VACCINATION OF GUINEA-PIGS AND HUMAN BEINGS AGAINST LEPTOSPIRAL INFECTIONS

By J. SMITH

From the City Hospital Laboratory, Aberdeen

During the past few years, increasing evidence has been obtained of the presence, in this country, of leptospiral infections associated particularly with operatives employed as miners (Buchanan, 1927; Swan & McKeon, 1935), sewer workers (Fairley, 1934; Alston, 1935; Alston & Brown, 1935), and fish workers (Davidson *et al.* 1934; Davidson & Smith, 1936; Smith & Davidson, 1936). With a view to minimizing the risks of contracting infection, it seemed feasible, therefore, that in circumstances where it is wellnigh impossible to eradicate the vectors of the disease, other methods such as active immunization of the workers might be practised. Such environmental conditions as exist in sewers and mines might make attempts at rat extermination valueless, whereas in the fish trade, with the expenditure of a little care and forethought, the factors which permit of infection could, it is believed, be entirely abolished.

It appeared important, therefore, to review the previous work on immunization of animals and man against infection with *Leptospira icterohaemorrhagiae*, and to re-study the problem particularly as regards the production of sufficient antibodies to give complete protection. Successful active immunization against leptospiral infections was first demonstrated by Ido *et al.* (1916). These workers immunized guinea-pigs with emulsions made from the livers of infected animals in which the leptospira were killed by 0·5 per cent phenol. Noguchi (1918) later showed that three injections of 0·5 c.c. of killed leptospiral cultures made guinea-pigs completely resistant to both homologous and heterologous strains. When smaller quantities were given, the immunity established was often only sufficient to protect the animals against the homologous strain, and not against heterologous types. Active immunity was found to persist for at least 8 weeks. Again, Berger (1923) successfully immunized guinea-pigs with leptospiral cultures, killed by treating them with copper foil. Following upon the guinea-pig experiments of Ido *et al.*, Ito & Matsuzaki (1916) immunized miners with killed cultures, and claimed satisfactory protection results. This method was employed by further Japanese workers, and Inado (1922) reported on the results obtained by Wani in immunizing 10,368 miners with a killed vaccine containing 30–75 million leptospira per c.c., and giving a dose of 1 c.c., followed by another of 2 c.c. Among the vaccinated miners the resulting morbidity rate was reduced to 0·12 per cent, or less than one-ninth of that occurring amongst the unvaccinated. Baermann & Zuelzer (1928) used a

https://doi.org/10.1017/S0022172400034963 Published online by Cambridge University Press

single dose of 5 c.c. of vaccine, or two doses of 1 and 2 c.c. at 5–8 day intervals. The vaccine was killed by heating to 65° C., and preserved with 0·5 per cent phenol. They immunized eighty men.

With a view to minimizing the losses which occur amongst dogs due to "yellows"—a leptospiral infection—Dalling & O'Kell (1926) vaccinated these animals with infected guinea-pig liver emulsion killed by 0·5 per cent phenol. The vaccinated animals were then inoculated with virulent *L. icterohaemorrhagiae*, and were found to be immune. Vaccination of dogs with killed cultures is now practised, and the procedure has also been used to control a similar disease which occurs, frequently in epidemic form, in fox farms (Dunkin & Laidlaw, 1926).

Vaccination experiments in guinea-pigs

Methods

The medium used for culturing the leptospira for the preparation of vaccines was that employed by Prof. Schüffner. The details of the preparation of this simple medium are as follows: To 1500 c.c. tap water add 1·5 g. Witte peptone; boil; add 300 c.c. Ringer's solution and 150 c.c. Sørensen buffer mixture, *p*H 7·2 ($M/15$ Na_2HPO_4 72 c.c., $M/15$ KH_2PO_4 100 c.c.); boil until precipitation is complete; place in ice chest to cool; filter; check *p*H; place 3 c.c. in clean, new test tubes; autoclave for 20 min.; add 0·3 c.c. fresh sterile rabbit serum; heat at 56° C. for ½ hour; incubate at 37° C. overnight for sterility.

