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EXPERIMENTAL INFECTION WITH MYCOBACTERIUM AVIUM, SEROTYPE 2, IN PIGS

1. INTRAVENOUS INOCULATIONS*

By
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JØRGENSEN, J. BERG: *Experimental infection with Mycobacterium avium, Serotype 2, in pigs. 1. Intravenous inoculations.* Acta vet. scand. 1977, *18*, 532—544. — A porcine strain of Mycobacterium avium, Serotype 2, was used for intravenous inoculation of pigs in doses 5, 1, 10^{-1}, 10^{-2} and 10^{-3} mg (1 mg = 78×10^6 viable units), 2 pigs per dose.

Dose 5 mg proved fatal for both of the inoculated pigs, which were killed in extremis 64 and 69 days, respectively, after inoculation. Dose 1 mg caused clinical disease in 1 of 2 pigs, but was not lethal. Post mortem, the clinically affected pigs showed a generalized granulomatous tuberculosis. The other pig given 1 mg and the pigs given smaller doses, showed no clinical signs, and lesions and presence of acid-fasts were mostly limited to the lymph nodes of the lung, liver and digestive tract.

All the pigs showed delayed hypersensitivity to avian PPD tuberculin (1000 t.u.) and some of them cross-reacted with human PPD tuberculin (1000 t.u.). The clinically affected pigs gave a very weak response to tuberculin, the others a strong response.

The smallest dose capable of establishing an infection and producing tuberculous lesions was not determined, but seems to be less than 10^{-3} mg (78000 viable organisms).

Mycobacterium avium, Serotype 2; pathogenicity; intravenous inoculation; pigs.

Though Mycobacterium (M.) avium infection in pigs was first recognized in 1904 (*Weber & Bofinger*) there is still uncertainty about the epidemiology of this infection. To provide

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a better understanding of the pathogenesis and epidemiology of the infection, it was decided to make a series of experiments on pigs. In the present paper the results of intravenous inoculations are reported, and subsequent papers will deal with oral inoculations, contact infections and the immunizing effect of B C G vaccine against infection with M. avium.

In the present work M. avium was inoculated i.v. in doses 5 mg, 1 mg, 10^{-1} mg, 10^{-2} mg and 10^{-3} mg.

MATERIAL AND METHODS

Experimental animals. Ten pigs 8—10 weeks old. The weights of the pigs at the start of the experiment are shown in Table 1.

Inoculation material. M. avium, Serotype 2, strain SSC 1323 of pig origin (*Jørgensen et al.* 1972) was grown in Dubos fluid medium with Tween 80 for 14 days. The density of the culture was adjusted to 0.1 in a Coleman spectrophotometer at 580 nm, corresponding to about 1 mg semidried bacteria per ml (10^7—10^8 viable units per ml). Further dilutions were made from this standard culture. Viable unit counts on dilutions 10^{-5} and 10^{-6} on Löwenstein-Jensen medium, 5 tubes per dilution, showed 78×10^6 viable organisms per ml of the standard culture after 8 weeks of incubation. Colonies on 7H10 agar were smooth-transparent (SmT) (*Fregnan & Smith* 1962). The virulence of the strain was tested on 2 hens and 2 rabbits by i.v. injection of 1 mg. The animals died after 29 and 45 days, and after 20 and 22 days, respectively.

Inoculation. The doses employed are indicated in Table 1. Two pigs were inoculated with each dose.

Clinical observations. The behaviour and appetite of the pigs were observed daily, and their weight recorded every 1 or 2 weeks.

Tuberculin tests were performed before inoculation and 12, 26, 40, 54, 68, 96 and 130 days after inoculation as comparative tests with human and avian tuberculin (1000 t.u. per 0.1 ml of Statens Seruminstitut PPD tuberculin RT23 (human), and 20 μg (1000 t.u.) per 0.1 ml of M. avium PPD sensitin RS10)*. A dose

* Thanks are due to Mr. Mogens Magnusson, head of the Tuberculin Department, Statens Seruminstitut, for supplying these preparations.

of 0.1 ml was injected intradermally at the base of the ear, human PPD on the left side, avian PPD on the right. Before the injection the thickness of the skin was measured with a pair of calipers, and reactions were read after 24, 48 and 72 hrs. by measuring the increase in skin thickness with an accuracy of 0.5 mm and the diameter of a possible erythema as accurately as possible.

