STUDIES ON CERTAIN BIOLOGICAL CHARACTERISTICS OF MALLEOMYCES MALLEI AND MALLEOMYCES PSEUDOMALLEI

II. Virulence and Infectivity for Animals

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As a natural infection, glanders occurs only in horses, mules, and donkeys. Other animals occasionally become infected from contact with infected solipeds. The most susceptible of these occasionally infected species are ferrets, moles, field mice, cats, and dogs. Sheep, goats, hogs, rabbits, white mice, and house mice are reported to be less susceptible, and cattle are immune (Hutyra and Marek, 1926). The guinea pig is considered to be the most susceptible laboratory animal. The virulence of various strains of *Malleomyces mallei* has been reported to vary widely (Bernstein and Carling, 1909; Dudgeon *et al.*, 1918), but in the virulence tests recorded in the literature large doses were given, and evaluation of virulence was made solely on the severity of disease produced.

The wild rats of southeastern Asia constitute a natural reservoir of melioidosis. The disease, chronic in the rat, is thought to be transmitted to other animals, and to man, by ingestion or inhalation of materials contaminated with rat excreta. Rabbits, dogs, and cats have been found infected naturally, and an occasional case of infection has been reported in other domestic animals. Guinea pigs, mice, and rabbits have been used in studies of the experimentally produced disease (Stanton and Fletcher, 1932). Guinea pigs were reported to be almost universally susceptible, dying of a fulminating infection within 24 hours after a massive inoculation and within 3 weeks after a smaller inoculation. Monkeys were more resistant, developing fatal infection only after ingestion of massive doses. Rabbits and guinea pigs were susceptible to inoculation by the intraperitoneal or subcutaneous routes and by ingestion and inhalation. Although the organisms were reported as highly virulent, the inoculum always contained thousands to millions of organisms (0.01 to 1 ml of a 48-hour broth culture—Stanton and Fletcher, 1932). No reference to a more quantitative evaluation of virulence by MLD or LD_{50} determinations was found in the literature.

The present studies include a comparison of the virulence of the several strains and their infectivity by various portals of entry, and a comparison of the susceptibility of several common species of laboratory animals. The source and characteristics of the strains of *Malleomyces* studied are given in the first paper of this series (Miller *et al.*, 1948). The pathological changes and pathogenesis

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of experimental glanders and melioidosis will be reported elsewhere (Miller, Smith, and Tanner, 1948).

COMPARATIVE VIRULENCE OF STRAINS

Since hamsters proved to be the most susceptible of the common laboratory animals tested, the virulence of the several strains was compared in this species. Decimal dilutions of a suspension of the growth from a 24-hour agar slant culture were inoculated intraperitoneally, using 5 to 8 animals for each dilution. The number of organisms injected was determined by triplicate plant count. All animals that died were autopsied and cultures of the infected organs were made. Survivors were held from 20 to 30 days, killed with nembutal, and examined for

TABLE 1

Comparative virulence for hamsters of strains of M. mallei and M. pseudomallei injected intraperitoneally

STRAIN	NUMBER OF ORGANISMS IN	
	MLD	LD_{50}
M. mallei 2MP	>500,000,000*	
M. mallei 3MP	>500,000,000*	
M. mallei 3PP	>500,000,000*	
M. mallei 2MP (serial passage)	7,000	
M. mallei C3	20	12
M. mallei C4	20	12
M. mallei C5	>900,000*	
M. mallei C6	>1,200	
M. mallei C7†	<26	2‡
M. pseudomallei W294	15	6
M. pseudomallei W295	>85,000,000*	

* Produced 50 to 90% mortality.

† Only one titration was done with this strain; see test.

‡ This value is only approximate; calculated dose of 2.6 organisms produced 80% mortality.

the presence of pathologic changes; cultures were made of the liver, spleen, lungs, and heart blood.

The least number of organisms that produced a 100 per cent morbidity and mortality was considered the minimum lethal dose (MLD). When possible, the LD_{50} dose was computed. The average results of several titrations are given in table 1.

It will be noted from table 1 that strains C3 and C4 of *M. mallei* and strain W294 of *Malleomyces pseudomallei* proved to be highly virulent. Animals receiving 30 or more organisms of these strains invariably succumbed to infection; death occurred within 14 days, and usually within 3 to 10 days, depending upon the size of the inoculum. Inoculation of approximately 10 organisms produced a 25 to 35 per cent mortality. Since the MLD was definite and easily determined, it was used in preference to the LD_{50}.

