Use of Stable, Sensitized Cells in an Indirect Microhemagglutination Test for Melioidosis

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Two evaluations were carried out in this study. The first was a comparison of the standard tube test with the automated microtitration test for the detection of antibodies to *Pseudomonas pseudomallei* by the indirect hemagglutination method. Data from this comparison indicated that the tests were equivalent. The second evaluation consisted of reproducibility studies on two lots of pyruvic aldehyde-stabilized sensitized erythrocytes in comparison with freshly prepared sensitized erythrocytes in the automated microtitration test. The influence of different types of the microtitration plates used was also examined. Results indicated that the use of stabilized antigens is feasible, and these antigens offer the advantage of being ready for immediate use.

Melioidosis has been increasingly recognized as an infectious disease in U.S. armed forces personnel returning from Southeast Asia (2, 5, 7). The clinical manifestations of this disease, caused by *Pseudomonas pseudomallei*, are diverse, ranging from subclinical infections to acute fulminating bacteremia (5). Although the incubation period has not been determined, McDowell and Varney (10) reported that from 5 to 17 years may elapse between initial exposure and recognition of chronic melioidosis. Other clinical investigators (1, 5, 7) suggested that the initial infections may be dormant or inapparent but, months to years later, these infections may be reactivated in fulminating disease. The diagnosis of melioidosis depends on either the isolation of the causative agent or serological demonstration of specific antibodies.

In 1970, Alexander and associates (1) confirmed the applicability of an indirect hemagglutination test (IHA) for the detection of antibodies to *P. pseudomallei* previously described by Ileri (6) and Strauss et al. (13). Since 1971, the IHA test has been successfully used by the Center for Disease Control to aid in the diagnosis of melioidosis, although the test has been infrequently requested for civilians. With the return of U.S. servicemen and the influx of Vietnamese refugees, the ability of a laboratory to perform the IHA test may be of value, since early recognition and institution of therapy is necessary for successful management of the disease (1), especially in the light of the recent confirmation of human-to-human transmission (9). A stable antigen for the indirect microhemagglutination (IMHA) test for melioidosis

would give the laboratory the capacity to perform the test readily upon request. The report presented here is comprised of two studies: (i) a comparison of the standard tube test (STT) with the automated microtitration test (AMT), with antigen attached to fresh sheep cells, and (ii) an evaluation of the use of pyruvic aldehyde (PA)-stabilized cells in the AMT.

MATERIALS AND METHODS

Preparation of erythrocytes. Citrated sheep erythrocytes (SRBC) were washed three times in 0.15 M NaCl and sensitized according to the method previously described (1); melioidosis antigen was supplied by A. D. Alexander. After being sensitized, cells were washed three times in saline and resuspended in saline to a 10% concentration, and a portion was retained for use as a fresh-cell control. These cells were packed and resuspended to a 10% concentration in isotonic phosphate-buffered saline, pH 7.2, containing 0.06% bovine serum albumin (PBS-BSA; fraction V, Armour Pharmaceutical Co., Kankakee, Ill.). The remainder of the cells were packed to be stabilized with PA (Pfaltz and Bauer, Flushing, N.Y.). For this study, fresh cells were prepared, on the average, every 3 days and stored at 4°C.

Stabilization of sensitized erythrocytes. Cells were stabilized with PA according to the method of Ling (8), except that the concentration of the PA used was 40% instead of 25%. After being stabilized, cells were washed and stored at a concentration of 10% in saline containing 0.01% thimerosal. For this study, two lots of PA-stabilized cells were prepared. The stability during storage of the PA cells was compared with fresh cells on the day after preparations, and at 1 week, 3 weeks, 3 months, 6 months, and 1 year. The PA cells were a year old when this study was initiated.

IHA and IMHA test procedures. (i) The STT was performed according to the method of Ileri (6), as modified by Strauss and associates (13). A single pipette was used to make serial dilutions. (ii) The AMT was performed as follows. A 0.5-ml drop of PBS-BSA diluent was placed in all except the first well of each row of a Cooke polyvinyl (Cooke Engineering Co., Alexandria, Va.) and/or a Linbro polystyrene (Linbro Chemical Co., New Haven, Conn.) microtitration plate. A 0.1-ml drop of preabsorbed (see below) serum at a 1:10 dilution was placed in the first well of each row. Twofold dilutions were carried out using the Cooke diluter with 0.05-ml Takatsky "tulip-type" loops (Cooke Engineering Co.). The loops were presoaked in diluent before the sera were transferred to prevent the carry-over effect (4). After dilution, 1 drop of antigen diluted to a 0.5% concentration in PBS-BSA was added to each well with the 0.025-ml dropper (Cooke Engineering Co.). Plates were shaken by hand, covered, incubated at room temperature and, after 2 h, read according to Stavitsky's pattern method (12).

