dreyer tube was then treated as described.

Viable count.—Except where stated, the number of spirochaetes given refers to motile organisms ; dead organisms were not counted unless total counts were required. Thus " count " means " viable count ".

Staining

Leptospira were stained in smears with Fontana and Giemsa techniques, and in sections by Dobell's modification of the Levaditi method, which allowed counterstaining of the tissues with haematoxylin and eosin (H.E.) if required.

Serological Methods and Controls

Production and titration of antisera

Rabbits previously shown to be free of antibodies to *L. icterohaemorrhagiae* were injected intravenously thrice weekly for three weeks with 12-day cultures of leptospira. Doses of approx. 10^7 organisms were used the first week, and the dose doubled each week as recommended by van Thiel (1948). The sera were titrated in depressions on porcelain tiles or in Dreyer tubes, using doubling dilutions. Live cultures were used as antigens for agglutination-lysis and formolized cultures for agglutination tests ; in each case the antigen suspension contained approx. 10^7 organisms per ml.

The rate of appearance of antibodies in rabbits

One rabbit was injected intravenously with 5×10^8 leptospira in 2·5 or 5 ml. of each of the following 12-day cultures : avirulent live, avirulent formolized, virulent live and virulent formolized. Each rabbit was bled a few drops from an ear vein hourly for the first day and at short intervals on successive days. The sera were titrated for agglutinins and lysins. They were produced in parallel in each animal 50–60 hr. after inoculation in each case. The agglutination and lysis titres of each serum rose to a maximum of 16,000 to 500,000 on the 8th–10th day after inoculation. There was no difference between the four animals in the rate of appearance of the antibodies. In each the titre had fallen to a constant level within 20 days. Thus it appears that a single large dose of leptospira will immunize rabbits as effectively as the elaborate schedule used at first. A second dose of an antigen preparation similar to the first was given 100 days after the first injection. There was no significant difference between the responses to virulent or avirulent leptospira.

Serological relationships of virulent and avirulent leptospira

Rabbit antisera prepared against virulent or avirulent leptospira showed no significant differences in agglutination or agglutination-lysis titres when titrated against either virulent or avirulent cultures. Volumes of 0·25 ml. of antiserum diluted 1/100 were cross-absorbed with concentrated washed suspensions containing 5×10^9 live heterologous-strain organisms in 1·25 ml., giving final antiserum concentrations of 1/600 in 1·5 ml. Agglutinins for both homologous and heterologous strains were completely removed. Absorption of lytic antisera (original titre 6400) with washed formolized leptospira completely absorbed both agglutinins and lysins.

Animals

Guinea-pigs of the stock laboratory strain were of stated weight, usually 100–250 g. They were fed on oats, bran and green-feed. They were apparently uniformly susceptible to infection with virulent *L. icterohaemorrhagiae*. Mice used in some experiments were of the stock laboratory strain. They were almost uniformly resistant to leptospiral infection, although some developed a renal carrier state. Rabbits used for immunization weighed 1·5–2 kg.

Peritoneal exudates containing polymorphonuclear leucocytes were produced by injecting intraperitoneally approx. 7·5 ml./100 g. body weight of sterile saline 15–18 hr. before the experiment, and a further 5 ml. 2 hr. before. Mononuclear exudates were produced by injecting intraperitoneally 2 ml. sterile liquid paraffin into guinea-pigs, or 0·2 ml. into mice, 3 days before the experiment.

Intraportal injection

Under ether anaesthesia an upper right paramedian incision 3 cm. long was made in a guinea-pig. The portal vein was exposed on the dorsal surface of the pancreas and ligated just above the entrance of the splenic vein. About 1 cm. of the end of a cannula of polythene tubing of approx. 1 mm. internal diameter and 6 cm. long, fitted tightly over a No. 19 needle attached to a syringe and filled to the tip with the material to be injected at 37°, was inserted and tied into the portal vein through a nick just proximal to the ligature.