Although the virulence of the *M. mallei* strains decreased after 1 to 3 months of cultivation on artificial media, it was quickly and easily restored to the original level by 1 or 2 passages through hamsters. Twenty serial passages in hamsters produced no evidence that continued passage would enhance or alter the original virulence of these strains, nor was there any change in the type of disease produced.

Strain C7 was received just before the experimental work was terminated, and only one titration was performed. Injection of 26 organisms (and all larger doses) gave 100 per cent mortality in an average of about 80 hours, and the injection of a calculated dosage of 2.6 organisms resulted in 80 per cent mortality. This strain, recently isolated from a fatal human case, was the most virulent of the *M. mallei* strains studied.

M. mallei strains 2MP, 3MP, and 3PP had a low degree of virulence when received. Large doses produced subacute or chronic infections that resulted in about 80 per cent mortality over a period of 2 to 3 months. The majority of the surviving animals showed evidence of infection (Straus reaction or symptoms of general illness) at some time after inoculation, but recovered spontaneously. A few animals showed no evidence of infection at any time. The virulence of strain 2MP was increased by 4 serial passages by the pulmonary route. The MLD of this passage strain was about 7,000 organisms.

M. mallei strains C5 and C6 and *M. pseudomallei* strain W295 also proved to be of low virulence and were not used further in animal studies.

INFECTIVITY BY VARIOUS PORTALS OF ENTRY

In view of the high infectivity of *M. mallei* and *M. pseudomallei* for the hamster by the intraperitoneal route, it appeared of interest to compare the infectivity by this route with that by others.

Titrations for infectivity by the subcutaneous route were performed according to the procedure previously described for intraperitoneal titrations.

Infectivity by the respiratory route was determined by producing infectious aerosols in a specially designed apparatus (Rosebury *et al.*, 1948). Groups of hamsters were exposed for varying periods of time to aerosols containing different concentrations of organisms in order to give graded doses of the inocula. Hamsters of uniform size were used, and the average volume of air inspired per minute under the experimental conditions employed was determined prior to the test runs (Rosebury *et al.*, 1948). The number of organisms inspired (inoculum) was computed from the total volume of aerosol inspired and the determined number of viable organisms per unit volume of aerosol. A total of 149 hamsters were exposed to aerosols of *M. mallei* and 256 hamsters to aerosols of *M. pseudomallei*.

The results in table 2 show that the LD_{50} (average of several titrations) by the subcutaneous route is the same, within experimental error, as that by the intraperitoneal route. The results of repeated virulence titrations by the respiratory route were somewhat variable. The figures in the table represent the average LD_{50} computed from all of the separate titrations. Infectivity by the respiratory route, although somewhat lower than that by the subcutaneous and intraperitoneal routes, was comparatively high.

Attempts were made to ascertain infectivity by the oral route by forced manual feeding of decimal dilutions of the organisms in broth. The morbidity and mortality after such inoculation of either species of *Malleomyces* were very irregular, and no accurate LD_{50} dosage could be determined. A dosage of 4 to 5 million organisms infected only 20 to 40 per cent of the animals, whereas doses of 400 to 500 organisms infected a similar percentage. Moreover, in several groups that were given doses between these two extremes, none of the animals became infected.

SUSCEPTIBILITY OF VARIOUS ANIMAL SPECIES TO INFECTION

Hamsters, guinea pigs, ferrets, rabbits, black mice, white mice, white rats, and monkeys were tested to determine their susceptibility to infection with *M. mallei* and *M. pseudomallei.*

Guinea pigs. It has been pointed out by several authors that when guinea pigs are injected with unknown biological materials for the isolation of *M. mallei,* several animals should be injected with each sample since some individuals are much more susceptible than others. On the other hand, the guinea pig has been

TABLE 2

Infectivity by various portals of entry (hamsters)

ORGANISM	NUMBERS OF ORGANISMS IN ONE LD_{50}		
	Intraperitoneal	Subcutaneous	Respiratory
M. mallei C3 and C4.....	12	15	160
M. pseudomallei W294.....	6	10	70

reported to be uniformly and highly susceptible to infection with *M. pseudomallei* (Stanton and Fletcher, 1932).