Preparation of sera. The sera were first diluted 1:5 in saline and then heated at 56°C for 30 min. After the sera were inactivated, a 1:10 dilution was prepared by adding an equal volume of a 2.5% concentration of fresh SRBC to the 1:5 serum dilution. This step preabsorbed the serum with normal SRBC. Sera to be used for the five pools employed in this study were tested by the STT and mixed with other individually tested sera into pools with approximate titers of 10, 40, 80, 160, and 1,280. After the pools were formed, each was repeatedly tested in the STT to determine a precise end point. In addition to these pooled sera, 33 individual human sera were tested in the STT and AMT.

Test design. In comparing titers in the STT with those in the AMT, the five pools of sera were tested on 5 consecutive days by the two test methods. Only antigen attached to fresh cells and only the Linbro plates used for microtechnique were employed in this comparison. Each pool was tested 12 times daily for a total of 60 times by each method. All testing was performed on a blind basis. The 33 individual sera were compared in the STT and AMT in both Cooke and Linbro microtitration plates.

The two lots of PA-treated antigen were compared with the fresh-cell antigen by using the same five

pools of sera in both Cooke and Linbro microtitration plates and tested for 5 consecutive days for a total of 360 determinations.

RESULTS

Although Alexander et al. (1) previously described a manual microtitration for melioidosis antibody determinations, we thought that it was feasible to evaluate the AMT against the STT before approaching the use of a stabilized antigen. The results of these comparisons on pooled sera are given in Table 1. The calculations are based on the following titers (the true end point dilution factor [titer] of each pool, as determined by the STT, is given in parentheses): pool A, 160 (300); pool B, 10 (≤ 10); pool C, $\geq 1,280$ (1,800); pool D, 40 (75); and pool E, 80 (150). Reproducibility throughout this article is defined as the percentage of results that are within one dilution of the geometric mean titer (GMT). GMTs were based on $\log_2$ values of the titers. Results of the test of two pools, C and D, indicated a significant difference between the GMTs of the STT and the AMT; however, the actual titers were within one dilution of the true end point titer. The 60 determinations for each pool during the 5 tests days showed the AMT to be consistently one dilution higher than the STT for pool C and consistently one dilution lower than the STT for pool D. Each method was 100% reproducible within a given day.

Of the 33 individual diagnostic sera compared in the STT and the AMT, 16 titers (48.5%) agreed exactly and 30 titers (90.9%) were within one dilution. Regardless of the type of microtitration plate used, the AMT result was always the lower titer. If a titer of 40 or greater is considered as indicative of melioidosis, as suggested by Alexander et al. (1), then all sera reactive in the STT were also reactive in the AMT. However, if a titer of 80 is considered diagnostic, then two of the specimens reac-

TABLE 1. Results of melioidosis antibody determinations on pooled sera to compare AMT and STT

Pool	Test	GMT	No. of determinations at each dilution										% Reproducibility
			<10	10	20	40	80	160	320	640	1,280	2,560	
A	AMT	240						25	35				100
	STT	256						19	41				100
B	AMT	5	58	2									100
	STT	5	60										100
C	AMT	2,470									3	57	100
	STT	1,305								7	44	9	88
D	AMT	41			1	56	3						98
	STT	68				15	44	1					98
E	AMT	88				2	48	10					97
	STT	96					44	16					100

tive in the STT would be nonreactive in the AMT.

Periodic testing of the two lots of PA-stabilized antigen with control sera of low, medium, and high titers indicated no decrease in antigen reactivity during storage. PA lot 1 antigen tended to produce results one dilution higher than the PA lot 2 antigens, but titers were always within one dilution of the titer produced with the fresh antigen.