Leptospirosis in guinea-pigs

Typically, guinea-pigs of 150 g. infected intraperitoneally with 10^6 or more virulent leptospira appeared ill on the 3rd–4th day. They became febrile, pale and anorexic. Jaundice usually appeared about the 4th–5th day, preceding death by about 12 hr. and increasing rapidly together with a terminal fall in temperature. The most prominent pathological appearance at autopsy, apart from jaundice, was widespread petechial, sometimes confluent haemorrhages, especially in the lung, abdominal wall muscle and retroperitoneal fat. Renal tubular necrosis was invariably present leading to death from uraemia ; nitrogen retention was demonstrable about 24 hr. after infection and rose terminally to a blood urea level of over 200 mg. per 100 ml. Spirochaetes could always be found in blood cultures throughout the infection. At death, the blood, liver and areas of haemorrhage were swarming with spirochaetes which were easily seen in smaller numbers throughout all tissues examined.

EXPERIMENTS

The Fate of Leptospira Injected Intraperitoneally

Two groups each of six guinea-pigs weighing 250 g. were injected intraperitoneally with 2×10^7 leptospira from a 5-day culture. One group received virulent organisms (6th culture passage), the other avirulent organisms. One animal in each group was killed with ether at intervals of 2, 26, 40, 66, 90 and 96 hr. after injection. Sections stained by Dobell's method and H.E. were prepared from skin, abdominal wall muscle, mesenteric lymph nodes, retroperitoneal fat, kidney, adrenal, liver, stomach wall, bladder, lungs and heart, and examined for the presence of spirochaetes and for lesions.

The results of the experiment using virulent spirochaetes are shown in the Table. Spirochaetes undergoing granular degeneration were commonly seen in

tissue sections taken at all stages of infection (Fig. 1). In the final stages of degeneration typical round black (argyrophil) granules, sometimes seen in phagocytes, represented the remains of the leptospira.

TABLE—*Time of Appearance of Spirochaetes and of Histological Lesions in* 250 *g. Guinea-pigs. Injected I.P. with* 2×10^7 *Virulent* L. icterohaemorrhagiae.

	First appearance (hr. after inoculation) of				
	Spirochaetes in				
	Tissue	Blood vessels	Degenerating spirochaetes	Haemorrhages	Degenerative lesions
Liver	2	2	2	40	40
Kidney	2	2	—	40	26
Lymph nodes	40	40	26	—	—
Retroperitoneal fat	66	26	66	40	—
Lung	90	26	26	26	—
Muscle	90	26	66	66	40
Spleen	90	90	90	—	—

— = not observed.

Avirulent spirochaetes were found in the liver, kidney and lymph node 2 hr. after injection. Twenty-six hours after injection spirochaetes could not be found at all, but degenerating forms and granules were found in the lymph node. Avirulent organisms caused no apparent histological changes. Neither virulent nor avirulent organisms produced any significant local inflammatory response. Although there was undoubtedly some phagocytosis in the lymph nodes and in Kupffer cells in the liver, virulent leptospira apparently grew to great numbers in the liver. They could be found throughout the infection in blood vessels in sections. Organs other than the liver, kidney, spleen and lymph nodes appeared to be invaded by spirochaetes carried into tissue spaces by blood extravasated in haemorrhages or in exudates. Leptospira appeared to be phagocytosed either in reticulo-endothelial tissues during massive bacteraemia, as in the terminal phases of infection, or by phagocytes active in areas of haemorrhage, which contained many organisms.