Preliminary observations with both species of *Malleomyces* in this laboratory indicated that there was marked individual variation in susceptibility to infection. An evaluation of guinea pig susceptibility to the virulent strains of *Malleomyces* available was, therefore, attempted. This was done by (1) determining the morbidity and mortality following a single large intraperitoneal dose of organisms, and (2) by injecting graded doses intraperitoneally in virulence titrations.

A large group of adult guinea pigs was given an intraperitoneal inoculation of 690,000 organisms of *M. mallei* (strain C4), and another like group was given a similar inoculation of 460,000 organisms of *M. pseudomallei* (strain W294). The day of death was recorded and autopsies were performed. Survivors were killed from 95 to 168 days after inoculation and autopsied. The results are shown in table 3. It will be noted that the results with the two species of *Malleomyces* were closely parallel. Some of the animals in each group died of acute glanders or melioidosis within 15 days; others died of subacute or chronic forms of the diseases after 20 to 100 days. Some of the survivors at autopsy were found to have an active chronic infection after 168 days, but others showed no evidence of active infection or of healed lesions.

A virulence titration was performed in guinea pigs with *M. mallei*, strain C7. This was the most virulent strain of *M. mallei*. The results are shown in table 4. All animals that died had the acute or subacute form of the disease. One or more animals in each group either recovered or showed no evidence of having been infected. Of the survivors only one was found to have foci of active infection at autopsy. It is evident that some individual animals were very resistant to infection. The fact that 2 of 3 animals given 26 organisms died of the disease shows that some individual animals were also very susceptible to infection with *M. mallei*. The calculated LD_{50} from this titration was 512 organisms. It is apparent that the chronic form of the disease did not develop with

TABLE 3

Results of intraperitoneal injection of a single large dose of M. mallei or M. pseudomallei in guinea pigs

ORGANISM	NO. OF ORGANISMS INJECTED (IP)	MORTALITY RATIO	SURVIVORS		PER CENT DEAD OF ACUTE DISEASE	PER CENT DEAD OF SUBACUTE OR CHRONIC DISEASE	PER CENT INFECTED WHEN KILLED	PER CENT RECOVERED OR NO EVIDENCE OF INFECTION
			No.	Killed after				
M. mallei C4	690,000	34/46	11	168 days	41	33	6	20
			1	95 days				
M. pseudomallei W294	460,000	27/32	4	168 days	34	49	3	13
			1	125 days				

TABLE 4

Results of a virulence titration of M. mallei, strain C7, in guinea pigs

NO. OF ORGANISMS INJECTED (IP)	MORTALITY RATIO	AVERAGE DAY OF DEATH	SURVIVORS		NO. STILL INFECTED	NO. RECOVERED OR NO EVIDENCE OF INFECTION
			No.	Killed after		
2,600,000	3/4	17	1	70 days	1	0
260,000	3/4	17	1	63 days	0	1
26,000	2/4	26	2	63 days	0	2
2,600	3/4	12	1	63 days	0	1
260	3/4	15	1	63 days	0	1
26	2/3	23	1	63 days	0	1

this highly virulent strain, as it did after the injection of a single large dose of strain C4. All except one of the animals either developed a fatal acute form of the disease or showed no evidence of infection.

A virulence titration was performed, using *M. pseudomallei*, strain W294, in graded doses intraperitoneally. The dosage given and the results are shown in table 5. Regardless of the size of the dosage, some animals died of the acute disease, some died of the chronic disease, and some either recovered or were never infected. The results of this titration parallel those after a single large dose. The calculated LD_{50} from this titration was 440 organisms.

Ferrets. Twelve ferrets were used to test the susceptibility of this species to

glanders and melioidosis. Four animals were given moderate doses intraperitoneally and the remaining 8 were given graded doses subcutaneously. All animals died of acute glanders or melioidosis between 8 and 15 days after inoculation. The smallest dosages given were 91 and 73 organisms of *M. mallei* and *M. pseudomallei*, respectively. It appears that the ferret is quite susceptible to both diseases and that the MLD values are less than 91 and 73 organisms for *M. mallei* and *M. pseudomallei*, respectively.

