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EXPERIMENTAL INFECTION WITH MYCOBACTERIUM AVIUM, SEROTYPE 2, IN PIGS

2. ORAL INFECTION WITH LARGE DOSES OF M. AVIUM*

By

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JØRGENSEN, J. BERG: *Experimental infection with Mycobacterium avium, Serotype 2, in pigs. 2. Oral infection with large doses of M. avium.* Acta vet. scand. 1977, *18*, 545—558. — Twelve pigs were inoculated orally with Mycobacterium avium. The doses used were 0.5, 2 or 10 mg daily for 5 days, or 10, 50 or 180 mg once (1 mg = 37×10^6 viable units). Two pigs were used per dose, 1 of which was sacrificed 3 days, the other 28/31 days after the last inoculation (Table 1).

Three days after inoculation, M. avium was found in the tonsils and in the intestinal mucosa of all 6 pigs, and in the mesenteric lymph nodes of 4. Viable unit counts for tonsils and intestinal mucosa were highest in pigs inoculated with 180 mg×1 and 10 mg×5. Histopathologically these pigs showed activation of the lymphoid tissue in the tonsils, Peyer patches and mesenteric lymph nodes. Twenty-eight/31 days after inoculation a spreading of the infection had taken place in all pigs, most often to the liver, less frequently to the spleen and the lungs. The kidneys and the musculature were not infected (Table 4). A correlation was apparent between the size of dose and the number of viable organisms in the tissues. Divided doses gave about 10 times higher viable counts than a single dose with the same total number of organisms (Table 5).

No gross lesions were found 28/31 days after inoculation. Microscopic granulomatous lesions were found in the tonsils of 6 pigs, in the intestinal mucosa of 4 pigs, in the mandibular and mesenteric lymph nodes of 6 pigs, in the retropharyngeal lymph nodes of 3 pigs, and less frequently in the parotid and hepatic lymph nodes (Table 3).

Five of 6 pigs were weakly sensitive to avian tuberculin PPD, 1000 t.u. per dose, when tested 22/25 days after inoculation; 1 of these pigs cross-reacted to human tuberculin (Table 2).

Mycobacterium avium, Serotype 2; pathogenicity; oral inoculation; large doses; pigs.

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In a previous work (*Jørgensen* 1977) a report was given of the results of intravenous inoculation of pigs with Mycobacterium (M) avium, Serotype 2. In continuation of this work a series of experiments have been made in which M. avium was given to pigs by the oral route. In the present study large doses were used, viz. 2.5, 10, 50, and 180 mg.

MATERIAL AND METHODS

Experimental animals. Twelve SPF pigs, the age, sex and weight of which are given in Table 1.

Inoculation material. M. avium, Strain SSC 1336 (ATCC 25291) of chicken origin. For a full description of the strain, see *Engbæk et al.* (1971). The procedure for standard culture preparation, viable unit counts, and checking of colony morphology has been described earlier (*Jørgensen* 1977). Viable unit counts showed 37×10^6 units per mg wet weight. Colonies were smooth-transparent (SmT). To control the virulence of the strain 1 hen and 1 rabbit were inoculated each with 1 mg i.v. The animals died after 41 and 19 days, respectively.

Inoculation. The culture, suspended in skimmilk, was administered individually by mouth in doses as indicated in Table 1. In the experiments with 10 and 50 mg the full dose was given to some pigs at once, while to others it was divided over 5 days. Dose 2.5 mg was given only as divided doses over 5 days, and 180 mg only as a single dose. Two pigs were used at each dosage level.

Tuberculin tests with avian and human PPD tuberculins, 1000 t.u. per 0.1 ml, were performed and evaluated as previously described (*Jørgensen*). The pigs were tested before inoculation and 22 days (single dose) or 25 days (5 doses) after inoculation.

Clinical observations. The pigs were weighed once a week and their appetite and general condition noted daily.

Duration of experiment. Of the 2 pigs inoculated at each dosage level, 1 was slaughtered after 3 days, the other after 28 days (single dose) or 31 days (5 doses). For pigs given 5 doses, the number of days is reckoned from the last inoculation (Table 1).