The rabbit's blood was collected from dilated ear veins, after making an incision by means of a scalpel, an electric light being kept close to the ear to maintain local heat. By this means 40 c.c. of blood could be collected with ease from large rabbits of the Belgian hare type. After the blood was collected, a few cubic centimetres of tap water were added to cause a certain amount of haemolysis, as the presence of haemoglobin in the serum tends to favour good growth. After clotting, the blood was centrifuged, and the serum sterilized by passing it through sterile Chamberland L_5 candles. The serum could then be stored in the refrigerator for considerable periods without any apparent deterioration in its growth-supporting properties. It should also be noted that certain rabbit sera are not satisfactory, but a simple test of the serum by means of Schüffner's sero-reaction will eliminate those specimens which are likely to cause lysis of the leptospira and thus prevent growth.

The various strains of leptospira were cultured in tubes containing 3 c.c. of the medium and incubated for 4 days at 30° C., and thereafter used for vaccination, in the case of non-virulent strains in the living state, and in the case of virulent strains after being killed by heat or chemical agents. The various types of vaccine employed are detailed in Tables I and II, and need no further explanation. The relative density of the organisms in the culture medium appeared to be approximately similar. The virulence of a strain was maintained by passage through guinea-pigs and then recovered by culture.

https://doi.org/10.1017/S0022172400034963 Published online by Cambridge University Press

In guinea-pig experiments it was found essential to use young animals, otherwise the results were irregular, since old animals frequently acquire a certain amount of immunity and survive inoculation with a virulent strain. Therefore, in all the following experiments, animals 6 weeks old at the commencement of the experiment were employed.

The method adopted in prophylactic vaccination was to give two doses of 1 c.c. of the vaccine subcutaneously at an interval of a week. After a further period of 2 weeks, each guinea-pig was given a subcutaneous injection of 0·2 c.c. of 4-day-old virulent culture. The control series of animals for each immunizing experiment was set aside from the same batch of animals as those which were vaccinated, and the control animals received a similar dose of the same virulent culture as employed on the vaccinated animals.

Results

In the first series of experiments, certain animals were vaccinated with a living non-virulent strain of *L. icterohaemorrhagiae* Weil and with killed vaccine prepared from a typical virulent strain recently isolated from a human case. In the second series of experiments, the guinea-pigs were vaccinated with vaccines prepared from *L. canicola*, *L. icterohaemorrhagiae* Rachmat (Indian strain), *L. hebdomadis*, and, for interest, *L. biflexa*. The first two strains were obtained through the kindness of Prof. Schüffner, and the third and fourth from the National Collection of Type Cultures at the Lister Institute.

Vaccination with strains of *L. icterohaemorrhagiae* Weil (Table I)

(1) *Vaccination with living non-virulent cultures* (Exps. 1, 2). In these experiments a total of thirty-six animals were vaccinated with two doses of 1 c.c. of a non-virulent culture of *L. icterohaemorrhagiae*, and eighteen animals were retained as controls. Four animals in the vaccinated series died of a

Table I. *Vaccination with* L. icterohaemorrhagiae *Weil*

		First vaccinated series				First control series			
Exp. no.	Type of vaccine	No. of guinea-pigs	No. dying of inter-current disease	No. surviving virulent culture	No. dying of leptospirosis	No. of guinea-pigs	No. dying of inter-current disease	No. dying of leptospirosis	No. surviving
1	Living non-virulent	6	0	6	0	6	0	6	0
2	Living non-virulent	30	4	26	0	12	0	12	0
3	Virulent culture killed at 55° C. for 30 min.	6	0	6	0	6	0	6	0
4	Virulent culture killed 0·5 % phenol	6	0	6	0	6	0	6	0
5	Virulent culture killed 0·5 % formalin	6	0	6	0	6	0	6	0
6	Virulent culture killed 0·5 % formalin	6	0	6	0	6	0	4	2
7	Virulent culture killed 0·5 % dettol	6	0	6	0	6	0	6	0

https://doi.org/10.1017/S0022172400034963 Published online by Cambridge University Press

Salmonella infection before the living virulent culture was administered. The result was clear-cut since all the immunized animals survived, while all the non-vaccinated died of a characteristic leptospiral infection.