Rabbits. Intravenous injection of as high as 300 million organisms of *M. mallei*, strain C4, did not infect rabbits. This species is apparently resistant to glanders. Rabbits given large doses of *M. pseudomallei*, strain W294, subcutaneously or intravenously developed acute fatal melioidosis. The degree of susceptibility of the species to this agent was not evaluated.

Mice. The susceptibility of black mice and white mice to virulent strains of *M. mallei* and *M. pseudomallei* was tested by the inoculation of graded doses of

TABLE 5

Results of a virulence titration of M. pseudomallei, strain W294, in guinea pigs

NO. OF ORGANISMS INJECTED (IP)	MORTALITY RATIO	DEAD OF ACUTE DISEASE		DEAD OF SUBACUTE OR CHRONIC DISEASE		NO. OF SURVIVORS*
		No.	Average day of death	No.	Average day of death	
4,400,000	4/5	2	8	2	22	1
440,000	5/5	1	10	4	45	0
44,000	5/5	2	11	3	46	0
4,400	4/5	1	13	3	31	1
440	3/5	1	10	2	37	2
44	1/4	0		1	26	3

* Through an oversight these animals were not autopsied when killed 2 months after inoculation.

the organisms. The results were somewhat irregular. Sixty to 75 per cent of the animals developed fatal acute or subacute forms of the diseases following intraperitoneal inoculations of 2.5 to 30 million organisms. Inoculation of less than 450,000 organisms produced no mortality, but a few animals killed 2 to 3 months after inoculation were found to have chronic foci of infection. Mice appeared to be relatively resistant to infection with *Malleomyces* organisms.

White rats. Thirty-five adult white rats were used to estimate the susceptibility of this species to glanders and melioidosis. Intraperitoneal inoculations of 1 million organisms of *M. mallei*, strain C4, failed to produce any demonstrable infection. Intraperitoneal doses of 30 million to 1 billion organisms of *M. pseudomallei*, strain W294, produced a subacute or chronic infection with 30 to 50 per cent mortality. Survivors showed no evidence of infection after inoculation or when killed and autopsied after 2 to 3 months. Smaller inocula produced no obvious evidence of disease, but foci of infection were found in an occasional animal killed for examination. White rats appear to be resistant to infection

with *M. mallei* and only slightly susceptible to infection with the only virulent strain of *M. pseudomallei* studied.

Monkeys. Six *Macaca mulata*, weighing 5 to 7 pounds, were tested for susceptibility by giving them graded doses of virulent *M. mallei* (strain C4) and *M. pseudomallei* (strain W294) subcutaneously. The animals were kept under close observation for 2 months; then they were killed with nembutal and carefully autopsied. The 2 animals given the largest doses (1.5 million organisms) of the 2 organisms developed subcutaneous abscesses at the site of inoculation 4 days after injection. In the monkey infected with *M. mallei*, the abscess was about 2 cm in diameter, drained spontaneously after 4 days, and healed completely after 3 weeks. During this time there was a daily temperature elevation of 1 to 3 degrees, a rapid sedimentation rate, and a marked increase in the white blood cell count with a moderate relative lymphocytosis. There was a loss of about 1 pound in body weight and the animal appeared moderately ill. In the monkey infected with *M. pseudomallei*, the abscess was about the same size,

TABLE 6

Comparative susceptibility of various species of laboratory animals to experimental glanders and melioidosis

SPECIES	GLANDERS		MELIOIDOSIS	
	Susceptibility	LD_{50} (no. of organisms strains C3 and C4)	Susceptibility	LD_{50} (no. of organisms strain W294)
Hamsters	Marked	12	Marked	6
Ferrets	Marked	<90	Marked	<73
Guinea pigs	Moderate	512	Moderate	440
Rabbits	Resistant		Moderate	Not determined
Mice	Slight		Slight	
White rats	Resistant		Slight	
Monkeys	Slight		Slight	

drained spontaneously in 4 days, and healed completely in 2 weeks. The temperature elevation, rapid sedimentation rate, and white blood cell elevation with relative lymphocytosis diminished to normal as soon as the abscess drained.