Post-mortem examination

Necropsy and histopathological examination were performed as described previously (*Jørgensen*). The tissues examined are

listed in Table 4. For pigs necropsied 3 days after inoculation histopathological examinations included tonsils, intestines and mesenteric lymph nodes only. An anterior, a middle and a posterior mesenteric lymph node were examined. In Table 4 they are indicated as ln. mesentericus I, II and III.

Cultures were made from the tissues listed in Table 4, and from 2 samples of intestinal contents, viz. 1 from the ileum (I) and 1 from the rectum or the posterior part of the colon (II).

Cultural technique and medium. The material, i.e. aseptically removed tissue (about 0.5 g) or intestinal contents (about 2 g) was homogenized in 4 ml Sauton medium diluted 1 in 4 with distilled water. This suspension was called dilution 10°. From

Table 1. Survey of experimental animals, dosage and weight gains.

Pig No., sex, age	Dosage	Necropsy, days after last inoculation	Weight, kg	Weight gain, kg
101 ♂ 70 days	10 mg 1 ×	3	19.5	
102 ♀ 80 days	10 mg 1 ×	28	15.5	11.5
103 ♂ 80 days	50 mg 1 ×	3	20.0	
104 ♂ 80 days	50 mg 1 ×	28	23.5	18.5
105 ♂ 80 days	180 mg 1 ×	3	18.5	
106 ♂ 80 days	180 mg 1 ×	28	17.5	14.5
107 ♂ 70 days	0.5 mg 5 ×	3	16.0	
108 ♂ 70 days	0.5 mg 5 ×	31	16.0	14.0
109 ♂ 70 days	2 mg 5 ×	3	17.5	
110 ♀ 70 days	2 mg 5 ×	31	18.0	15.0
111 ♂ 70 days	10 mg 5 ×	3	19.0	
112 ♂ 70 days	10 mg 5 ×	31	19.0	18.0

each sample, 0.1 ml was inoculated onto each of 4 tubes of Löwenstein-Jensen medium. From mesenteric, tracheobronchial and hepatic lymph nodes inoculations were made from dilutions 10^{-1} and 10^{-2}. Liver, lung, tonsil, intestinal mucosa and intestinal contents were decontaminated by treatment with 6 % sulphuric acid for 10 min. Viable unit counts were made after incubation for 8 weeks at 37°C.

RESULTS

Clinical observations. No clinical effect was observed. Weight gains after 28 days are recorded in Table 1.

Tuberculin tests. The pigs showed no reaction before inoculation. The results of testing at 22/25 days after inoculation are shown in Table 2. Reactions, characterized by erythema with no detectable induration, developed in all pigs except No. 104. There was reaction only to avian tuberculin, except in Pig 110 which cross-reacted with human tuberculin. The reactions to avian tuberculin were detectable only at the 24-hr. reading, except for Pig 110 which was positive also at the 48-hr. reading, though with a weaker reaction.

Table 2. Comparative tuberculin tests with PPD avian and human, 1000 t.u. per dose, 22/25 days after inoculation.

Pig No.	Inoculation	Days after inoculation	Increase in skinfold thickness, mm		Erythema, diameter in mm			
			avian	human	avian		human	
					24-hr.	48-hr.	24-hr.	48-hr.
102	10 mg 1 ×	22	0	0	7.8	0	0	0
104	50 mg 1 ×	22	0	0	0	0	0	0
106	180 mg 1 ×	22	0	0	13.1	0	0	0
108	0.5 mg 5 ×	25	0	0	9.3	0	0	0
110	2 mg 5 ×	25	0	0	10.2	9.5	0	8.5
112	10 mg 5 ×	25	0	0	11.5	0	0	0

No reactions were observed after 72 hrs.

Necropsy. None of the pigs showed gross lesions.

