COMPLEMENT FIXATION TEST IN EXPERIMENTAL CLINICAL AND SUBCLINICAL MELIOIDOSIS

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ABSTRACT

NIGG, CLARA (University of California, Berkeley), AND MARGARET M. JOHNSTON. Complement fixation test in experimental clinical and subclinical melioidosis. J. Bacteriol. **82**:159–168. 1961.—Soluble stable antigens prepared from *Pseudomonas pseudomallei* gave 4+ complement fixation reactions in a dilution of 1 to 8,000 when tested with specific rabbit antiserum diluted 1 to 10,000.

The complement fixation reaction was positive in 100% of experimentally infected rabbits 9 to 11 days postinfection. Infected guinea pigs and monkeys showed similar results.

Monkeys inoculated with very small infecting doses of *P. pseudomallei* developed positive complement fixation reactions in the absence of clinical manifestation of infection. An anamnestic complement-fixing antibody response could be induced in such monkeys, after the titer had dropped to approximately the preinfection level, by inoculating very small doses of viable *P. pseudomallei* or larger doses of killed melioidosis vaccine.

The complement fixation test described appeared to be both sensitive and specific, and should be of value in human melioidosis which cannot be diagnosed on the basis of clinical manifestations alone.

It is suggested that subclinical infections may play a role in the epidemiology of human melioidosis. The potential application of the complement fixation test to serological surveys in areas where melioidosis occurs endemically is discussed.

Melioidosis, caused by *Pseudomonas pseudomallei*, was first recognized in Burma in 1911 by Whitmore and Krishnaswami (1912). The course of the disease may be acute, with death occurring as early as 3 days after onset of symptoms, or it may be subacute with death delayed for several months. The mortality is reported to be approximately 95% (Pons, 1930; Stanton and Fletcher, 1932; Souchard, 1932; Dunlop, 1952). A few chronic cases which survived for several years have been recorded (Prevatt and Hunt, 1957).

Because of the high mortality and the fulminating course of acute melioidosis, rapid diagnosis is most urgent so that effective chemotherapy can be instituted as early as possible. But the literature emphasizes repeatedly that the variable clinical forms of the disease make it impossible to arrive at a diagnosis on the basis of clinical findings alone.

Bacteriological examinations afford the most reliable diagnostic procedure, but the results of such tests may not be available in time to institute effective therapy. Moreover, the organism, which assumes a variety of colonial forms (Nigg et al., 1956), may be discarded as a contaminant by the uninitiated. Once the organism has been isolated, its identity can be established by cultural and serological methods.

Skin tests and various serological tests such as agglutination and complement fixation have been used for diagnostic purposes, but the reports of low titers, the lack of satisfactory specificity and the failure to obtain positive reactions in certain proved cases, have discouraged the use of such diagnostic tests (Stanton and Fletcher, 1932; Fournier et al., 1953; Brygoo, 1953). Moreover, the presence of agglutinins for *P. pseudomallei*, in fairly high titer, in a high percentage of sera from normal persons, both in nonendemic (Cravitz and Miller, 1950) and endemic areas (Brygoo, 1953), along with their rather slow development in melioidosis (Stanton and Fletcher, 1932; Brygoo, 1953), emphasizes the limited usefulness of agglutination tests for diagnostic purposes.

Because of the urgency of early diagnosis, it seemed worthwhile to examine again the possibility of developing a satisfactory serological diagnostic test, specifically a complement fixation test. Such a test would appear to be useful since

it has been reported that a 1 to 10 dilution of serum from normal persons does not give complement fixation reactions with *P. pseudomallei* antigen (Cravitz and Miller, 1950). Having no human cases of melioidosis at our disposal, the development of the complement fixation reaction was studied in experimentally infected animals.

MATERIALS AND METHODS

Strains of P. pseudomallei. The following strains, used either in the preparation of the complement fixation antigen and antisera or for infection of animals, were all isolates from cultures received from the Institute for Medical Research at Kuala Lumpur, Malaya, through the courtesy of S. S. Elberg: 114-8, a smooth isolate after 27 serial passages through mice of a culture labeled "Str. Ipoh 10.2.53"; 111-9, a rough isolate from a culture labeled "Str. Wong Fook Mook 10.2.53"; 111-13, another rough isolate obtained after 13 serial passages through monkeys of the preceding original culture; and 111-14, a rough isolate from strain 111-9.