Duration of the experiment was planned to maximum 6 to 8 months, or until the pigs had reached a weight of 90—100 kg.

Post-mortem examination comprised the tissues listed in Tables 2 and 3.

Histopathology. Materials from the tissues (Table 2) were fixed in 4 % formalin and sections stained with haemalum-eosin and by the van Gieson and Ziehl-Neelsen (Z-N) methods.

Culture. After aseptic removal the tissues (about 0.5 g) were homogenized in 3 ml Besredka's fluid medium. Material from the tonsil and intestinal mucosa was decontaminated with 5 % sulphuric acid for 10 min. From each tissue suspension, 0.1 ml was inoculated onto each of 3 tubes of Löwenstein-Jensen (L-J) medium, L-J medium with 2.5 µg isonicotinic acid hydrazide (INH) per ml (*Plum* 1952) and Besredka's fluid medium. After incubation at 37°C for 8 weeks the cultures were read, and the results recorded as + or —.

Microscopy of Z-N stained smears was performed on all tissues.

RESULTS

Clinical observations. Table 1 shows the weights of the pigs at the start of the experiment and at 9 and 19 weeks after inoculation, and the weekly and total weight gains within the same period. After 9 weeks, 1 of the pigs given 5 mg (No. 13) had lost 0.4 kg in weight, while the other (No. 14) had gained 1.8 kg. Of the pigs given 1 mg, No. 15 had gained 16.3 kg after 19 weeks, while No. 16 had gained 75 kg. The rest of the pigs (Nos. 17—22) showed an average weight gain of 71.5 kg after 19 weeks. Loss of appetite and progressive emaciation were observed in Pigs 13 and 14, which were killed after 69 and 64 days, respectively. Pig 15 (dose 1 mg) showed unthriftiness and moderate emaciation. The rest of the pigs showed no clinical signs. Table 1 shows duration of experiment and weight at slaughter for each pig.

Table 1. Survey of experimental animals.

Pig No.	Dose, mg	Weight at start of the experiment, kg	Weight after inoculation, weeks p.i.	Weight after inoculation, kg	Weight gain total	Weight gain per week	Necropsy days after inoculation	Necropsy weight, kg
13 ♀	5	14.4	9	14.0	—0.4	—0.04	69	14
14 ♂	5	13.2	9	15.0	1.8	0.2	64	15
15 ♀	1	19.7	19	36.0	16.3	0.9	138	41
16 ♂	1	14.0	19	89.0	75.0	3.9	140	95
17 ♂	10^{-1}	15.6	19	93.0	77.4	4.1	144	95
18 ♀	10^{-1}	17.5	19	92.0	74.5	3.9	146	105
19 ♀	10^{-2}	18.3	19	93.0	74.7	3.9	153	108
20 ♀	10^{-2}	17.5	19	93.0	75.5	4.0	154	105
21 ♀	10^{-3}	18.0	19	83.5	65.5	3.5	158	104
22 ♀	10^{-3}	17.4	19	79.0	61.6	3.2	160	96
Average for Pigs 17—22		17.4		88.9	71.5	3.8		

Tuberculin tests. The criterion of a delayed hypersensitivity reaction was an increase in skin thickness of at least 1 mm and/or an erythema at least 5 mm in diameter. Non-infected pigs might show an induration of 0.5 mm and an erythema not exceeding 5 mm. None of the pigs reacted to tuberculin before inoculation. Pigs 13 and 16 first showed reaction on Day 12, Pig 20 on Day 40, and the rest of the pigs on Day 26 after inoculation. In Fig. 1 the 24-hour readings of skin thickness and erythema are shown graphically in relation to the time after infection. Each graph represents the mean reactions of 2 pigs inoculated with the same dose of organisms, except in the case of the pigs inoculated with 1 mg, which showed widely differing reactions and therefore are dealt with separately. The 2 pigs dosed with 5 mg (Nos. 13 and 14) showed weak and transient reactions, and also for Pig 15 (dose 1 mg) the reactions tended to be transient. The rest of the pigs showed a pronounced reactivity, which persisted until the last test on Day 130, though with a tendency to decrease. The hypersensitivity was highest to avian tuberculin, but cross reactions to human tuberculin were seen in all the pigs except Nos. 13 and 14 (dose 5 mg). Further details about the tuberculin tests in this series of experiments will be presented in a separate report (*Jørgensen & Weis Bentzon*, in preparation).