Histopathology. Pigs necropsied 3 days after inoculation showed hyperplasia of the lymphoid tissue in the tonsils, Peyer patches and mesenteric lymph nodes. The intestinal propria mucosae was moderately infiltrated with plasma cells, lymphocytes and eosinophils. Small accumulations of macrophages were seen in the mesenteric lymph nodes of 2 pigs (107 and 111) inoculated 5 times with, respectively, 0.5 and 10 mg (Fig. 1). In Ziehl-Neelsen (Z-N) stained sections no acid-fast rods were seen. Pigs necropsied 28/31 days after inoculation all showed epithelioid and giant cell infiltrations, especially in the tonsils and the mandibular and mesenteric lymph nodes, which were positive in all the pigs. The intestines were positive in 4 of the 6 pigs. Infiltrations were found less frequently in the hepatic, medial retropharyngeal and parotid lymph nodes (Table 3). No specific lesions were found in the spleens, livers, lungs and kidneys. Small lymphocyte accumulations were found in the liver of Pig 106 and in the lung of Pig 108. In the intestinal mucosa the lesions

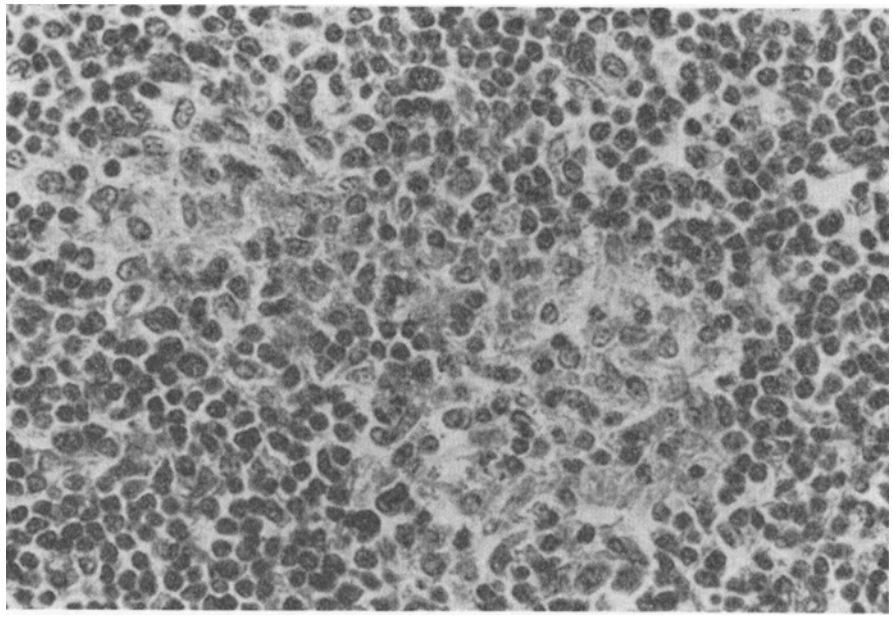

Figure 1. Pig 111. Section of mesenteric lymph node. Macrophage accumulation in the lymphoid tissue. Magnification: approx. 480 ×. Staining: Haemalum-eosin.

Table 3. Histopathological findings 28/31 days after inoculation.

Tissue	Pig No. and dosage					
	102 10 mg	104 50 mg	106 180 mg	108 0.5 mg×5	110 2 mg× 5	112 10 mg×5
Positive lymph node No.*	1, 11	1, 11, 13	1, 2, 4, 11	1, 4, 11	1, 4, 11, 13	1, 11
Liver	0	0	?	?	0	0
Lung	0	0	0	?	0	0
Tonsil	+	+	+	+	+	+
Intestinal mucosa (Peyer patch)	0	+	+	+	0	+
Spleen and kidney	0	0	0	0	0	0

* For lymph node No., cf. Table 4.
\+ = Tuberculous lesions.
? = Lymphocyte accumulation.
0 = No lesions.

were located to the Peyer patches (Fig. 2). Just 1 pig (106, dose 180 mg×1) showed epithelioid cell infiltration in the propria mucosae, with many intracellular acid-fast rods (Fig. 3). In Z-N stained sections acid-fast rods could be demonstrated in the mesenteric lymph nodes and tonsils of all the pigs. No dose-dependent difference in the severity of the tuberculous infiltrations could be seen except in the tonsils, intestines and mesenteric lymph nodes. In Pig 112 (dose 10 mg×5) the most pronounced infiltrations were found in the tonsils and in the mesenteric lymph nodes, and in Pig 106 (dose 180 mg×1) in the intestinal mucosa and the mesenteric lymph nodes. It was characteristic that lesions were most pronounced in the middle and posterior mesenteric lymph nodes and often were found in these nodes only.