(2) *Immunization with heat-killed cultures* (Exp. 3). In this experiment virulent cultures were killed by heating at 55° C. for 30 min. Six animals were treated, and six were used as controls. Inoculation with virulent cultures showed the vaccinated to be completely immune and the non-vaccinated to be all susceptible to infection.

(3) *Immunization with virulent cultures killed by chemical agents* (Exps. 4, 5, 6, 7). For these experiments cultures were treated with 0·5 per cent phenol, 0·5 per cent formalin, and 0·5 per cent dettol, and set aside for 48 hours, when microscopic and cultural examination failed to show the presence of any living forms. Six animals were vaccinated with the phenolized culture, twelve with the formalinized, and six with the dettolized. Here again the results were entirely satisfactory since all vaccinated animals showed complete protection, while the control animals, with two exceptions, all died of a typical leptospiral infection.

Vaccination with heterologous strains (Table II)

It has been shown by various workers that strains of leptospira isolated from patients and animals in different parts of the world differ in their serological characteristics (Fletcher 1928; Taylor & Goyle, 1931; Hindle, 1931; Ruys & Schüffner, 1934). Thus *L. canicola* has been recovered from dogs and human patients by Schüffner, who has shown by agglutination and agglutinin absorption tests that this organism differs from *L. icterohaemorrhagiae* Weil. Similarly Indian, Sumatran and Japanese strains have also been found to differ from the true Weil strain. Thus for this series of experiments cultures of *L. canicola* (dog), *L. icterohaemorrhagiae* Rachmat (Indian strain), *L. hebdomadis* (Japanese strain), and *L. biflexa* (saprophytic type) were employed. All these strains were apparently non-virulent for guinea-pigs even when living cultures were injected in 1 c.c. amounts (Table II).

Table II. *Vaccination with heterologous strains of* Leptospira

		Second vaccinated series				Second control series			
Exp. no.	Type of vaccine	No. of guinea-pigs	No. dying of inter-current disease	No. surviving virulent culture	No. dying of lepto-spirosis	No. of guinea-pigs	No. dying of inter-current disease	No. dying of lepto-spirosis	No. surviving
8	*L. canicola* living	6	0	6	0	6	0	5	1
9	*L. canicola* killed 0·5 % formalin	6	1	5	0	6	0	6	0
10	*L. icterohaemorrhagiae* Rachmat living	6	0	5	1	6	0	5	1
11	*L. icterohaemorrhagiae* Rachmat living	10	2	7	1	6	0	6	0
12	*L. hebdomadis* living	10	1	6	3	6	0	6	0
13	*L. biflexa*	10	0	0	10	6	0	5	1

https://doi.org/10.1017/S0022172400034963 Published online by Cambridge University Press

(4) *Vaccination with* L. canicola. In Exps. 8 and 9 *L. canicola* was employed in the form of a living vaccine, and also as vaccine killed by 0·5 per cent formalin, each vaccine being used to vaccinate six animals. Eleven surviving animals all appeared to be resistant to infection with the virulent form of *L. icterohaemorrhagiae*, whereas eleven out of the twelve control animals died.

(5) *Vaccination with the Indian strain* L. icterohaemorrhagiae Rachmat. In Exps. 10 and 11, sixteen guinea-pigs were vaccinated with living non-virulent cultures of the Indian strain. Two animals died in the course of vaccination, two died of a leptospiral infection, and twelve survived the test dose of virulent culture of *L. icterohaemorrhagiae*, whereas eleven out of twelve control animals died.