Preparation of rabbit antisera. Rabbits were inoculated with a mixture of equal volumes of a formalinized suspension of *P. pseudomallei* strain 111-9 (standardized to contain ca. 10^9 organisms/ml) and 1 to 4 or 1 to 10 dilution of an aqueous extract from the same strain prepared in the same manner as the complement fixation antigen. The rabbits were given several courses of intravenous doses of 2 ml each at 3-day intervals with 1 week of rest between courses. Sera were obtained 7 to 10 days after the last inoculation of each course. Those sera with the highest titer were selected for subsequent use.

Sera from infected animals. Blood samples were obtained from all animals before infection and at intervals after infection. All sera were sterilized by filtration through Millipore filters.

Preparation of complement fixation antigen. The antigens, prepared by two methods, were aqueous extracts of disintegrated *P. pseudomallei* strain 114-8 grown on beef heart infusion agar in Roux bottles at 37 C for 2 days. The growth was washed off with distilled water with the aid of glass beads. The centrifuged bacterial sediment was washed one or two times.

In the first method (antigen 1), the wet weight of the sediment was determined and the latter resuspended in a minimal amount of water. This suspension, inactivated in the autoclave, was subjected to disintegration in an Omnimixer (model 500 B, 14,000 rev/min). The sediment, after disintegration, was collected by centrifugation, resuspended, and again subjected to disintegration and centrifugation. The two supernatants were combined and filtered through a Selas 03 candle to give a cell-free preparation. The amount of water used throughout the processing was measured and kept to a minimum. Water was added just before filtration to give a final ratio of 10 ml water (final volume) to 3 g of the initial bacterial sediment.

In the second method (antigen 2), the initial washed bacterial sediment, resuspended in a small amount of water (about 100 ml for the bacterial sediment from 20 Roux bottles), was disintegrated in a sonic oscillator (Raytheon, 10 kc/sec, 250 watts at −8 to 0 C for ½ hr). The sonically treated suspension was sedimented in a refrigerated angle head centrifuge. The supernatant was saved and the sediment resuspended in ca. 25 ml water and again centrifuged. The two supernatants of the sonically treated suspension were combined, clarified by filtration through a Selas 02 candle, and sterilized by filtration through a Selas 03 candle. The final volume of filtrate, prepared from the growth of 20 Roux bottles, was usually about 90 ml.

The final opalescent filtrate, which constituted the antigen, was kept frozen in a Dry Ice cabinet. Such antigens have retained their full activity during storage for at least 2 years.

Complement fixation tests. The human sera were inactivated at 56 C for 30 min; the animal sera at 60 C for 20 min. Mixtures of 0.2 ml diluted serum, 0.2 ml diluted antigen (1 unit), and 0.2 ml complement (2 units) were incubated in a water bath at 37 C for 1 hr after which there was added 0.2 ml 2% washed sheep red blood cells and 0.2 ml (2 units) of appropriately diluted commercially prepared antisheep hemolysin. The results, read after an additional incubation of ½ hr, were recorded in terms of degree of fixation from 0 to 4+. Appropriate controls were included with all tests. The sheep blood for the hemolytic system was collected aseptically in sterile Alsever's solution. The diluent for all of the reagents was a Veronal-NaCl buffer (Kabat and Mayer, 1948) containing 0.1 g $MgCl_2 \cdot 6H_2O$ and and 0.04 g $CaCl_2 \cdot 2H_2O$ per liter (Mayer, Croft, and Gray, 1948; Browne, Michelbacher, and Coffey, 1954).