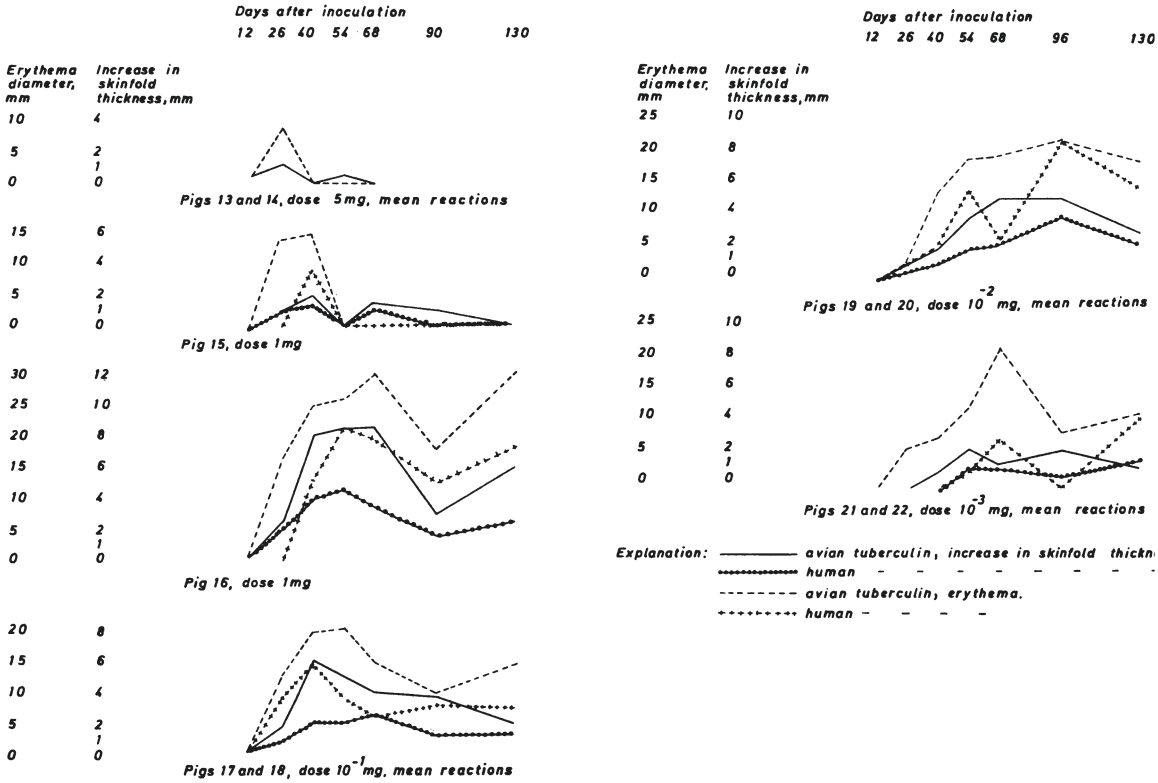

Figure 1. Comparative tuberculin tests with PPD avian and human, 1000 t.u. per dose, in pigs after intravenous inoculation with M. avium. Readings after 24 hrs.

Necropsy. Table 2 shows the distribution and number of macro- and microscopic lesions. Pigs 13 and 14 (dose 5 mg) were extremely emaciated, with reddish, consolidated areas in the lungs, most pronounced in the anterior lobes (Fig. 2). There were numerous white, pin-point nodules in the kidneys, and hyperplasia of some of the lymph nodes. Pig 15 (dose 1 mg) was underdeveloped, but not emaciated. In the liver there were many and in the kidneys innumerable whitish, pin-point nodules, and in the lungs scattered consolidated lobuli. Several lymph nodes were hyperplastic. The rest of the pigs showed either no gross lesions, or solitary from few to many whitish, well-defined nodules, 0.5—1 mm in diameter, of fleshy, caseous, or calcareous appearence. The tissues involved were livers and kidneys, and mesenteric, tracheobronchial and hepatic lymph nodes (Table 2).

Table 2. Pathological findings.

