BRUCELLA SUIS INFECTION OF GUINEA-PIGS BY THE RESPIRATORY ROUTE.

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THERE is no evidence to show that *Brucella suis* either injected into or inhaled by a susceptible animal produces any early pathological change in lung tissue. On the other hand the organism is highly infectious when inhaled ; an estimated inhaled dose of about thirty viable cells produces a fifty per cent chance of infection in the guinea-pig (Elberg and Henderson, 1948). A constant and very early pathological change shown by such animals is enlargement of the bronchial or cervical lymph nodes, according to the size of the particle in which the organisms are presented to the host (Druett, Henderson and Peacock, unpublished work) ; the lymph nodes also show massive invasion by *Br. suis*.

In the light of these findings it seemed possible that the organism deposited on the alveolar surface was capable of multiplying thereon without penetrating lung tissue to produce pathological change in it. If this were so then as numbers increased so too would the chance of one or more cells being phagocytosed and transported to a nearby lymph node. This site could then be that from which there was a welling over of organisms into the circulation to produce generalised infection.

The experiments described in this paper are primarily concerned with an examination of this hypothesis.

MATERIALS AND METHODS.

Preparation of infecting suspension of Br. suis.

Dried cultures of *Br. suis* (Strain PS III) were taken up in Zobell's (Zobell and Zobell, 1932) fluid and placed on slopes of fortified tryptose agar (F.T.A.) (McCullough, Mills, Herbst, Roessler and Brewer, 1947). The 48-hr. growth from one slope was used to inoculate a larger slope (8-oz. (226·8 g.) bottle containing 30 ml. F.T.A.). After 48 hr. incubation at 37° the resulting growth was washed off with 25 ml. Zobell's fluid, the suspension examined for purity, and viable counts carried out. A fresh batch of suspension was prepared for each experiment.

Animals.—Guinea-pigs of 400–500 g. weight from a healthy stock were used.

Method of infecting animals.—Animals were exposed to a measured concentration of a single-cell aerosol of *Br. suis* in the Henderson (1952) apparatus for 1 min. The animals were housed 5 in a cage.

Post-mortem procedure.

Guinea-pigs were killed and the following examination made.

Body weight determined ; heart blood removed for culture and serology ; the appearance of the organs noted ; bone marrow, liver, spleen, bronchial and cervical lymph nodes and lungs cultured ; spleen and lungs removed to sterile 1-oz. bottles for viable counts ; bronchial lymph nodes removed for grinding and culture (Exp. 3 only).

Direct culture of tissues.

Tissues, with the exception of bone marrow, were cultured by rubbing a cut surface over half a plate of F.T.A. to which was added 1 part in 8 million brilliant green and 4 units/ml. polymyxin (Burroughs Wellcome) to prevent overgrowth with contaminants encountered in undiluted lung samples.

Viable counts on lungs and spleen.

Tissue weights were measured and Zobell's fluid added. For the purpose of determining total volumes of tissue suspensions the S.G. of tissue was taken as 1·0. Spleens were made up to 4, 5, 10 or 15 ml. according to spleen weight and expected count. Lungs were suspended in 10 ml. of fluid.

The tissues were disintegrated for 2 min. in specially designed high-speed emulsifiers. Preliminary test had shown this period to be satisfactory and to have no effect on the viability of dilute suspensions of *Br. suis*. Spleens emulsified well. Lungs needed a preliminary mincing with scissors; the tissue was not as completely broken down as spleen tissue, but made workable suspensions.

Tissue suspensions were diluted, in tenfold steps, in 0·1 per cent tryptose saline, and 0·2 ml. volumes spread over the surface of F.T.A. plates. Duplicate or triplicate plates were used for each dilution.

Grinding of bronchial lymph nodes.

In one experiment two or more bronchial lymph nodes from each animal were removed to a sterile 3 in. × $\frac{3}{8}$ in. (7·62 × 0·95 cm.) test-tube containing a layer of washed sterile sand, and were broken up by grinding with a sterile glass rod after adding 0·4 ml. Zobell's fluid. The samples were cultured by direct plating of the bulk of the suspension and adding fortified tryptose broth to the remainder. The broth cultures were plated on F.T.A. after 3, 7, 14 and 21 days' incubation at 37°.