TABLE 1. *Titration of antigens prepared from Pseudomonas pseudomallei by different methods*

Antigens from strain 114-8			CF[a] reactions[b] with heterologous[c] antiserum (R496) diluted:			
Method of disintegration	Antigen no.	Dilution	1 to 4,000	1 to 6,000	1 to 8,000	1 to 10,000
Omnimixer	1	1 to 500	4	4	4w	2w
		1 to 1,000[d]	4	4	4	4w
		1 to 2,000	4	4	4w	3
		1 to 4,000	1w	1w	0	0
Sonic oscillator	2	1 to 1,000	4	3st	2w	0
		1 to 2,000	4	4	4w	2w
		1 to 4,000	4	4	4	3w
		1 to 6,000	4	4	4	3-4
		1 to 8,000[d]	4	4	4	4w
		1 to 10,000	4	4	4	3st
		1 to 12,000	4	4w	4w	3st

[a] CF = complement fixation.
[b] w = weak; st = strong.
[c] Strain 111-9.
[d] Dilution of antigen representing 1 unit.

RESULTS

Table 1 shows that antigen prepared by either of the methods described above was satisfactory for complement fixation tests. The titer, expressed as the highest dilution (in a volume of 0.2 ml) that gave the strongest reaction with the test antiserum, was 1 to 1,000 for the antigen prepared by disruption of killed bacteria in the Omnimixer and 1 to 8,000 for that prepared by sonic treatment of viable bacteria. Although these two antigens differed in their antigenic content, as shown by their titer, 1 unit of each (i.e., 1 to 1,000 and 1 to 8,000 dilutions, respectively) gave approximately the same complement fixation titer of the test antiserum (i.e., 1 to 10,000). Because of the lower activity of the antigen in the presence of antigen excess (antigen 2), only 1 unit of antigen was used in the subsequent titration of sera.

Development of the complement fixation reaction in experimentally infected animals. 1) In rabbits:—Since fully virulent strains of *P. pseudomallei* produce a rapidly lethal infection in rabbits, a strain (111-14) of moderate virulence was used for infecting most of the rabbits in this study. A few were infected with a strain (114-8) of high virulence. The infecting dose of each strain was kept low in attempts to induce minimal infections. This procedure was used to prolong the survival time to obtain maximal production of complement fixation antibodies. Chemotherapy (Hezebicks and Nigg, 1958) was administered to some rabbits to suppress infection to extend the life of the animals. All rabbits were infected intravenously. They were maintained throughout the entire period of observation in closed chambers (Reyniers type) and all manipulations were made through glove ports to protect personnel from infectious ocular and nasal discharges which may develop in rabbits.

Of 17 experimentally infected rabbits, only 10 survived for 1 week or more, and these were killed when they appeared to be in the terminal stages of infection. Table 2 summarizes the complement fixation reactions with the sera from these 10 rabbits. Over 70% (5 of 7) gave positive complement fixation reactions at 7 or 8 days postinfection and 100% (8 of 8) were positive at 9 to 11 days postinfection or earlier. All showed pathological changes characteristic of those in experimental melioidosis.

Table 3 shows examples of the effect of chemotherapy (chloramphenicol) on the development of complement fixation reactions. In rabbit 112, early therapy, with dosages intended to suppress but not cure the infection, inhibited the development of complement fixation antibodies, but after therapy was discontinued on day 20, the titer increased, presumably with progression of the infection, from 1 to 40 (on day 18 and day 25) to 1 to 640 at 5 weeks postinfection, when the experiment was terminated.

To permit the development of a maximal complement fixation titer, therapy was withheld from rabbit 212 until day 17, at which time the titer had reached 1 to 320, where it remained (with therapy) until day 23 when the rabbit was killed.

2) In guinea pigs:—Ten guinea pigs were infected intracardially or intraperitoneally with ca. 10^5 organisms of the virulent strain 114-8. Relatively large doses were required to produce clinical infection in this species. Only one of nine guinea pigs tested 3 to 7 days postinfection gave positive complement fixation reactions. At 10 or 11 days postinfection, three of four survivors were positive. Table 3 shows the development of the complement fixation reaction, with a final

TABLE 2. *Development of complement-fixing antibodies in rabbits infected intravenously with Pseudomonas pseudomallei*[a] *(CF*[b] *antigen was prepared from strain 114-8)*