(6) *Vaccination with the Japanese strain* L. hebdomadis. In Exp. 12, ten guinea-pigs were vaccinated with living non-virulent cultures of *L. hebdomadis*, and six animals were retained as controls. One of the vaccinated group died before immunization was completed, and when the remaining nine were inoculated with a virulent culture of *L. icterohaemorrhagiae* six animals survived and three died of typical leptospirosis. In the control series all died of infection.

(7) *Vaccination with the saprophytic strain* L. biflexa (Exp. 13). Ten guinea-pigs received two doses of vaccine, but when inoculated with virulent *L. icterohaemorrhagiae* all succumbed to infection, while in the control series for this experiment five out of six animals died. Vaccination with *L. biflexa* failed, then, to give any demonstrable protection against *L. icterohaemorrhagiae*.

Comment

It is obvious from the above experiments that a solid immunity can be developed when guinea-pigs are vaccinated with living non-virulent strains, or killed emulsions of virulent strains of *L. icterohaemorrhagiae*. Further, similar results can be obtained with certain leptospiral strains which differ serologically from *L. icterohaemorrhagiae* Weil. Thus, vaccination with *L. canicola* produced effective protection against *L. icterohaemorrhagiae* Weil, whereas, when vaccines of *L. icterohaemorrhagiae* Rachmat and *L. hebdomadis* were employed, the results were not so satisfactory, as three out of nine vaccinated animals died of typical leptospirosis.

Production of immune bodies as a result of vaccination

It has been shown repeatedly that as a result of infection with *L. icterohaemorrhagiae*, immune bodies demonstrable as agglutinins, lysins, and complement-fixing bodies appear rapidly after convalescence has been established, and a similar result must be produced by vaccination. In analogy with other bacterial infections it is presumed that the leptospiral agglutinins are not true indicators of immunity, but the demonstration of the lytic titre of a serum by Schüffner's sero-reaction may indicate the true immune properties. On the other hand, when the microscopic serological test is carried out, it is found that

https://doi.org/10.1017/S0022172400034963 Published online by Cambridge University Press

the lytic titre of a serum for living cultures is practically identical with the agglutinin titre for killed formalinized cultures. A series of guinea-pigs were inoculated with, in one instance, living non-virulent cultures, and in the other instance, phenolized cultures of a virulent strain, and 2 weeks after the second injection blood samples were obtained and tested. The results are detailed in Table III. They show that the lytic titres in the series inoculated with the living strains were definitely higher than in the animals treated with the phenolized vaccine. The titres in both series ranged from 1/6 to 1/30, but this was apparently sufficiently high to give protection against a subsequent injection of 0·2 c.c. of virulent culture.

Table III. *Production of immune bodies in vaccinated guinea-pigs*

Type of vaccine	Exp. no.	Guinea-pig no.	Titre	Result of inoculation with 0·2 c.c. of virulent culture
Two doses of 1 c.c. non-virulent *L. icterohaemorrhagiae*	14	1	1/10	Survived
		2	1/100	,,
		3	1/30	,,
		4	1/100	,,
		5	1/30	,,
		6	1/30	,,
		7	1/10	,,
		8	1/10	,,
		9	1/30	,,
		10	1/30	,,
Two doses of 1 c.c. of 0·5 % phenolized culture	15	1	1/10	Survived
		2	1/6	,,
		3	1/10	,,
		4	1/10	,,
		5	1/6	,,
		6	1/6	,,
		7	1/10	,,
		8	1/10	,,
		9	1/10	,,
		10	1/6	,,