Blood cultures.

By means of capillary pipettes 2 to 3 ml. of heart blood were collected and added to 20 ml. fortified tryptose broth containing 1 part in 12 million of brilliant green. (The growth from an inoculum of 42 cells of *Br. suis* had been tested in this medium and found satisfactory.) Cultures were incubated at 37° and subcultured on F.T.A. after 3, 7, 14 and 21 days' incubation.

From some animals quantitative blood cultures were made by inoculating F.T.A. plates with 1/12th ml. of blood, spreading, and incubating at 37°. The blood counts were expressed as viable cells/ml.

Marrow cultures.—The muscles were dissected from the right femur, the bone broken with bone forceps, and a sterile loop passed into the medullary cavity. The marrow removed was spread over a plate of F.T.A.

Serology.

Agglutination tests were done with serum from test animals separated from heart blood collected in dry sterile tubes. Twofold dilutions from 1/5–1/5120 were mixed with equal volumes of *Br. suis* suspension and the results read after 24 hr. in a water bath at 56°. The heat-killed suspension of the infecting strain was diluted to opacity 3 (Brown's Scale).

Identification of Br. suis.—All cultures presumed to be *Br. suis* by colonial appearance were confirmed morphologically and by slide agglutination.

RESULTS.

Three experiments were made and in each a group of 100 guinea-pigs was exposed. The dosage was varied to meet the needs of each test and Table I shows the details. The estimated intake of *Br. suis* is based on a respiratory minute volume of 150 ml. and the lung retention is taken as 50 per cent of this value (Harper and Morton, 1953).

In recording results, quantitative data are given only for lung tissue. The more qualitative method of direct plate culture was used as an indicator of the path

of infection to other tissues. To examine the validity of this procedure quantitative data available from lung and spleen viable counts were compared with the qualitative results of testing these tissues. When the viable count reached about 10^4 cells per g. of tissue, lung or spleen, then only occasional colonies were

TABLE I.—*Dosage of* Br. suis *Used in Experiments* 1, 2 *and* 3.

Experiment number.	Cloud concentrations (cells/litre).	Estimated respiratory intake.	Estimated lung retention.	Calculated* ID.
1	450	68	34	80%
2	3,110	467	234	100%
3	774	116	58	90%

* From regression line data of Elberg and Henderson (1948) and unpublished experiments in this Department.

observed by the direct plate method. When the count in these tissues reached about 5×10^5 per g. of tissue, a confluent growth was obtained by the direct plate method. It follows that a negative finding obtained by this latter method is not proof of absence of infection. Nevertheless the method gives useful guidance to the degree and the route of infection. For example, if positive bronchial lymph nodes are found in a series of animals in which all tissues other than lung are negative, then we may reasonably assume that at least one route leading to generalised infection is *viâ* these lymphatic glands.

Development of Generalised Infection.

Experiment 1.—One hundred animals were exposed to a cloud concentration expected to produce about 80 per cent of infected animals within 28 days. Through accident and non-specific deaths only 93 remained for examination. Fifty-five animals were killed in groups of 5 at short intervals from 4 to 28 days after exposure to find the most profitable time for a more intensive examination of the initiation of the disease. The remaining animals were killed at later stages to examine the progress of the established disease. The results are given in Table II. The bacterial counts are expressed as the arithmetic mean in each group. There was, however, a wide scatter around the mean (see Table V).

The first three lots of animals examined between 4 and 11 days after exposure showed a marked increase in the number of *Br. suis* in lung tissue over the few organisms originally retained. While this occurred, there was no infection detected by the direct plate method in the regional lymph nodes, spleen or liver. The first evidence of infection in these organs occurred on the 13th day, by which time the viable count in the lungs had risen to $2–3 \times 10^4$—an increase of about 1000-fold over the number originally inhaled and retained. After the infection had reached the bronchial lymph nodes the spread to other tissues was rapid, and the animals developed typical lesions which were well established by the 19th day. These preliminary findings helped to plan a second experiment designed to give more information about the early stages of the disease.