No.	Tissue	Pig No. and dosage									
		13 5 mg	14 5 mg	15 1 mg	16 1 mg	17 10^{-1} mg	18 10^{-1} mg	19 10^{-2} mg	20 10^{-2} mg	21 10^{-3} mg	22 10^{-3} mg
1	Ln. mandibularis	(++)	(+++)	hyp. (+++)							
2	„ parotideus	hyp. (+)	(+++)	(++)							
3	„ retropharyng. lat.	hyp. (++)	(+++)	(+)							
4	„ „ med.	(++)	(+++)	(++)							
5	„ cervicalis sup. dors.	hyp. (++)	(+++)	(+)							
6	„ „ „ ventr.	(++)	(+++)	(+)							
7	„ subiliacus	(+)	(+++)	(+)							
8	„ popliteus	(+)	(+++)	(+)							
9	„ inguinalis prof.	(+)	(+++)	(+)							
10	„ „ superf.	(+)	(+++)	hyp. (+)							
11	„ mesentericus	(+++)	(+++)	hyp. (+++)			++ (++)	+ (+)	+ (+)	(+)	+ (+)
12	„ tracheobronchalis sin.	hyp. (+++)	(+++)	hyp. (+++)			++ (++)	+ (+)	(+)	(+)	
13	„ hepaticus	(+++)	(+++)	hyp. (+++)	(+)		++ (++)	+ (+)	++ (++)	(++)	+ (++)
14	Spleen	(+++)	(+++)	hyp. (+++)							
15	Liver	(+++)	(+++)	++ (+++)	+ (+)		+ (+)				
16	Lung	+++ (+++)	++ (+++)	+ (++)	(++)	(+)	(+)				
17	Kidney	+++ (++)		+++ (++)	+ (+)		+ (+)				
18	Myocardium										
19	Musc. long. dorsi		(+)								
20	„ triceps brachii										
21	„ biceps femoris										
22	„ psoas major										
25	Tonsil	(++)	(++)	(+++)							
27	Intestinal mucosa (Peyer patch)	(++)	(+++)	(+)	(+)	(+)					

Explanations: Symbols without brackets indicate gross lesions.
Symbols in brackets indicate microscopic lesions.

\+ = Solitary lesions.
++ = Many lesions.
+++ = Innumerable lesions.
hyp. = Hyperplasia.
Blank space = No abnormalities.

Lesions of doubtful tuberculous nature were found in the livers of Pigs 17, 19, 20 and 21.

Histopathology. In Pigs 13 and 14 (dose 5 mg) the majority of the tissues were infiltrated with epithelioid cells and a few giant cells, especially the lymphoid tissue of lymph nodes, spleens, tonsils and Peyer patches, and livers and lungs. In the livers such infiltrations were found both in the parenchyma and in the interlobulary tissue, together with accumulations of lymphocytes and leukocytes, especially eosinophils (Fig. 3). In the lungs there were heavy peribronchial, perivascular, and interalveolar accumulations of epithelioid and giant cells and lymphocytes (Fig. 4). In the consolidated areas of the lungs the inflammatory reaction was of the same nature, but more intense, especially as regards the lymphocyte accumulations. In the bronchi and alveoli an exudate consisting of epithelial cells, leukocytes, and macrophages was found. There was no caseation or demarcation of the pathological tissue. In Z-N stained sections acid-fast rods, most of them intracellular, were demonstrated in almost all of the tissues examined. In Pig 15 (dose