Culture. In pigs necropsied 3 days after inoculation, cultures from the tonsils and the intestinal mucosa were invariably positive, and the mesenteric lymph nodes were positive in 4 of the 6 pigs. The other tissues were negative. Viable unit counts are recorded in Table 4. Pigs necropsied 28 or 31 days after inoculation showed growth from the sites of primary infection, i.e. the tonsils and the intestinal mucosa, and from the corresponding lymph nodes, i.e. the mandibular, medial retropharyngeal, and

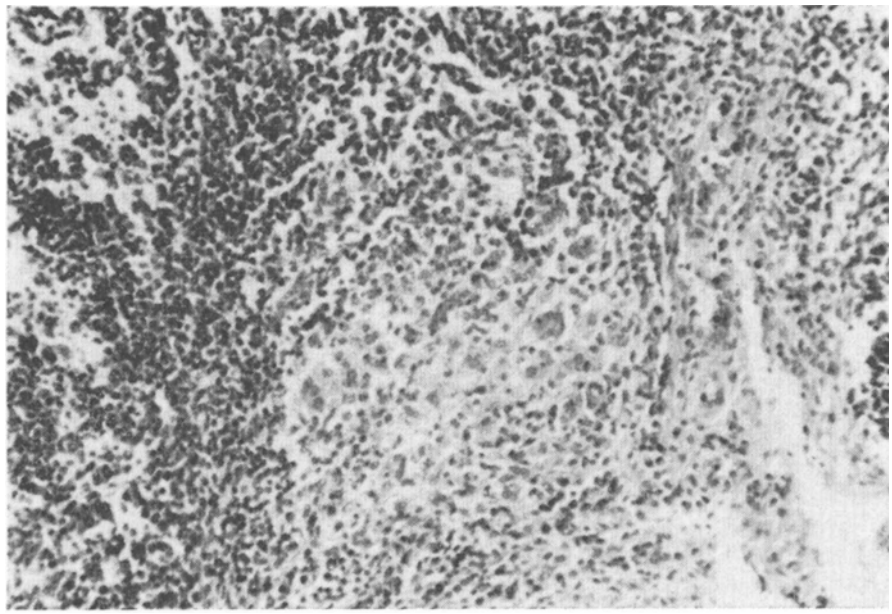

Figure 2. Pig 104. Section of Peyer patch. Epithelioid and giant cell infiltration (granuloma) in the lymphoid tissue. Magnification: approx. 150 ×. Staining: Haemalum-eosin.