The Fate of Leptospira Injected Intravenously

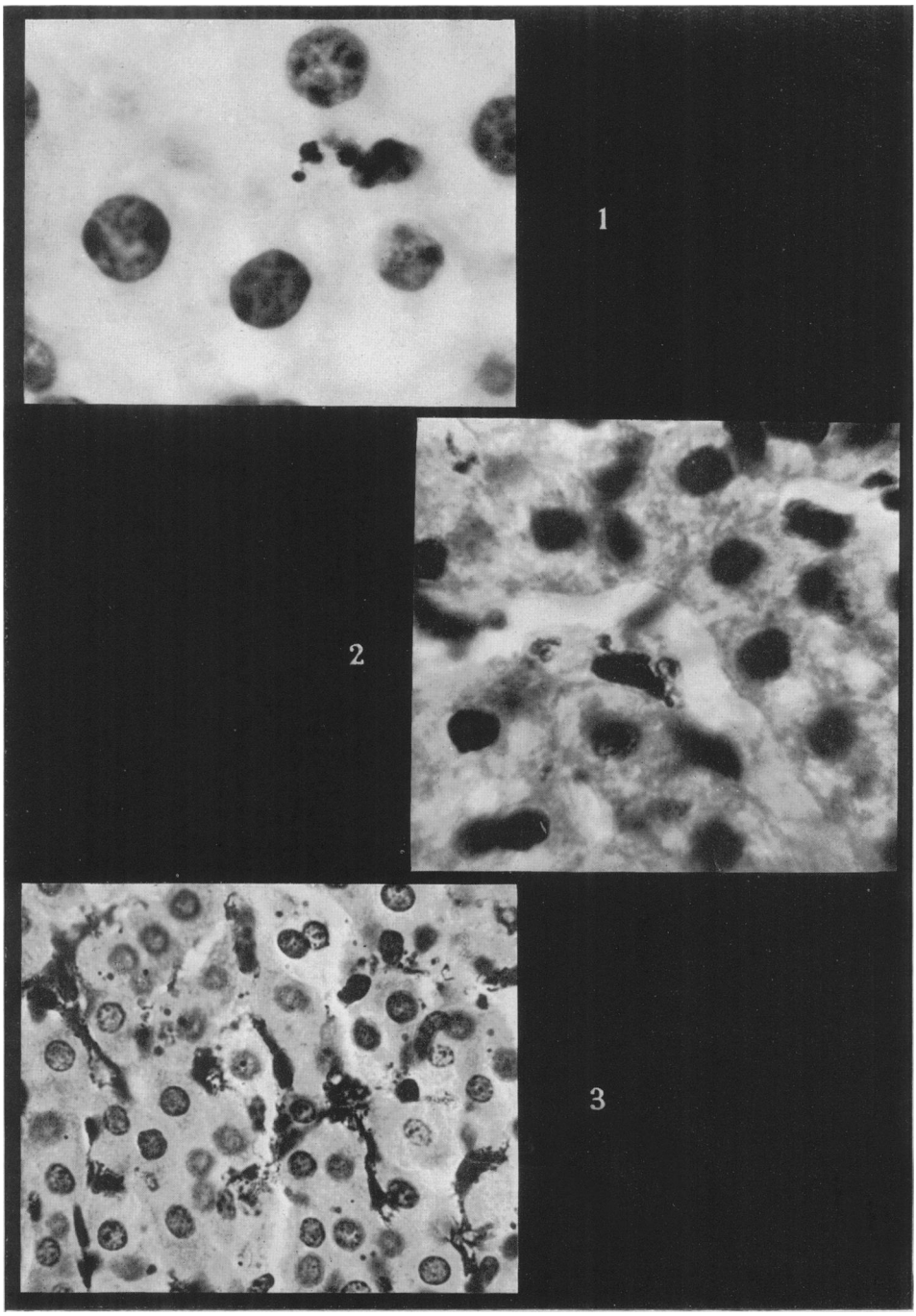
In an experiment similar to the foregoing, groups of guinea-pigs were injected intravenously. Of a concentrated even saline suspension of leptospira (second culture passage from a 10-day virulent culture), 1 ml. containing 10^9 leptospira was injected intravenously into each of three 250 g. guinea-pigs. A similar injection of avirulent organisms was made into three other guinea-pigs. Degeneration forms were very scarce in these suspensions. One of each group of animals was killed with ether 30 min., 90 min. and 5 hr. after injection. Sections stained by

EXPLANATION OF PLATE

FIG. 1.—A degenerating leptospira curling up into a granule in the liver. Dobell and H.E. × 1500.