Experiment 2.—Animals in this group were exposed to a dose giving an estimated lung retention of 250 cells, which would be expected to infect about 100 per cent of guinea-pigs. The animals were sacrificed in groups of 10 at 2-day intervals.

Table III shows that the same pattern of events occurred as in the first experiment, but that the whole process is accelerated. The gross pathology was typical in all animals killed on or after the 14th day. The possibility that lightly infected bronchial glands were not being detected by direct plate culture led to the third experiment.

TABLE II.—*Experiment* 1 : *Results of Exposure of* 93 *Guinea-pigs to a Cloud of* Br. suis (*Estimated Lung Retention* 34 *Cells* $\equiv ID_{80}$). *Animals Killed in Groups of* 5.

Days after exposure.	Lungs. Number positive.	Lungs. Mean viable count $\times 10^{-4}$	Direct plates, number positive. Bronchial.	Cervical.	Spleen.	Liver.	Bone marrow.	Blood culture. Number positive.	Blood culture. Count/ml.	Agglutinins. Number positive.	Agglutinins. Titre.
4	1	0·44	0	0	0	0	0	0	—	0	—
7	2	0·12	0	0	0	0	0	0	—	0	—
11	4	0·95	0	0	0	0	0	1	—	0	—
13	5	2·7	5	2	1	1	0	1	—	0	—
15	5	80·8	2	1	2	2	2	2	—	2	180
17	3	68·3	3	1	1	1	1	2	—	1	320
19	5	52·4	3	3	3	3	3	3	—	3	213
21	5	250·5	5	5	5	5	5	5	—	5	512
24	4	101·2	4	4	4	4	4	4	—	4	1280
26	4	367·0	4	4	4	4	4	4	—	4	560
28	5	732·0	5	5	5	5	5	5	—	5	960
35	4	60·0	4	4	4	4	4	3	93	4	2080
42	2	41·9	2	2	2	1	1	2	6	2	5120
56	4	39·9	4	5	3	1	0	2	0	5	2816
84	5	141·2	2	2	2	1	1	2	48	5	2240
96	5	2060·0	3	3	1	0	0	0	—	5	2040
126	5	1008·0	3	2	1	0	0	0	—	5	1536
154*	2	121·0	1	0	1	0	0	0	—	4	1920
215*	2	1350·0	1	0	0	0	0	0	—	4	1280

* Groups of 4 animals killed at these times.

In this and succeeding Tables mean viable counts are per organ.

TABLE III.—*Experiment* 2 : *Results of Exposure of* 100 *Guinea-pigs to a Cloud of* Br. suis. (*Estimated Lung Retention* 234 *Cells* $\equiv ID_{100}$). *Animals Killed in Groups of* 10.

Days after exposure.	Lungs. Number positive.	Lungs. Mean viable count $\times 10^{-4}$.	Direct plates number positive. Bronchial.	Cervical.	Spleen.	Liver.	Bone marrow.	Blood culture. Number positive.	Blood culture. Count/ml.	Agglutinins. Number positive.	Agglutinins. Titre.
2	9/9*	0·09	0	0	0	0	0	0	—	0	—
4	8/9*	1·84	0	0	0	0	0	0	—	0	—
6	10	12·3	3	0	0	0	0	0	—	0	—
8	9/9*	38·1	6	2	1	0	0	1	<12	0	—
10	10	24·9	9	6	4	3	0	4	<12	0	—
12	10	40·2	8	3	7	4	1	6	<12	1	10
14	9	157·2	9	5	8	6	7	9	340	5	224
16	10	106·0	9	9	9	9	9	9	512	9	446
18	10	290·0	10	9	10	10	10	10	806	10	356
20	10	209·0	10	10	10	10	10	10	898	10	584

* Cultures lost due to contaminations.