Carrier conditions in guinea-pigs following upon vaccination

It is known that 20–30 per cent of rats in this country harbour *L. icterohaemorrhagiae* in their kidneys without any harmful effects. It was thought that the foregoing experiments, in which the animals were vaccinated and then treated with virulent cultures, might produce similar conditions in guinea-pigs. So 16 days after the vaccinated animals had been treated with virulent cultures and survived, they were killed, their kidneys were removed, emulsified in saline, and inoculated in groups into pairs of young animals (Table IV). Thus the kidneys from thirty-two surviving animals in Exps. 1 and 2 were divided into seven groups (three of four pairs and four of five pairs), and inoculated into seven pairs of guinea-pigs. Among thirty-two animals inoculated with living non-virulent *L. icterohaemorrhagiae* and six with living non-virulent *L. canicola*, no carriers were detected. In Exps. 3, 4, 5, 6 and 7, where animals were treated with heat-killed, phenolized, formalinized, and dettolized vaccine

https://doi.org/10.1017/S0022172400034963 Published online by Cambridge University Press

of *L. icterohaemorrhagiae*, carriers of virulent leptospira were demonstrated amongst the animals protected by means of the formalinized and dettolized emulsions. Again, in Exp. 9 formalinized emulsion of *L. canicola* was used, and in Exps. 10 and 11, where the living form of *L. icterohaemorrhagiae* Rachmat was employed, further evidence of carrier conditions was obtained in both series. The foregoing results are difficult to interpret, and would require further experiments for their elucidation. It is notable, however, that carrier conditions did not occur amongst animals vaccinated with living *L. icterohaemorrhagiae* or *L. canicola*, whereas they occurred amongst those vaccinated with living *L. icterohaemorrhagiae* Rachmat. In the vaccination

Table IV. *Carrier conditions in guinea-pigs following upon vaccination*

Exp. no.	Type of vaccine	No. of guinea-pigs completely immunized	Kidneys of surviving animals inoculated into further guinea-pigs	Result of inoculation tests
1, 2	*L. icterohaemorrhagiae* living non-virulent	32	7 pairs	No leptospiral infection
3	*L. icterohaemorrhagiae* heat-killed virulent	6	1 pair	No leptospiral infection
4	*L. icterohaemorrhagiae* phenolized virulent	6	1 pair	No leptospiral infection
5, 6	*L. icterohaemorrhagiae* formalinized virulent	12	2 pairs	One pair showed leptospiral infection
7	*L. icterohaemorrhagiae* dettolized virulent	6	1 pair	Both animals showed typical leptospiral infection
8	*L. canicola* living non-virulent	6	1 pair	No leptospiral infection
9	*L. canicola* formalinized	5	1 pair	Both animals died of leptospiral infection
10, 11	*L. icterohaemorrhagiae* Rachmat living non-virulent	12	4 pairs	Three pairs died leptospirosis. One pair survived

tests it was demonstrated that better immunity against virulent *L. icterohaemorrhagiae* Weil was obtained with living non-virulent *L. icterohaemorrhagiae* Weil and living non-virulent *L. canicola* than with living non-virulent *L. icterohaemorrhagiae* Rachmat. Further immunization with chemically killed vaccines of virulent strains of *L. icterohaemorrhagiae* Weil were less successful in preventing this carrier condition than the living non-virulent forms. This result might be due to two causes, first, a higher degree of immunity might be obtained with the living non-virulent homologous form of vaccine than with the killed form, and second, the living form may have some additional immunizing property which is absent from the killed form. It should be noted, however, that rats have been encountered in which the lytic titre of the serum was 1/10,000 and yet those animals were carriers of virulent leptospira.

https://doi.org/10.1017/S0022172400034963 Published online by Cambridge University Press