Rabbit no.	Infection[c]		Chemotherapy[d]	Postinfection CF titer[e] on days:						Gross pathological changes
	Dose (organisms)	Termination		7-8	9-11	13-14	17-19	23-25	35	
402	2.4×10^3	S8	None	20						Moderate
403	2.4×10^3	S8	None	20						Moderate
453	2×10^3	S9	Sulfa D2-4, 7-9	40	80					Advanced
112[f]	3×10^3	S35	Chlor D3-7, 10-14, 18, 19		20		40	40	640	Moderate
113	3×10^3	S35	Chlor D3-7, 10-14, 18, 19		10		10	10	50	Moderate
315	2.8×10^3	S25	Chlor D3-7, 13, 14, 17-21	25		80	400	400		Minimal
316	2.8×10^3	S7	Chlor D3-6	10						Minimal
317	2.8×10^3	S19	Chlor D3-7	<10		50	100			Moderate
212	2.2×10^2	S23	Chlor D17, 18, 21		80		320	320		Moderate
311	42	S24	Chlor D3-7	<10		100		100		Moderate

[a] Rabbit 311 was infected with strain 114-8, all the others with strain 111-14.
[b] CF = complement fixation.
[c] S8 = sacrificed on day 8.
[d] Chlor = chloramphenical, sulfa = sulfadiazine, D2-4 = on days 2 to 4, inclusive.
[e] Titer is expressed as the reciprocal of the highest serum dilution which showed 2+ or more fixation. None of the preinfection sera fixed complement in a dilution of 1 to 5.
[f] Serum inactivated 56 C, 30 min.

titer of 1 to 640, in a guinea pig (206) that survived for 4 weeks postinfection.

3) In *Macaca irus* monkeys:—The results in infected monkeys were of special interest and significance. Table 4 shows the development of complement fixation antibodies in monkeys infected with aerosols of strain 111-13. The low infecting doses, which varied from 1.3×10^2 to 3.7×10^3 organisms, extended survival, making it possible to follow the development of complement fixation antibodies. No chemotherapy was administered at any time during the observation period. In experiment 1, only one (203) of the nine monkeys died before the termination of the experiment on day 20, although another (192) was moribund at that time. None of the remaining seven monkeys showed signs of clinical infection at termination, 20 days postinfection, although all showed characteristic lesions, varying in extent. All but one (200) of the nine monkeys developed complement fixation antibodies. None of the remaining eight monkeys was positive at 6 days postinfection, but the majority of these were positive at 13 days and all at 19 days postinfection, with titers ranging from 1 to 20 to 1 to 640. The lower titers might have increased had the animals not been killed. In general, the titers were correlated with the extent of the pathological changes which varied from minimal to extensive.

To follow the development of complement fixation antibodies after a smaller inhalatory dose, monkey 3 (experiment 2) was infected with 130 organisms in the form of an aerosol. Complement fixation antibodies were not demonstrable at 7 days postinfection, but showed a titer of 1 to 40 at 16 days. The titer increased to 1 to 640 at 62 days, at which time the monkey appeared to be weak. During the next few days there was evidence of progressive clinical infection. At 68 days, this monkey was moribund and therefore killed. The pathological changes were characteristic of those in chronic melioidosis in experimentally infected animals.

It was assumed that all of the above monkeys, infected by the inhalatory route, would have died if the observation period had been extended. An attempt was therefore made to induce milder infections, with recovery, by the intraperitoneal inoculation of even smaller infecting doses ($\frac{1}{100}$ LD_{50}) of the same strain. Chemotherapy also was withheld from these monkeys.

In monkey 1 (Table 5), complement fixation antibodies were not demonstrable at 11 days postinfection, but were demonstrable at 18 days, although in low titer which gradually dropped to

TABLE 3. *Effect of chemotherapy on the complement fixation reactions of animals infected with Pseudomonas pseudomallei*