Experiment 3.—Animals were exposed to a dose giving an estimated lung retention of about 60 cells and were examined in groups of 10 at daily intervals commencing 24 hr. after exposure. In this series special attention was paid to the bronchial lymph nodes, which were cultured by a method designed to detect small numbers of *Br. suis* (see Materials and Methods).

Tables IV and V show that *Br. suis* multiplies in the lungs before infection of the bronchial lymph nodes can be detected by the most sensitive culture method. It will be noted that in some of the animals examined on the 7th and 9th days after infection growth of *Br. suis* was obtained from the cervical lymph nodes when the bronchial nodes were sterile ; one animal had a lung viable count of only 91 cells. All the cultures of spleen suspensions made on the 9th day were sterile and the lung viable count showed a marked fall-off. No explanation can be found for these results which are contrary to the findings in Exp. 1 and 2. Technical error cannot positively be ruled out.

It is of interest that of the 23 bronchial lymph node samples which gave positive cultures 16 were detected by direct plating, 21 by plating of tissue suspensions and 23 by the fluid culture technique.

Progress of the Established Disease.

Animals surviving after the 28th day in Exp. 1 were used to observe the progress of the established disease. The results are shown in Table II. There were no deaths in this group. Animals examined in the early stages of the disease looked emaciated and had lost weight ; those examined later looked well and put on about the same amount of weight as would normal guinea-pigs under similar conditions.

Between the 35th and 84th day lung viable counts were about 1/10th of the peak value reached on the 28th day. Tests on the 96th day showed a sharp rise in counts which persisted to the 215th day. This was associated with lung abscesses found in animals killed at these times.

Between 35 and 215 days after infection the spleen viable count showed a decline to about 0·1 per cent of the peak value recorded on the 28th day (see Table VI). The spleen suspension was the only tissue that was positive in all the 4 animals examined on the 215th day. Two animals had positive lung cultures and one a scanty infection in the bronchial lymph nodes at this time. Cultures made from the cut surface of spleens were sterile.

A change in the character of the lesions was noted in animals held for 56 days or longer. There was then a marked tendency for the cervical lymph nodes to contain numerous small abscesses, which later coalesced to form one large abscess filled with thick creamy pus. Spleens showed a few large abscesses in contrast to the more numerous small ones seen in animals examined earlier. The liver (except in one animal killed on the 154th day, which contained one large abscess) showed shallow pits over the surface corresponding in distribution with the small abscesses seen in animals killed earlier in the disease. The spleen size, in proportion to the body weight, showed a decline in the later stages of the disease, but after 215 days was still twice the normal size. Lungs measured on the same basis showed little variation except when abscesses were present late in the disease ; such lungs were 2–3 times the normal weight.

TABLE IV.—*Experiment* 3: *Results of Exposure of* 100 *Guinea-pigs to a Cloud of* Br. suis. (*Estimated Lung Retention* 58 *Cells* $\equiv ID_{90}$). *Animals Killed in Groups of* 10.

Days after exposure.	Lungs. Number positive.	Lungs. Mean viable count $\times 10^{-4}$	Direct plates number positive. Bronchial.*	Cervical.	Spleen.	Liver.
1	6	0·002	0	0	0	0
2	9	0·041	0	0	0	0
3	6	0·035	0	0	0	0
4	7	0·78	1	0	0	0
5	7	0·77	2	1	0	0
6	9	5·70	1	1	0	0
7	10	8·29	2	3	0	0
8	9	36·6	6	0	0	0
9	6	2·50	4	2	0	0
10	9	7·0	7	3	2	2

* Includes positive cultures from emulsified lymph nodes and broth cultures.

TABLE V.—*Experiment* 3: *Lung Viable Counts in* 100 *Guinea-pigs Exposed to a Cloud of* Br. suis. (*Estimated Lung Retention* 58 *Cells*). *Animals Killed in Groups of* 10.