Vaccination of human beings

As the results of the guinea-pig experiments appeared very satisfactory, it was resolved to test out the effect of leptospiral vaccines on various patients in hospital. The patients selected for this purpose were mild cases of scarlet fever whose illness had, to all intents and purposes, terminated within 48 hours of admission to hospital. They were for the most part children between the ages of 8 and 14 years. On admission, samples of blood were obtained, and later, if the blood gave a negative sero-reaction with *L. icterohaemorrhagiae*, and if the patient's condition warranted it, they were vaccinated. Tentative trials with killed leptospiral emulsions, grown on the medium already described, showed that the reactions produced by the subcutaneous inoculation of 1 c.c., and later 2 c.c., amounts of the vaccine were entirely negligible. Slight local reactions appeared at the site of inoculation, but in not a single case was there any degree of generalized disturbance as evidenced by fever, sickness or increased pulse rate. It was therefore decided to vaccinate the patients with two doses of vaccine at weekly intervals. By the time vaccination was completed the patients were ready to return to their homes, so by arrangement with the parents the children were brought back to hospital in order that blood samples might be obtained 2–3 weeks after the final dose of vaccine had been administered.

First series. For this purpose, a vaccine was prepared by growing a virulent strain of *L. icterohaemorrhagiae* in the medium already described at 30° C. for 4 days. The cultures were then treated with 1/5000 merthiolate, and after 2 days were tested for the presence of living leptospira by cultural and animal inoculation tests. These tests showed that this dilution of the antiseptic was sufficient to sterilize the emulsion of leptospira. The patients were then inoculated with 1 c.c. of the killed vaccine (undiluted culture), and a second dose of 2 c.c. was given a week later. In all, eighty-two patients whose sero-reactions were negative, even in a dilution of 1/10, were so vaccinated. The results, so far as the production of demonstrable immune bodies, were not satisfactory. Thirty-three individuals out of the total of eighty-two returned for the blood examination, and of these, twenty-four gave entirely negative tests. In seven individuals the serum reacted with *L. icterohaemorrhagiae* to a titre of 1/10, and in only one case to a titre of 1/30.

Second series. In this series, forty individuals were vaccinated in the same way as those in the first series, but instead of using the killed virulent strain a merthiolate-killed emulsion of a non-virulent strain of *L. icterohaemorrhagiae* was employed. Only fifteen of this series returned to give blood samples, and when these were tested only four reacted to a titre of 1/10 with *L. icterohaemorrhagiae*.

Third series. In this series, a leptospiral vaccine prepared by a firm specializing in immunological products was tried. The vaccine is prepared for the purpose of vaccinating dogs and foxes against leptospiral infections, and

https://doi.org/10.1017/S0022172400034963 Published online by Cambridge University Press

the dosage recommended is two of 1 c.c. at an interval of 1 week. In all, twenty-eight patients were vaccinated with two doses of vaccine, 1 and 2 c.c. respectively. The reactions produced by the vaccine were again entirely negligible. Of those vaccinated, thirteen returned to give blood for the sero-reaction. When these were tested only three samples reacted with *L. icterohaemorrhagiae* and that only in a 1/10 dilution.

Comment

It will thus be seen that vaccination of human beings by the methods described did not stimulate the production of demonstrable immune bodies to the extent that might have been expected. It would appear from the guinea-pig experiments that a lytic titre of 1/10 would give ample protection against a virulent strain of *L. icterohaemorrhagiae*. When patients in the early stage of a leptospiral infection are treated with anti-leptospiral serum in even 40–80 c.c. amounts, there is definite clinical evidence of its curative effect, and yet it is not possible to demonstrate the presence of the foreign immune bodies in blood samples by the sero-reaction, the dilution being too great. It would appear, therefore, that the presence of small amounts of immune bodies in the tissues would be sufficient to give ample protection. At any rate it would seem very desirable indeed to vaccinate workers whose daily duties exposed them to a possible attack of leptospirosis—as in sewer workers. The foregoing experiments have demonstrated that this type of vaccination is well tolerated. In comparison with the guinea-pig experiments, however, the dosage in these series of human vaccinations is entirely out of proportion, and it would appear probable that increased dosage might give a better production of immune bodies, and consequently a higher grade of immunity. The final test for the efficacy of the method would require to rest on a comparison of the incidence of Weil's disease in a series of vaccinated and unvaccinated individuals who were exposed to possible infection.