Serum samples	Infecting strain	CF[a] reactions with serum diluted:							Therapy with chloramphenicol day-dosages in mg
		1 to 10	1 to 20	1 to 40	1 to 80	1 to 160	1 to 320	1 to 640	
Rabbit 112[b]	111-14								
Preinfection		0							3-100, 100
Postinfection:									
4 days		½							4-100, 100; 5-100, 200; 6-200, 200; 7-200, 200; 8 and 9-0
10 days		4	2–3	0					10-100, 100; 11-100, 100; 12-200, 200; 13-200, 200; 14-200, 200 15, 16, and 17-0
18 days		4	4	1–2	0				18-200; 19-200
25 days		4	4	3–4	0				
35 days		4	4	4	4	4	4	2	
Rabbit 212[c]	111-14								
Preinfection		1							
Postinfection:									
4 days		0							
11 days		4	4	4	3–4	1	0		
17 days		4	4	4	4	4	4	½	17-200; 18-100, 100; 19 and 20-0
21 days		4	4	4	4	4	3	0	21-100, 100
23 days		4	4	4	4	4	3–4	0	
Guinea pig 206[c]	114-8								
Preinfection		0							
Postinfection:									7-100
11 days		3–4	3	2–3	0				
14 days		4	4	4	3–4	0			
18 days		4	4	4	4	3–4	0		
21 days		4	4	4	4	4	1	0	
28 days		4	4	4	4	4	4	2	

[a] CF = complement fixation.
[b] Sera inactivated 56 C, ½ hr.
[c] Sera inactivated 60 C, 20 min.

the base line at approximately 2½ months postinfection. The primary antibody response in this monkey, although low in titer, indicated that the organisms of the first infecting dose must have multiplied, since it seems most unlikely that the 194 organisms inoculated constituted sufficient antigen to induce demonstrable antibody response. Such presumptive evidence of multiplication would, in turn, imply the development of subclinical infection. Complete recovery was indicated by the return, at 77 days postinfection, of the complement fixation titer to the preinfection level. Reinfection at this time with 1 LD_{50} induced, within 1 week, an abrupt high rise in titer which increased to 1 to 500 and persisted at this level for some weeks. The sharp rise in titer, after this second small infecting dose, appeared to be an anamnestic response since it seemed unlikely that 20,000 organisms were sufficient to induce the titer observed within 8 days.

The maintenance of the high complement fixation titer after the small second infecting dose could possibly have been a manifestation of progressive low-grade chronic infection although clinical signs were absent. This possibility could have been established only by killing the animal. Instead, it seemed more desirable to determine whether immunity had developed along with complement fixation antibodies as the result of two small successive infecting doses. The monkey was therefore challenged 32 days after the second infecting dose with 10 LD_{50}. The complement

TABLE 4. *Complement fixation reactions of monkeys infected with aerosols of Pseudomonas pseudomallei strain 111-13 (no chemotherapy)*

Expt no.	Monkey no.	Infecting dose (organisms)	Termination[a]	Serum samples	CF[b] reactions[c] with serum diluted: 1 to 10	1 to 20	1 to 40	1 to 80	1 to 160	1 to 320	1 to 640	Gross pathological changes
1	194	4.2×10^2	s20	Preinfection	0							Minimal
				Postinfection:								
				6 days	0							
				13 days	2w	0						
				19 days	3st	2w	0					
	195	4.2×10^2	s20	Preinfection	0							Minimal
				Postinfection:								
				6 days	0							
				13 days	0							
				19 days		3w	2w	tr				
	192	4.7×10^2	m20	Preinfection	0							Extensive
				Postinfection:								
				6 days	0							
				13 days		3w	2st	1	0			
				19 days			3st	2–3	1st	0		
	193	4.7×10^2	s20	Preinfection	0							Moderate
				Postinfection:								
				6 days	0							
				13 days	4	4	3st	1w	0			
				19 days	4	4	4	4	4w	3w	tr	
	199	2.6×10^3	s20	Preinfection	0							Extensive
				Postinfection:								
				6 days	0							
				13 days	4	4	4	3st	tr			
				19 days	4	4	4	4	4	4w	3st	
	200	3.3×10^3	s20	Preinfection	0							Minimal
				Postinfection:								
				6 days	0							
				13 days	1	0						
				19 days	tr	0						
	201	3.3×10^3	s20	Preinfection	0							Extensive
				Postinfection:								
				6 days	0							
				13 days	4	4	4w	3w	1st			
				19 days	4	4w	3w	0				
	202	3.7×10^3	s20	Preinfection	0							Moderate
				Postinfection:								
				6 days	0							
				19 days	4	4	4	4	4w	tr		
	203	3.7×10^3	d13	Preinfection	0							Moderate
				Postinfection:								
				6 days	0							
				13 days	4	4	4	2	0			
2	3	1.3×10^2		Preinfection	0	0						Extensive (delayed)
				Postinfection:								
				7 days	0	0						
				16 days	3w	3w	2w	0				
				28 days	4	4	4	4	3st			
			m68	62 days	4	4	4	4	4	4	3	

[a] s20 = sacrificed on day 20; m20 = moribund on day 20; d13 = dead on day 13.
[b] CF = complement fixation.
[c] tr = trace, w = weak, st = strong.