Days after exposure.	Lung viable counts $\times 10^{-2}$				
1	0	0	0	0	0·1
	0·1	0·1	0·2	0·3	0·4
2	0	0·7	1·2	1·2	1·6
	2·5	4·1	4·9	5·1	9·6
3	0	0	0	0	0·4
	0·6	0·6	2·3	7·9	9·0
4	0	0	0	0·8	10·7
	13·3	21·2	43·0	**47·8**	413·0
5	0	0	0	2·5	18·7
	25·4	27·6	**56·0**	71·9	**334·0**
6	0	1·0	27·1	27·6	29·4
	57·7	62·1	74·0	148·0	**3650**
7	0·9*	6·7	8·3	27·8	**75·1**
	75·4*	113·0	139·0*	221·0	**7620**
8	0	9·5	27·5	**30·1**	**97·6**
	368·0	716·0	**7220**	**12,100**	**12,700**
9	0	0	0	0	**12·7**
	72·2	91·7	**138·0**	**303·0**	895·0*
10	0	**18·8**	24·0	**67·9**	77·6
	78·2	**145·0**	**382·0**	**1600**	**3800**

Heavy type = Bronchial glands yield growth of *Br. suis*.

* Cervical glands yield growth of *Br. suis*; bronchial glands negative.

TABLE VI.—*Spleen Viable Counts Experiment* 1.

Days after exposure.	Viable count $\times 10^{-4}$.
4	0
11	0·57
15	956·0
19	600·0
24	845·0
28	3180·0
42	196·0
84	388·0
126	0·54
215	2·02

Cultures made from animals examined later than the 42nd day showed a fall-off in growth on the direct plates and by the 154th day the majority of plates made from tissues showing typical lesions were sterile. Victor and Valliant (1952) also noted a fall-off in positive cultures made from tissues containing lesions in animals held 15–20 weeks after infection with *Br. suis*.

One animal examined 84 days after exposure was in marked contrast to the other animals killed at the same time. The tissue appearance and cultural findings were consistent with an infection of shorter duration (including positive blood and marrow cultures). This animal may have had a much longer incubation period than its fellows or may have been cross-infected from animals put in the same room 28 days later. The latter possibility seems slight as we have no evidence of cross-infection amongst animals exposed to a single cell aerosol of *Br. suis*.

Blood and Bone Marrow Cultures.

Cultures were made from all animals in Exp. 1 and 2; quantitative blood cultures were made on animals examined on and after the 35th day in Exp. 1 and on all animals in Exp. 2. The results are shown in Fig. 1 and 2. A positive blood picture appeared from the 11th day in Exp. 1 and from the 8th day in 2 (where the dosage was higher). The proportion of infected animals yielding positive blood cultures reached 100 per cent on the 21st and 14th days respectively. By the 96th day the blood was sterile although tissues were still yielding positive cultures. The degree of infection in the blood rose from less than 12 cells/ml. on the 8th day to a mean count of about 900/ml. on the 20th day. (Individual animals had as many as 2000 ml. on the 18th day.) By the 56th day counts had again fallen to less than 12/ml. The one higher value obtained on the 84th day was associated with the anomalous results already referred to in the last section.

The bone marrow cultures followed the same trend as the blood cultures but in both experiments positive results were not obtained until the blood had been positive for 4 days. As with the blood, the marrow was sterile by the 96th day. The method used for culturing blood was more sensitive and it may be that the direct plating of marrow did not detect all infected samples.

Agglutinins.

Sera from all animals in the first two experiments were examined for the presence of specific agglutinins. The results are shown in Fig. 3 and 4. The presence of agglutinins was first noted on the 15th day in Exp. 1 and on the 12th day in 2.