Specific agglutination and complement fixation tests became positive in both animals within 8 to 14 days after inoculation and the titers rose progressively to a maximum 4 weeks later. Agglutinin titers reached 1:2,560 in the glandered monkey and 1:1,280 in the monkey with melioidosis. The complement-fixing titer reached 1:640 in both animals. Intradermal skin tests using a 1:10 dilution of commercial mallein were negative in both animals 4 and 6 weeks after inoculation.

Both animals regained their normal health and vigor after the abscesses healed and appeared completely normal when killed 2 months after inoculation. Autopsy showed complete healing of the skin lesions and no evidence of specific pathological change elsewhere. Cultures of all organs were negative for *M. mallei* and *M. pseudomallei*.

The 4 animals receiving smaller inocula showed no clinical or laboratory evidence of infection at any time and pathological and cultural examinations at autopsy were negative. It is apparent that rhesus monkeys were only slightly susceptible to subcutaneous inoculation with the virulent strains of *Malleomyces* studied.

Hamsters. The average MLD values for virulent cultures of *M. mallei* and *M. pseudomallei* were found (see comparative virulence of strains) to be approximately 20 and 15 organisms, respectively, by the intraperitoneal or subcutaneous routes. Because of this high susceptibility, together with the fact that hamsters are easily obtained and simple to care for in large numbers, this species was chosen as the most suitable animal for experimental studies. Although adult male hamsters were sometimes preferred because of the development of the easily recognized Straus reaction, comparative studies showed that they were no more susceptible than were adult females.

COMPARATIVE SUSCEPTIBILITY OF THE VARIOUS SPECIES

The results of the studies in the preceding sections of this paper are summarized in table 6 to facilitate comparisons.

DISCUSSION

M. mallei strains 2MP, 3MP, and 3PP were of very low virulence and produced subacute or chronic infection in hamsters. The moderately virulent strains C3 and C4 produced acute fulminating infections in hamsters and ferrets but only subacute or chronic infections in the majority of guinea pigs. The highly virulent strain C7 produced acute fulminating forms of the disease in both hamsters and guinea pigs. It appears that the virulence of the strain has a great deal to do with the type of disease that is produced. This is undoubtedly a partial explanation for the occurrence in horses of all gradations of the disease from acute fulminating glanders to the chronic low-grade form of farcy. It may also serve to explain why the disease never became widespread in man, but occasionally in the presence of outbreaks of fulminating infections in horses a large number of human cases developed.

The two strains of *M. pseudomallei* were reported to be avirulent stock laboratory cultures. Strain W295 proved to be avirulent for experimental animals, but strain W294 appeared to be highly virulent.

The relatively low MLD values in hamsters by subcutaneous, intraperitoneal, and respiratory routes emphasized the high degree of infectivity of virulent *Malleomyces* organisms in a susceptible host. The erratic results after oral inoculation suggests that these organisms are often readily destroyed after ingestion. However, the fact that a small oral inoculum produced infection in 20 to 40 per cent of the animals indicated that this may be an important route of natural infection.

The uniformly high degree of susceptibility of hamsters to glanders and melioidosis compared with the irregular susceptibility of guinea pigs emphasizes the value of hamsters for experimental work. The irregular infectivity of *M.*

pseudomallei (strain W294) for guinea pigs contrasts with the uniform infectivity reported by Stanton and Fletcher (1932). Since their strains were freshly isolated from cases of the natural disease, this discrepancy may be due entirely to a difference in virulence of strains. The low degree of susceptibility of rats to melioidosis and the development of chronic forms of the disease emphasizes the role this species may play as a natural reservoir of infection.

SUMMARY

The various strains of *Malleomyces mallei* and *Malleomyces pseudomallei* studied varied greatly in their virulence. The strains of low virulence tended to produce subacute or chronic forms of the diseases, whereas strains of high virulence produced acute fulminating infections. The virulence of one strain (2MP) was increased by serial passage in hamsters.

Hamsters were easily infected by inoculation by the intraperitoneal, subcutaneous, and respiratory routes. Oral inoculation gave irregular results, but some animals became infected after the ingestion of relatively small doses.

Of the laboratory animals tested hamsters were found to be the most susceptible to both diseases. Ferrets were also very susceptible, but guinea pigs were only moderately susceptible and individual animals varied a great deal in the degree of susceptibility. Rabbits, mice, rats, and monkeys were least susceptible.

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