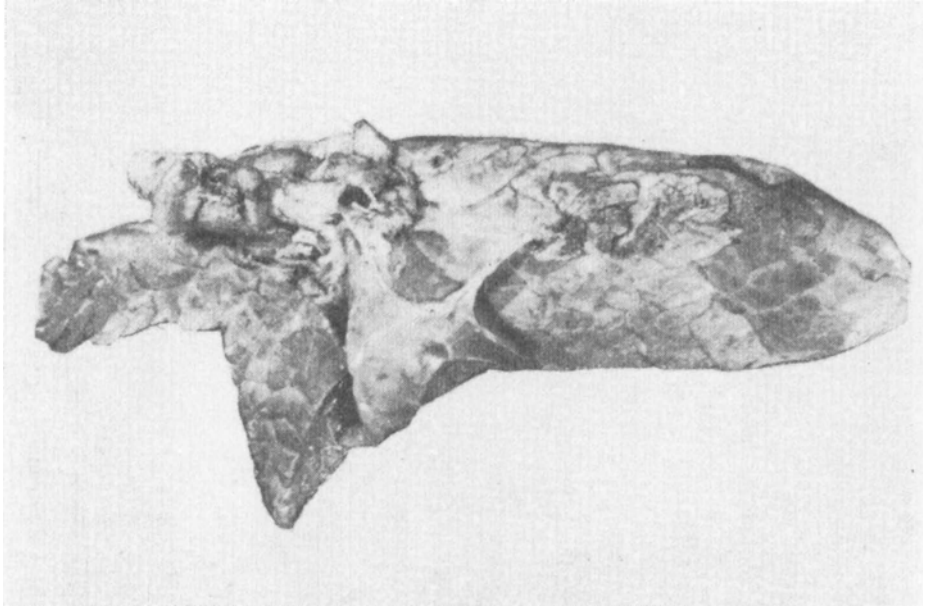

Figure 2. Pig 13. Lung with areas of consolidation in the apical and cardiac lobes and in the posterior section of the diafragmatic lobe.

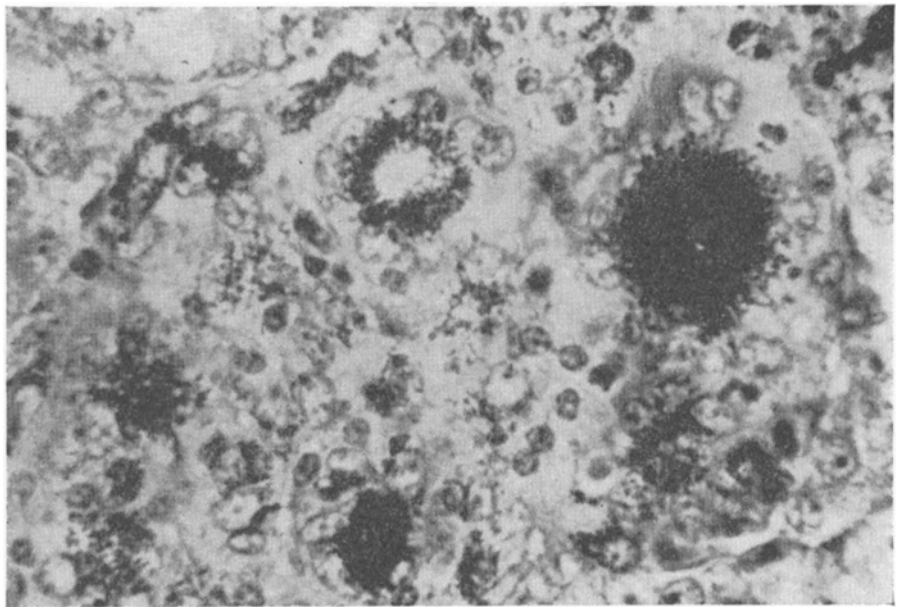

Figure 3. Pig 14. Section of liver. Granulomatous infiltration with epithelioid and giant cells, heavily loaded with acid-fast rods, here black. Magnification: approx. 590 ×. Staining: Ziehl-Neelsen.

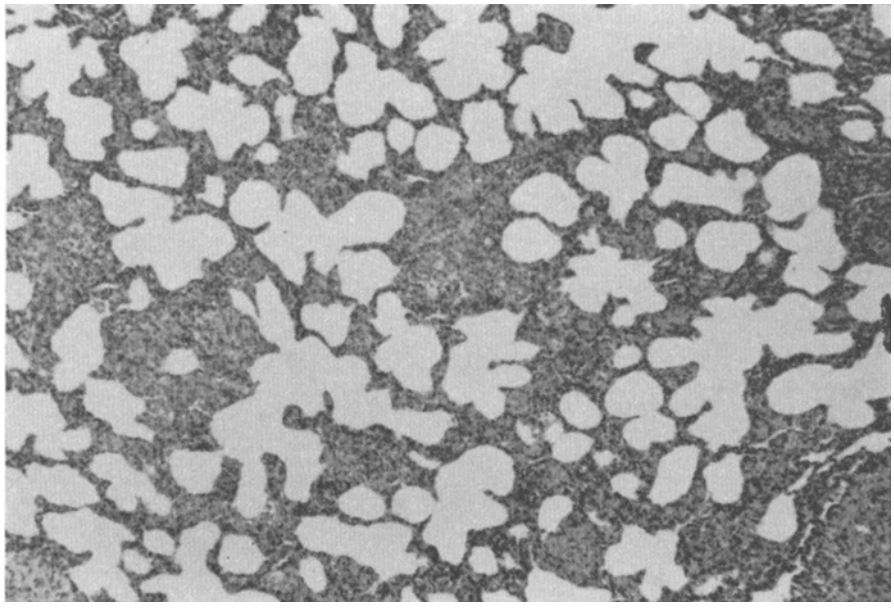

Figure 4. Pig 14. Section of lung. Microgranulomas in the interalveolar tissue. Magnification: approx. 60 ×. Staining: Haemalum-eosin.