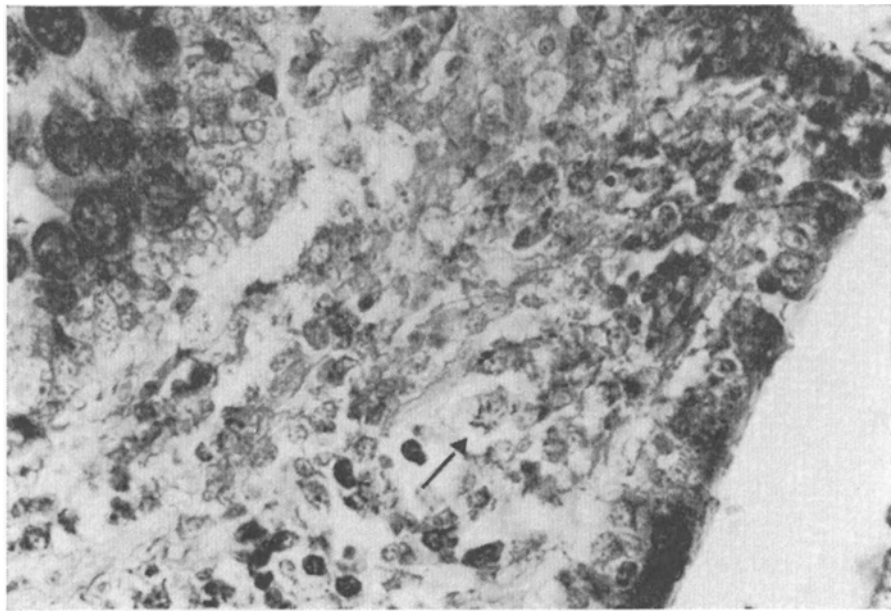

Figure 3. Pig 106. Section of intestinal propria mucosae. Epithelioid cell infiltration, intracellular acid-fast rods, here black (arrow). Magnification: approx. 480 ×. Staining: Ziehl-Neelsen.

Table 4. Results of culturing.

No.	Tissue	Pig No., dosage, and duration of experiment											
		101 10 mg 3 days	103 50 mg 3 days	105 180 mg 3 days	107 0.5 mg ×5 3 days	109 2 mg ×5 3 days	111 10 mg ×5 3 days	102 10 mg 28 days	104 50 mg 28 days	106 180 mg 28 days	108 0.5 mg ×5 31 days	110 2 mg ×5 31 days	112 10 mg ×5 31 days
1	Ln. mandibularis	cont.*		cont.	cont.		cont.		3620	6800	1790	3920	> 4000
2	„ parotideus												
4	„ retropharyngeus medialis								2250	40	1680	50	770
11	„ mesentericus I								131000		320000	∞	∞
11	„ „ II		100	60	100	100		6600	21000	∞	419000	81000	∞
11	„ „ III			100				27000	92000	∞	404000	19100	∞
12	„ tracheobronchal. sin.												> 4000
13	„ hepaticus							20	2970	80		780	> 4000
14	Spleen									70			50
15	Liver							10	100	40		180	40
16	Lung									10	10		10
17	Kidney												
19	Musculus longissimus dorsi												
25	Tonsil	300	370	3600	280	20	300	1200	150000	300	1460	2100	∞
27	Intestinal mucosa (Peyer patch)	160	50	900	20	90	480	1700	35000	260000	6200	5000	174000
28I	Intestinal contents				cont.			30	1290	410	10	130	70
28II	„ „							20			280	10	90

* cont. = Contaminated.
Blank space = No growth.
Figures indicate viable units per 0.5 g tissue or 2 g faeces.
∞ = > 400000 units.

mesenteric lymph nodes. The parotid lymph nodes were negative. A more or less pronounced spreading had occurred to the spleen, liver and lung, and to the tracheobronchial and hepatic lymph nodes. The kidneys and the musculature were not infected. Viable unit counts for tissues outside the primary complex were low except in Pig 112 (dose 10 mg×5), which showed high viable unit counts in the tracheobronchial and hepatic lymph nodes. Cultures were positive from the contents of the ileum in all of the 6 pigs, and from the contents of the rectum in all but 2 pigs (104 and 106) (Table 4). For pigs necropsied 28/31 days after inoculation the effect of the size of dose and the difference in effect between a single dose and the same dose divided in 5 was roughly evaluated by adding up the viable unit counts for all tissues examined, and for the mesenteric lymph nodes separately. These lymph nodes were chosen because of their connection with the port of entry of the infection and because they had been subjected to no decontaminating treatment. Counts recorded in Table 4 as "innumerable", i.e. more than 400,000, were entered in the calculation as 400,000. The results of the evaluation are shown in Table 5. In the group of animals dosed once, a positive

Table 5. Summed up viable unit counts, 28/31 days after inoculation.