FIG. 2.—A Kupffer cell in a guinea-pig 30 min. after the intravenous injection of virulent leptospira. Ingested leptospira may be seen beginning to curl up into granules. × 950.

FIG. 3.—Kupffer cells containing ingested leptospira in the liver of a guinea-pig after intraportal injections of washed virulent leptospira followed by 10 ml. saline. × 550.



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Dobell's method and H.E. were prepared from liver, kidney, spleen, lung and a mesenteric lymph node of each animal, and examined for spirochaetes and for lesions.

The results with virulent and avirulent organisms were almost identical. Thirty minutes after injection intact and degenerating forms of spirochaetes were found mainly interstitially and in Kupffer cells in the liver (Fig. 2) but also sparsely in lung, kidney and spleen. A few degenerating forms were seen phagocytosed in the lymph node. After 90 min. fewer of either form of leptospira were found in the liver. Degenerated forms only were seen in the lymph node, lung and kidney. In the spleen there was a greatly increased number of intact and degenerating organisms, the latter mainly inside phagocytes. After 5 hr. the distribution was still the same, except that the spleen now appeared to be infiltrated with numbers of mononuclear phagocytes containing many black granules representing phagocytosed degenerated spirochaetes.

It is concluded from this experiment that virulent and avirulent leptospira were phagocytosed by Kupffer cells in the liver and by macrophages in other tissues, both before and after degeneration into granular forms, soon after entry into other tissues. The phagocytes, carrying with them ingested spirochaetal débris, migrated to the spleen or lymph node, where they could be found after 5 hr. By this time the concentration of organisms in the liver had decreased. Surviving virulent leptospira remained to establish themselves in the liver. Degenerated leptospira appeared to behave like any other injected foreign particles.

Phagocytosis of Leptospira

Phagocytosis by Kupffer cells

Of concentrated saline suspensions of virulent 8-day cultures (2nd and 3rd culture passage) or of avirulent cultures, 2–5 ml. containing approx. 10^9 leptospira were injected over 10 min. into the portal vein of 200-g. guinea-pigs. The results with virulent or avirulent organisms were similar. Sections of liver excised 5–10 min. after injection showed large numbers of spirochaetes in the sinusoids, mainly trapped in Kupffer cells. Sections of spleen and lung showed few spirochaetes and no spirochaetal débris. In a similar experiment the injection of spirochaetes was followed by an injection over 10 min. of 10 ml. of warm saline through the same cannula. Leptospira were found attached to and apparently inside Kupffer cells in sections of liver excised at the end of the experiment.

Phagocytosis by leucocytes

Negative results were obtained in many experiments *in vivo* and *in vitro* where phagocytic leucocytes were mixed with leptospira under conditions designed to promote phagocytosis. Stavitsky (1948) has reviewed conflicting evidence concerning phagocytosis of leptospira. In view of the lack of confirmation of the only positive observations of phagocytosis of leptospira by leucocytes (Corrales, 1919 ; Martin and Pettit, 1919) the details are of interest.

Experiments in vivo.—Negative results were obtained in two series of experiments : (*a*) Guinea-pigs injected intraperitoneally with saline 18 hr. beforehand were injected intraperitoneally with 8–12 day cultures of virulent (2nd, 3rd and 6th culture passage) or avirulent leptospira. Samples of the peritoneal exudate, rich in polymorphs, were removed at intervals from 15 min. to $4\frac{1}{4}$ hr. after

infection and examined (*i*) directly under dark-ground in sealed preparations at 37°, and (*ii*) by Fontana or Giemsa staining of smears. Intraperitoneal injection of 0·1 ml. or 1·0 ml. of immune rabbit serum (agglutinin titre 5000) caused lysis of the spirochaetes without inducing phagocytosis. (*b*) Similar experiments done on guinea-pigs with a mononuclear peritoneal exudate 3 days old.