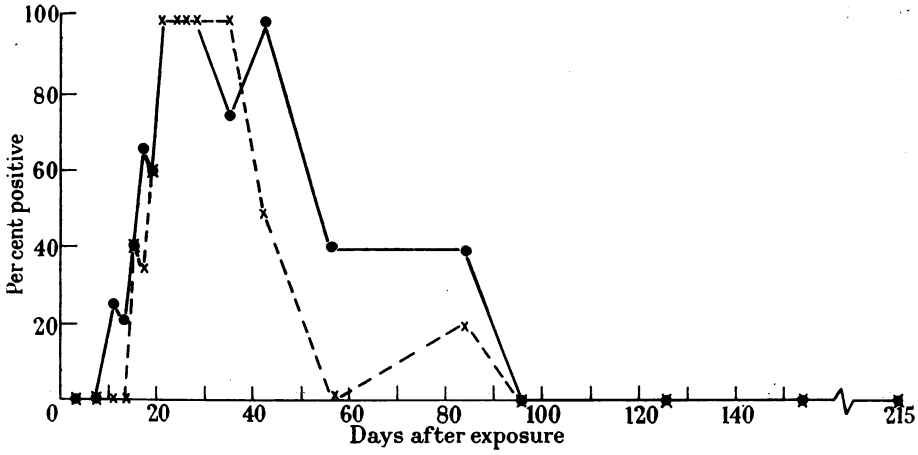

FIG. 1.—Exp. 1. Blood and marrow cultures: per cent positive of animals yielding *Br. suis* from other tissues.

● ———— ● = Blood culture.
×— — — —× = Marrow culture.

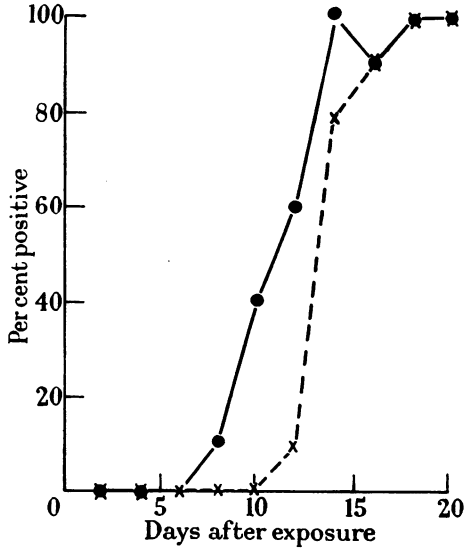

FIG. 2.—Exp. 2. Blood and marrow cultures: per cent positive of animals yielding *Br. suis* from other tissues.

● ———— ● = Blood culture.
×— — — —× = Marrow culture.

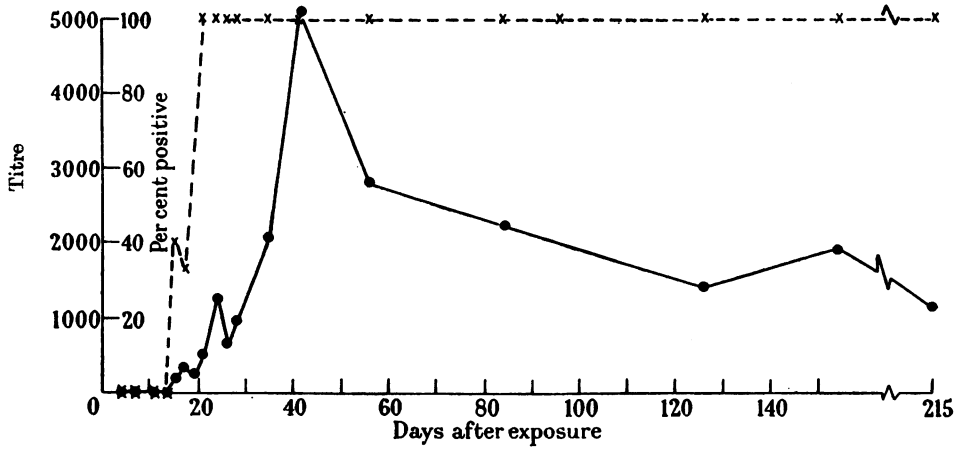

FIG. 3.—Exp. 1. Agglutinins.

● ———— ● = Titre.

×— — — —× = Per cent positive.