Table 3. Bacteriological findings.

No.	Tissue	Pig No. and dosage						
		13 5 mg	14 5 mg	15 1 mg	16 1 mg	17 10^{-1} mg	18 10^{-1} mg	19 10^{-2} m
1	Ln. mandibularis	+++ (+)	+++ (+)	+++ (+)	(+)			(+)
2	„ parotideus	++ (+)	+++ (+)	+ (+)	(+)			
3	„ retropharyng. lat.	+++ (+)	+++ (+)	(+)			(+)	
4	„ „ med.	++ (+)	+++ (+)	+ (+)				(+)
5	„ cervicalis sup. dors.	++ (+)	++ (+)	(+)				
6	„ „ „ ventr.	++ (+)	+ (+)	(+)				
7	„ subiliacus	++ (+)	+++ (+)	(+)				
8	„ popliteus	++ (+)	+++ (+)					
9	„ inguinalis prof.	+ (+)	+++ (+)	(+)				
10	„ „ superf.	+++ (+)	+++ (+)	(+)				
11	„ mesentericus	+++ (+)	∞ (+)	+++ (+)	(+)		+ (+)	+
12	„ tracheobronchal. sin.	++ (+)	∞ (+)	+ (+)		(+)	+ (+)	+ (+)
13	„ hepaticus	+++ (+)	∞ (+)	+++ (+)		(+)	+ (+)	+ (+)
14	Spleen	+++ (+)	∞ (+)	+ (+)	(+)			
15	Liver	∞ (+)	∞ (+)	+ (+)				
16	Lung	+++ (+)	+++ (+)	+++ (+)	(+)		(+)	
17	Kidney	(+)	+ (+)	(+)				
18	Myocardium	(+)	++ (+)	(+)				
19	Musc. long. dorsi	(+)	(+)	(+)				
20	„ triceps brachii	(+)	+ (+)	(+)				
21	„ biceps femoris	(+)	(+)	(+)				
22	„ psoas major	(+)	+ (+)					
24	Joint	(+)	(+)	(+)				
25	Tonsil	++ (+)	+++ (+)	+++ (+)				
26	Bone marrow	++ (+)	+++ (+)	(+)				
27	Intestinal mucosa (Peyer patch)	++ (+)	+++ (+)	+++ (+)				

Explanations: Symbols in brackets indicate growth.
Symbols without brackets indicate results of microscopy.
+ = 1—50.
++ = 50—300 acid-fasts, observed within 4 min.
+++ = 2—20 per field.
∞ = innumerable.
No symbols = No findings.

Table 3 (continued).

No.	Tissue*	Pig No. and dosage 20 10^{-2} mg	21 10^{-3} mg	22 10^{-3} mg
11	Ln. mesentericus	+		
		(+)	(+)	
12	„ tracheobronchal. sin.	(+)		
13	„ hepaticus	+++		
		(+)	(+)	(+)
16	Lung	(+)		

* Only positive tissues indicated.

1 mg) the findings were similar as in Pigs 13 and 14, but more lymphocytes, eosinophils and giant cells were seen. There was no caseation, and acid-fast rods were fewer than in Pigs 13 and 14. In Pigs 16—22 the lesions consisted of epithelioid and giant cell granulomas with a distinct tendency to demarcation or obliteration by fibrous tissue, and to caseation and calcification. No acid-fast rods were found. In the livers of Pigs 17, 19, 20 and 21 an interstitial hepatitis was found, with eosinophilia, fibrosis and lymphocyte accumulations of the type described as lymphadenopathia nodularis hepatis (*Roneus* 1966, *Bindseil* 1967).

Microscopy. Pigs 13 and 14 (dose 5 mg) showed many acid-fast rods in nearly all the tissues, Pig 15 (dose 1 mg) in 11 of the 27 tissues examined. The rest of the pigs were negative, or positive in the mesenteric, hepatic and tracheobronchial lymph nodes only.

Culture. The 2 pigs on the 5-mg dose were positive in all the tissues examined. The same result was found in Pig 15 (dose 1 mg) except that the popliteal lymph node and the major psoas muscle were negative. In the other pigs the infection was confined to the lymph nodes of the digestive tract, the hepatic and tracheobronchial lymph nodes, and the spleen and lungs. The positive tissues are indicated in Table 3.