TABLE 5. *Complement fixation reactions with sera from monkeys infected intraperitoneally with Pseudomonas pseudomallei strain 111-13 (no therapy)*

Monkey no.	Inoculation	Blood sample	CF[a] reactions[b] with serum diluted:							
			1 to 10	1 to 20	1 to 40	1 to 80	1 to 160	1 to 320	1 to 500	1 to 640
1	18 May: 1.9×10^2 organisms (ca. $\frac{1}{100}$ LD_{50})	Preinfection	0							
		Days after 1st infection:								
		11	0							
		18	3	2	tr					
		25	2	1	tr					
		77	0							
	3 Aug: 2×10^4 organisms (ca. 1 LD_{50})	Days after 2nd infection:								
		8	4	4	4	4	3			
		14	4	4	4	4	4	4	3-4	
		28	4	4	4	4	4	4	3-4	
	4 Sept: 1.5×10^5 organisms (ca. 10 LD_{50})	Days after 3rd infection:								
		7					4	4		1
		14					4	4		4w
		56					4	4		4w
		65	(Dead)							
4	6 Jan: 2×10^2 organisms (ca. $\frac{1}{100}$ LD_{50})	Preinfection	1-2	0						
		Days after infection:								
		7	2	tr	0					
		14	4	4	3st	0				
		28	4	4	4	1	0			
		63	4	4w	1w	0				
		187	4w	1-2	0					
	11 July: 1 ml vaccine ip[c]	Days after vaccine:								
		7	4	4	4w	1w	0			
		21	4	4	4	1st	0			

[a] CF = complement fixation.

[b] None of the sera was anticomplementary in the lowest dilution tested. tr = trace, w = weak, st = strong.

[c] ip = intraperitoneally.

fixation titer was not increased at 7 days postchallenge but at 14 days it had increased to 1 to 640 and persisted at this level for the remainder of the observation period. There were no clinical signs of infection within 8 weeks postchallenge. However, in the ninth week the monkey became clinically ill and died 65 days postchallenge. The pathological changes were entirely typical of those in chronic melioidosis in experimental animals. The total observation period for this monkey was somewhat more than 5 months.

Although no clear-cut conclusions can be drawn from a single monkey, it would seem that any immunity which may have developed after the first two infecting doses was inadequate to protect against a challenge of 10 LD_{50}, although the course of the challenge infection may have been modified so as to suppress acute infection and permit the development of chronic infection instead.

Monkey 4 (Table 5), which was similarly infected, showed no increase in complement fixation titer within 7 days postinfection but at 14 days the titer was 1 to 40. At 187 days the titer had dropped to 1 to 10 where it remained for about 3 more months. The appearance of complement fixation antibodies and subsequent drop in titer in this monkey were also interpreted as evidence of subclinical infection since no signs of clinical disease developed at any time during the observation period. About 6 months after infection, approximately 6×10^8 organisms in the form of a killed bivalent melioidosis vaccine were inoculated intraperitoneally. Within 7 days the

titer rose from 1 to 10 to 1 to 40 where it remained until termination of the experiment. This prompt increase in titer was also interpreted as an anamnestic response, the significance of which is discussed below.

Four control monkeys showed no significant increase in complement fixation antibodies 7 days after intraperitoneal inoculation of 1 ml of the same vaccine.

DISCUSSION

The complement fixation test described is not only sensitive but also specific since the reactions were negative with 1 to 10 dilutions of all the normal sera tested to date, including 47 human, 27 sheep, 52 rabbit, and 16 monkey sera. Weak reactions were encountered in lower dilutions of a few of these sera.