	Pig No. and dosage					
	102 10 mg×1	104 50 mg×1	106 180 mg×1	108 0.5 mg×5	110 2 mg×5	112 10 mg×5
All tissues	3.7×10^4	4.4×10^5	$>1.1\times10^6$	1.2×10^6	$>5.1\times10^5$	$>1.8\times10^6$
Mesenteric lymph nodes	3.4×10^4	2.4×10^5	$>8.0\times10^5$	1.1×10^6	$>5.0\times10^5$	$>1.2\times10^6$

correlation was apparant between size of dose and viable unit counts. Such correlation was not obvious in the group dosed 5 times, probably because by equalling "innumerable" with 400,000 the number of organisms in the tissues was underestimated. About 10 times higher viable unit counts were observed after dosing 5 times than after dosing once.

DISCUSSION

Since the first isolation of M. avium from pigs (*Weber & Bofinger* 1904) a number of oral inoculation experiments have been

reported. *Feldman* (1938) gave a survey of the experiments up to 1938. In most of these and later experiments tuberculous tissues or poorly defined doses of cultured organisms were used for inoculation. Only a few workers have used defined doses of culture (*Griffith* 1911, *Kauker & Zettl* 1964, *Ray* 1966). In experiments lasting from 2 to 12 months the tuberculous lesions were mostly located to the lymph nodes of the digestive tract, and the tendency to generalization was little (*Jong* 1905, *Titze* 1907, *Bang* 1913, *Hastings & Halpin* 1913, *Schalk et al.* 1935, *Ray* and others). Recently results have been published of a series of oral inoculation experiments with Group III mycobacteria (*Ray, Kleeberg & Nel* 1969), M. intracellulare Serotype 6 (*Tammemagi & Simmons* 1971, *Tuffley et al.* 1973) and M. avium Serotype 8 (*Thoen et al.* 1976). In these experiments, the material for inoculation was cultured organisms in amounts specified by weight or viable units.

In the present work it was found that 3 days after inoculation the organisms were present in tonsils and intestinal mucosa and might even have reached the mesenteric lymph nodes, and that 28/31 days after inoculation they had spread to other organs as well, most pronouncedly in the animals given respectively 180 mg$\times$1 and 10 mg$\times$5, in which spreading had occurred to liver, spleen and lung. In the animal given 0.5 mg$\times$5 the infection had spread to the lungs, and in the rest of the animals (doses 10 mg $\times$1, 50 mg$\times$1 and 2 mg$\times$5) to the liver only (Table 4).

The slight tendency even for large doses (10 mg and 50 mg) to cause generalization is consistent with the results of a number of previous experiments with either culture material (*Jong, Titze, Griffith, Ray*), tuberculous tissues (*Mohler & Washburn* 1908, *Hastings & Halpin, Schalk et al., Gwatkin & Mitchell* 1952) or contact with tuberculous poultry (*Schalk et al., Plum* 1948).

The obvious correlation between the number of viable organisms in the tissues and the size of dose was not surprising. What may be of epidemiological significance, however, is that divided doses seemed to result in considerably higher viable unit counts than the full dose given at once.

No gross lesions being found at 31 days after inoculation, the patho-morphological features of the tuberculous infection could be studied only histologically.

The early tissue reaction to invasion of tubercle bacteria is an accumulation of polymorphonuclear leukocytes, which dis-

appear within 24 hrs. to be replaced by mononuclear phagocytes, s.c. macrophages, which are considered to be the first stage of the epithelioid cell tubercle (*Vorwald* 1932). A similar tissue reaction has been described in experimental M. lepraemurium infection in mice (*Closs & Haugen* 1975).

In pigs sacrificed early after inoculation, the tissue reaction consisted mostly in hyperplasia of the lymphoid tissue in the Peyer patches, the tonsils and the mesenteric lymph nodes. Yet small macrophage accumulations were found in the mesenteric lymph nodes of 2 pigs killed 3 days after a course of 5 inoculations, i.e. 8 days after the first inoculation. A similar length of the histogenesis of macrophage-epithelioid-cell granulomas was observed by *Dannenberg et al.* (1968) and *Itoh* (1974).