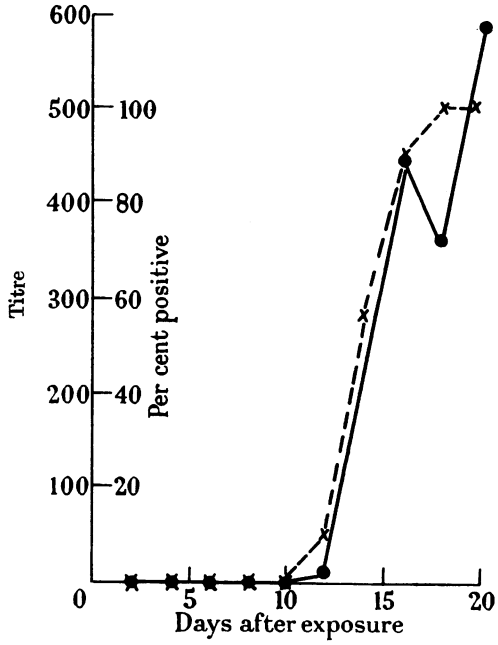

FIG. 4.—Exp. 2. Agglutinins.

● ———— ● = Titre.

×— — — —× = Per cent positive.

The proportion of infected animals showing agglutinins reached 100 per cent on the 21st and 18th days respectively. The titre rose to a peak of 5120 after 42 days and then slowly declined, but was still 1280 after 215 days. The rises in titre in the first 20 days in the two experiments were roughly parallel, the rise commencing earlier in the second (where the dosage was higher). Throughout the remainder of the 215-day period all infected animals retained agglutinins in their sera.

In all animals with a titre of 80 or over there was evidence of splenic enlargement and heavy infection. Positive sera were obtained only from animals with evidence of splenic infection. In the developing disease the threshold for obtaining a positive serological reaction is the presence of 10^6 bacterial cells/g. of spleen tissue and a spleen weight of about 0·24 per cent of the body weight (twice the normal spleen size).

DISCUSSION.

The experiments offer clear evidence of the multiplication of *Br. suis* retained in the lungs of guinea-pigs exposed to a single-cell aerosol. Initially, as one might expect, the rate of increase in numbers of viable cells is dependent on the size of the retained dose ; when a concentration of 10^3 viable cells/g. of lung tissue is reached the subsequent rate of increase is independent of the size of the retained dose. Before any other tissues can be shown to be infected the lung viable count has risen to about 300 times the respiratory ID_{50}. The order of infection of other tissues suggests that the spread is *viâ* the regional lymph nodes and blood stream. Once infection can be demonstrated in regional lymph nodes the spread throughout the body is rapid.

These findings support the hypothesis, put forward by Elberg and Henderson (1948), that *Br. suis* retained in the lungs does not give rise to demonstrable lesions but multiplies at the site of deposition until phagocytosis takes place and viable brucella are transported to the nearby lymph nodes. From this site infection of the blood stream takes place which leads to generalised infection. Macroscopic lung lesions were only seen in animals held for 96 days or longer and were one of the features of the later stages of the disease.

Once established the disease becomes chronic, and although the blood and marrow become sterile by the 96th day after exposure, and the numbers of viable brucella in the tissues decline, after 215 days the spleen and some of the other tissues are still enlarged, contain abscesses and yield viable brucella. These findings are in contrast with those of de Ropp (1945) who found with guinea-pigs infected with large numbers of *Brucella abortus* that although agglutinins were still present in the blood, the spleen was sterile and had returned to normal size 30 weeks after infection. It would appear that guinea-pigs can rid themselves more readily of *Br. abortus* than of *Br. suis*.

SUMMARY.

Multiplication of *Br. suis* in the lungs of guinea-pigs exposed by the respiratory route to small numbers of organisms has been shown to be actively in progress as early as 48 hr. after the exposure. In the absence of any evidence of invasion of lung tissue the findings are in keeping with the hypothesis that the multiplication takes place on the surface of lung epithelium and that organisms are transported by phagocytes to lymph nodes from which sites the infection becomes generalised.

Observations on the persistence of *Br. suis* (Strain PS III) in the infected animal showed that tissue was still actively infected 215 days after exposure by the respiratory route to a minimal infecting dose.

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