The test appears to have diagnostic value since more than 70% of the infected rabbits in this study developed positive complement fixation reactions within 7 or 8 days postinfection and 100% were positive at 9 to 11 days. This latter interval would probably coincide with the incubation period in human melioidosis. If so, the complement fixation test should be invaluable in making an early diagnosis so that effective chemotherapy may be instituted early in the disease.

The development of a positive complement fixation reaction in monkeys many weeks, even months, before clinical signs were observed, is further evidence of the sensitivity and diagnostic value of the test (monkeys 1, 3, and 4). The complement fixation test may also be useful in determining the effectiveness of therapy since in experimentally infected rabbits, the complement fixation titer dropped as chemotherapy was intensified, and increased as chemotherapy was interrupted to permit the infection to progress. From the observations to date in this laboratory, the persistence of a high-titered complement fixation reaction would seem to indicate persistence of infection.

The complement fixation test would also appear to be useful in population surveys in endemic areas for detecting evidence of subclinical infection. If subclinical infection can occur in some species of animals, as it apparently did in monkeys and guinea pigs, it probably can also occur in man. It is, however, likely that residual complement fixation antibodies following subclinical infection in man might be too low in titer to be detectable directly. But if an anamnestic reaction could be produced in man as it was in monkey 4, by inoculating one dose of killed vaccine, this would constitute a method of obtaining evidence of subclinical or undiagnosed infection in those areas where the disease occurs naturally. This, in turn, could throw some light on the epidemiological aspects of melioidosis.

Almost nothing is known about the epidemiology of this disease, although text books state that rodents constitute the natural reservoir of infection. The organism was isolated by Stanton and Fletcher (1932) from an occasional rat with natural infection, but the systematic examination of thousands of rats in areas where human melioidosis occurred has resulted only once in the isolation of *P. pseudomallei* from this species (Denny and Nicholls, 1927; Vaucel, 1937; Harries et al., 1948). Contaminated water supplies have been suggested as possible reservoirs for the organism (Vaucel, 1937; Chambon, 1955). Such water, used for irrigation, could presumably contaminate food stuffs as well as infect agricultural workers. It is of more than passing interest that three clinical cases of melioidosis have developed in persons who had been in automobile accidents in which the victims, thrown into muddy water, had suffered lacerations contaminated with mud. All three died of melioidosis 8 to 24 days after the accidents (Ragiot and Delbove, cited by Huard and Long, 1937; Marque and Raynal, 1935; LeMoine and Nguyen-Duc-Khoi, 1937). It is known that *P. pseudomallei* can persist in water for many weeks (Stanton and Fletcher, 1932; Vaucel, 1937) and that infection can be easily accomplished experimentally by applying the organisms to skin wounds made, e.g., by scarification (Stanton and Fletcher, 1932; Vaucel, 1937). Moreover, *P. pseudomallei* was actually isolated in Indochina in 1955 from 5 of 150 samples of water and mud obtained from parks, gardens, arroyos, paddy fields, etc. (Chambon, 1955). It would also seem to be significant, from an epidemiological point of view, that no contact cases or epidemiological relationships between cases have ever been reported (Dunlop, 1952).

These and other observations suggest strongly, first, that the organism which causes melioidosis may be widely distributed in nature in the endemic areas and secondly, that mild unrecognized forms of the disease may actually be fairly

prevalent. In this respect, the situation may be analogous to that in poliomyelitis.

Melioidosis is very frequently nosocomial, arising as a complication in patients hospitalized for some time for apparently unrelated, even noninfectious or surgical illnesses (Roques and Dauphin, 1943; Alain, Saint-Etienne, and Reynes 1949). Such observations suggest that individuals may harbor the organism and that it produces overt infection only when appropriate debilitating conditions develop.

The 95% mortality rate in untreated cases of melioidosis as reported in the literature may be very unrealistic. This figure is derived from the cases that come to the attention of physicians only because they have reached the clinical stage. It would seem that there must be many more subclinical and mild infections which remain entirely unrecognized (Dunlop, 1952). If so, the true mortality figure may drop to a rather low one, depending on the incidence of subclinical and unrecognized infections.

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