All pigs necropsied 28/31 days after inoculation showed typical tuberculous cellular infiltrations (Table 3). The tonsils were positive in all of the 6 pigs, the intestinal mucosa in 4. The infiltrates were chiefly located in the lymphoid tissue of the Peyer patches, and in only 1 case (Pig 105, dose 180 mg$\times$1) was tuberculous tissue found in the propria mucosae as well. Although the pigs given the highest doses had the most extensive lesions of the tonsils and the intestinal mucosa, with many intracellular acid-fast organisms, lesions were found even in the pig on the lowest dose. In the mesenteric lymph nodes lesions were most pronounced in the middle and posterior nodes, as is usually the case also in natural infections (*Nitschke* 1963, *Lautrup-Nielsen* 1975).

Tuberculin tests at 22/25 days after inoculation showed that 5 of 6 pigs were sensitive to avian tuberculin, and that 1 of these reacted to human tuberculin as well (Table 2). It is remarkable that 4 of the 5 pigs showed reaction only at the 24-hr. reading, and that the reaction was characterized by erythema without induration.

No clinical effect or dose-dependent differences in weight gains were observed in the present study. *Kauker & Zettl* observed clinical illness in pigs a few days after the last of 20 daily inoculations with 25 or 50 mg M. avium, and 1 pig inoculated with 50 mg daily died 10 days after the start of the experiment. These doses must be regarded as unusually large, and the course of the disease was even shorter than that observed after intravenous inoculation of 50—100 mg (*Griffith*).

In 1 pig given an oral dose of 100 mg, *Griffith* found a decreased growth rate during an observation period of 101 days. At necropsy caseated nodules were found in the tonsils and in the mesenteric and hepatic lymph nodes, but histological examination showed a septicaemic spread of the infection (*Eastwood* 1911) similarly to that found by *Jørgensen* (1977) after intravenous inoculation of 5 mg cultured organisms. It is possible that with a prolonged period of observation a similar course of the infection might have been observed in the present work in the pigs given the largest doses.

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SAMMENDRAG

Infektionsforsøg på svin med Mycobacterium avium, Serotype 2. 2. Peroral infektion med store doser af M. avium.

Tolv grise blev podet peroralt med M. avium i doserne 0,5, 2 og 10 mg daglig i 5 dage eller 10, 50 og 180 mg givet een gang (1 mg= 37×10^6 viable units). Der blev podet 2 grise pr. dosis, hvoraf den ene blev slagtet 3 dage og den anden 28/31 dage efter sidste podning (Tabel 1).

Ved undersøgelse 3 dage efter podning kunne M. avium dyrkes fra tonsiller og tarmslimhinde fra alle 6 grise og fra lnn. mesenterici fra 4 grise. I tonsiller og tarmslimhinde fandtes de højeste kimtal hos grise podet med 180 mg×1 og 10 mg×5. Ved histologisk undersøgelse af Peyerpletter, tonsiller og lnn. mesenterici fandtes en aktivering af det lymfoide væv, og hos 2 grise podet 5 × kunne påvises makrofagansamlinger. Ved undersøgelse 28/31 dage efter podning fandtes hos alle grisene en spredning af infektionen, oftest til lever og mindre hyppigt til milt og lunge, medens nyre og muskulatur ikke var inficerede (Tabel 4).

Der fandtes korrelation mellem dosisstørrelse og antal bakterier i vævene. Fraktioneret dosering gav ca. 10 gange større kimtal end den samme totaldosis indgivet på een gang (Tabel 5).

Der fandtes ikke makroskopiske forandringer 28/31 dage efter podning, men mikroskopiske processer kunne påvises i tonsiller og lnn. mandibulares og mesenterici hos 6 grise, tarmslimhinde hos 4 grise, lnn. retropharyngei med. hos 3 grise og mindre hyppigt i ln. parotidei og hepatici (Tabel 3).

Fem af 6 grise var svagt positive for aviært PPD tuberkulin, 1000 enh. pr. dosis, 22/25 dage efter podning. Hos een gris fandtes krydsreaktion med humant tuberkulin (Tabel 2).

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