Experiments in vitro.—In two series of experiments, 0·1 ml. of 10-day isotonic (*a*) live or (*b*) heat-killed cultures were each incubated at 37° with 1 ml. vol. each of whole polymorphonuclear peritoneal exudate from guinea-pigs, a saline suspension of cells from the exudate, and the supernatant, to give final concentrations of leptospira in two groups of (*i*) $3{\cdot}5 \times 10^6$ per ml. and (*ii*) 2×10^3 per ml. In no case was there a significant reduction of numbers after standing, rotating or centrifuging, for up to 1 hr. Negative results were also obtained in similar experiments using mouse peritoneal exudates. Phagocytosis was never observed in the following preparations: (*a*) that of Nelson (1953) for demonstrating immune adherence, using either human or guinea-pig blood; (*b*) peritoneal exudates to which 10^8 leptospira per ml. had been added, the exudate being allowed to clot between a slide and coverslip and observed under dark-ground at 37°; (*c*) preparations on a thin slide spread with a thin film of 50 per cent serum agar on which a small drop of peritoneal exudate and spirochaetes was placed and covered with a No. 0 coverslip; using a 4 mm. objective, flattened amoeboid polymorphs and motile spirochaetes could be observed in contact, in one plane of focus; (*d*) fibrin mesh preparations for observing chemotaxis (Harris, 1953), in which a small drop of centrifuged leptospira was deposited in the centre of the preparation; chemotaxis was not seen.

In all the above preparations active phagocytes avidly engulfed any other bacteria added. The addition of neither immune serum, which caused eventual lysis of the leptospira, nor crystalline lysozyme to a final concentration of 1/1000 (Gladstone and Johnston, 1955) induced phagocytosis.

No tests were done *in vitro* with mononuclear peritoneal exudates.

DISCUSSION

Standard methods demonstrated no serological differences between virulent and avirulent leptospira which might determine virulence. More elaborate methods appear to be less sensitive than those of agglutination and agglutination-lysis (Schneider, 1955; Chang and McComb, 1954). There appeared to be no difference between the abilities of virulent and avirulent organisms either to penetrate from the peritoneal cavity after intraperitoneal injection or to be engulfed by Küpffer cells in the liver. The sole significant difference appears to be in the ability to survive in the infected animal.

Phagocytosis of leptospira was not observed directly in numerous studies by Stavitsky (1945) although Corrales (1919) and Martin and Pettit (1919) reported its occurrence. These reports could not be confirmed in numerous experiments. However, degeneration of leptospira in tissues into granules has been known since the earliest observations (Kaneko and Okuda, 1917). Zuelzer (1918) and Basile (1921) stated that these granules could be found in phagocytes in tissue sections. These results have been confirmed here, but phagocytosis of leptospira by leucocytes could not be seen either *in vivo* or *in vitro* under a variety of conditions favourable for phagocytosis of other bacteria. Apparently fixed phagocytes

such as Kupffer cells are able to engulf leptospira, at least under the experimental conditions provided, and probably also whenever there is a high local concentration of circulating spirochaetes such as is seen in the terminal phases of infection.

It is significant that chemotaxis could not be demonstrated, using a reliable technique, especially as there is apparently neither phagocytosis nor an inflammatory response to the presence of the spirochaetes. Such leucocytic infiltration as may be seen occasionally in the liver or kidneys may well be a result of the products of tissue breakdown as it does not occur early in the infection even in the presence of large numbers of organisms.

SUMMARY

The fate of virulent and avirulent *Leptospira icterohaemorrhagiae* injected into young guinea-pigs was followed histologically. Both strains behaved in the same way except that virulent organisms survived while avirulent organisms did not.

Using suitable techniques, phagocytosis of or chemotaxis by virulent or avirulent organisms was not seen *in vitro*. Both strains were taken up *in vivo* by fixed phagocytes in reticulo-endothelial tissues, but apparently not by leucocytes. Virulent and non-virulent organisms appeared to be serologically identical.

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Fig. 1 was photographed by Mr. B. H. Glass, Fig. 2 and 3 by Mrs. Daphne Lemon.

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