DISCUSSION

The pathogenicity of M. avium for pigs has been the object of several experiments, most often with oral inoculation (*Titze* 1907, *Mohler & Washburn* 1908, *Griffith* 1911, a. o.), not so often with intravenous, intraperitoneal, subcutaneous or intradermal

inoculation (*Griffith, Graham & Tunnicliff* 1926, *Schalk et al.* 1935, *Ray* 1966).

The only experiments comparable to the present work are those of *Griffith*, who used cultured organisms in defined doses of 10 and 50 mg to infect pigs i.v. Both doses proved lethal to the pigs, which died 47—70 days after inoculation. Post-mortem examinations showed generalized infection and, as a constant finding, areas of consolidation in the lungs.

In the present work the virulence of M. avium was titrated by using doses ranging from 10^{-3} to 5 mg (Table 1). A dose of 5 mg proved fatal for 2 pigs, which were killed in extremis after 64 and 69 days, respectively. Necropsy showed reddish areas of consolidation in the lungs and numerous pin-point lesions of the kidneys. Histological and bacteriological examinations showed tuberculous, granulomatous inflammation and heavy infection of almost all the tissues examined (Tables 2 and 3). A dose of 1 mg (78×10^6 viable units) caused clinical disease in 1 of 2 pigs, but was not lethal. Post mortem, the findings were essentially the same as in the pigs on dose 5 mg, except that fewer bacteria were present in the tissues. The rest of the pigs, including Pig 16 (dose 1 mg), showed no clinical signs, and post-mortem examination revealed lesions and bacteria in but few tissues, chiefly the lymph nodes of the lung, liver and digestive tract (Tables 2 and 3).

Tuberculin reactions and clinical observations were correlated in the way that hypersensitivity was weak in the pigs with clinical signs and strong in the rest of them. The finding that M. avium will provoke delayed hypersensitivity to both avian and mammalian tuberculin is in agreement with the findings of *Luke* (1951) after intraperitoneal and of *Ray* after intradermal inoculation.

One mg was the lowest dose capable of causing clinical disease, but the fact that only 1 of 2 pigs given that dose became clinically affected would seem to indicate that other factors influenced their susceptibility. A similar difference in susceptibility seems to have existed between the 2 pigs on dose 10^{-1} mg, as judged by the pathological and bacteriological findings.

By titration of the virulence of M. avium for pigs on intravenous inoculation a minimal infective dose was not determined, since the smallest dose used, i.e. 10^{-3} mg or 78000 viable organisms, was still capable of establishing an infection and lesions.

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SAMMENDRAG

Infektionsforsøg på svin med Mycobacterium avium, Serotype 2. 1. Intravenøs infektion.

En M. avium stamme, Serotype 2, isoleret fra en gris, blev anvendt til intravenøs podning på grise i doserne 5, 1, 10^{-1}, 10^{-2} og 10^{-3} mg, 2 grise pr. dosis.

Dosis 5 mg havde letal effekt på begge grise, som blev aflivet i stærkt afmagret tilstand henholdsvis 64 og 69 dage efter podning.

Dosis 1 mg var ikke letal, men forårsagede klinisk påviselig sygdom hos een af 2 podede grise. Post mortem undersøgelse af disse grise viste udbredt granulomatøs tuberkulose med mange bakterier i de undersøgte vævsprøver, mest udtalt for grise podet med 5 mg. De øvrige grise, inklusive den anden gris podet med 1 mg, viste ingen kliniske symptomer, og post mortem undersøgelser viste en tendens til, at tuberkuløse processer og bakterier koncentreredes til leverens, lungens og fordøjelseskanalens lymfekirtler.

Alle grisene reagerede ved tuberkulinprøver med aviært PPD tuberkulin, 1000 enh. pr. dosis og eventuelt med humant PPD tuberkulin, 1000 enh.. De klinisk syge dyr havde svage reaktioner, medens de øvrige dyr havde kraftige reaktioner.

Det lykkedes ikke at påvise den mindste dosis, der var i stand til at etablere en tuberkuløs infektion og forårsage tuberkuløse processer. Denne dosis er sandsynligvis mindre end den mindste dosis (10^{-3} mg = 78000 bakterier) anvendt i dette forsøg.

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