SUMMARY

A. *Guinea-pig experiments*

1. Guinea-pig vaccination experiments have shown that these animals can be rendered completely immune from infection with virulent strains of *L. icterohaemorrhagiae* when they are treated with non-virulent living vaccines, or formalinized, heat-killed, phenolized, and dettolized suspensions of virulent cultures.

2. Vaccination with homologous strains of *L. icterohaemorrhagiae* produced better immunity than when heterologous strains such as *L. hebdomadis* or *L. icterohaemorrhagiae* Rachmat are employed.

3. Immune bodies are demonstrable in the serum of guinea-pigs vaccinated with *L. icterohaemorrhagiae* Weil, and a lytic titre of 1/6 appears to indicate that sufficient protective antibodies are present.

4. Following upon vaccination and inoculation with virulent cultures,

https://doi.org/10.1017/S0022172400034963 Published online by Cambridge University Press

carrier conditions have developed in animals treated with chemically killed vaccines of *L. icterohaemorrhagiae* Weil and with living non-virulent suspensions of the heterologous strain *L. hebdomadis* and *L. icterohaemorrhagiae* Rachmat.

B. *Human vaccination experiments*

1. Killed vaccines prepared from non-virulent and virulent forms of *L. icterohaemorrhagiae* Weil have been employed in vaccination experiments on human beings.

2. These vaccines given subcutaneously in doses of 1 and 2 c.c. amounts have been well tolerated, and gave rise to little local reaction and to no general disturbance.

3. Blood samples obtained from the vaccinated show that, when tested for lytic antibodies, the amounts produced are relatively small. The findings indicate the need for increased dosage to stimulate the further production of immune bodies.

The author is indebted to Dr Douglas Bell, Senior Resident Medical Officer, for much assistance in the vaccination of the human cases.

REFERENCES

Alston, J. M. (1935). *Lancet*, i, 806.
Alston, J. M. & Brown, H. C. (1935). *Brit. Med. J.* ii, 339.
Baermann, G. & Zuelzer, M. (1928). *Trop. Dis. Bull.* **25**, 602.
Berger, H. (1923). *Ibid.* **21**, 276.
Buchanan, G. (1927). *Med. Res. Council, Special Report Series*, No. 113.
Dalling, T. & O'Kell, C. C. (1926). *J. Path. Bact.* **29**, 131.
Davidson, L. S. P., Campbell, R. M., Rae, H. J. & Smith, J. (1934). *Brit. Med. J.* ii, 1137.
Davidson, L. S. P. & Smith, J. (1936). *Quart. J. Med.* **5**, 263.
Dunkin, G. W. & Laidlaw, P. P. (1926). *Dept. Med. Res. Council*, 1924–5, No. 31.
Fairley, N. H. (1934). *Brit. Med. J.* ii, 10.
Fletcher, W. (1928). *Trans. Roy. Soc. Trop. Med.* **21**, 265.
Hindle, G. (1931). *System of Bacteriology*, **8**, 29.
Ido, Y., Hoki, R., Ito, H. & Wani, H. (1916). *J. Exp. Med.* **24**, 485.
Inado, R. (1922). *Trop. Dis. Bull.* **20**, 141.
Ito, T. & Matsuzaki, H. (1916). *J. Exp. Med.* **23**, 557.
Noguchi, H. (1918). *Ibid.* **28**, 561.
Ruys, C. & Schüffner, W. A. P. (1934). *Nederl. Tijd. v. Geneesk*, **78**, 3, 27.
Smith, J. & Davidson, L. S. P. (1936). *J. Hygiene*, **36**, 438.
Swan, W. G. A. & McKeon, J. A. (1935). *Lancet*, ii, 570.
Taylor, J. & Goyle, A. N. (1931). *Indian Med. Res. Memoirs*, No. 20, p. 73.

(*MS. received for publication* 24. xi. 1936.—Ed.)

https://doi.org/10.1017/S0022172400034963 Published online by Cambridge University Press