The data generated from the 5 test days and 360 determinations in the AMT were examined in a number of ways. Table 2 shows the within-day and among-day reproducibility for each pool. All titers were included in the tabulation, regardless of the antigen or microtitration plate used. The three antigens are compared over the 5-day test period in Table 3, again without regard to the microtitration plate used. The greatest variations in titer were shown with the PA lot 1 antigen with pool A. Daily GMTs ranged from 281 to 538 for this lot of antigen with pool A. Both the PA lot 2 antigen and the fresh antigen produced inconsistent results with pool D. Daily GMTs with pool D ranged from 31 to 40 with PA lot 2 antigen and from 44 to 55 with fresh antigen. However, the fresh antigen showed the most reproducible results, with an overall reproducibility of 97.8%. Table 4 shows the comparison of the two types of microtitration plates. Antigen type was discounted for this tabulation. Reproducibility of titer was consistently better in the Linbro plates.

The GMTs were in close agreement with those achieved in the comparison of the AMT with the STT, as shown in Table I. A summary of all the AMT comparisons by antigen and plate type for the most variable pools, A, D, and

E, is presented in Table 5. For pools B and C, no matter which antigen or plate combination was used, the GMTs were constant, and the within-day and among-day reproducibilities were 100%. Considering all variables, the best agree-

TABLE 2. Observed within-day and among-day reproducibility of AMT for five serum pools

Pool	Day	GMT	Avg GMT	Within-day % reproducibility	Among-day % reproducibility
A	1	290.0		76.3	
	2	229.1		94.4	
	3	221.3	227.9	98.6	90.8
	4	203.7		93.1	
	5	195.4		91.7	
B	1	5.1		100.0	
	2	5.0		100.0	
	3	5.1	5.1	100.0	100.0
	4	5.1		100.0	
	5	5.0		100.0	
C	1	1278.0		100.0	
	2	1278.0		100.0	
	3	1278.0	1272.8	100.0	100.0
	4	1278.0		100.0	
	5	1252.0		100.0	
D	1	44.9		95.8	
	2	43.7		100.0	
	3	43.1	44.2	93.1	96.4
	4	48.5		98.6	
	5	40.9		94.4	
E	1	88.0		76.4	
	2	70.0		97.2	
	3	87.4	87.5	76.4	88.1
	4	94.3		97.2	
	5	97.7		97.2	

TABLE 3. Results of melioidosis antibody determinations on pooled sera for comparison of three antigens

Pool	Antigen	GMT	No. of determinations at each dilution								% Reproducibility	
			<10	10	20	40	80	160	320	640		1,280
A	PA 1	306.6					2	30	67	15	6	80.8
	PA 2	177.3	1				6	84	29			94.2
	Fresh	232.3					3	49	68			97.5
B	PA 1	5.1	115	5								100.0
	PA 2	5.0	120									100.0
	Fresh	5.1	117	3								100.0
C	PA 1	1,278.0									120	100.0
	PA 2	1,261.0							2		118	100.0
	Fresh	1,278.0									120	100.0
D	PA 1	52.4		1		73	43	3				96.7
	PA 2	35.5	1	3	13	102	1					95.8
	Fresh	46.9				93	27					100.0
E	PA 1	111.4				4	59	53	3	1		93.3
	PA 2	68.1			1	37	72	9				90.8
	Fresh	86.2				10	87	23				91.7

TABLE 4. Results of melioidosis antibody determinations on pooled sera for comparison of microtitration plates

Pool	Plate manufacturer	GMT	No. of determinations at each dilution								% Reproducibility	
			<10	10	20	40	80	160	320	640		1,280
A	Cooke	219.8					8	94	68	7	3	90.0
	Linbro	247.3	1				3	69	96	8	3	91.7
B	Cooke	5.1	174	6								100.0
	Linbro	5.0	178	2								100.0
C	Cooke	1,269.0								2	178	100.0
	Linbro	1,278.0									180	100.0
D	Cooke	41.1	1	4	13	131	31					90.0
	Linbro	47.5				137	40	3				98.3
E	Cooke	84.5			1	34	98	44	3			78.9
	Linbro	89.3				17	120	41	1	1		89.4

TABLE 5. Summary of all AMT comparisons

Pool	Plate manufacturer	Antigen	No. of determinations at each dilution									GMT (range)	Within-day % reproducibility	Among-day % reproducibility
			<10	10	20	40	80	160	320	640	1,280			
A	Linbro	PA 1						14	35	8	3	320 (200-639)	75-100	81
	Cooke	PA 1					2	16	32	7	3	294 (212-452)	75-100	80
	Linbro	PA 2	1				2	42	15			175 (151-226)	58-100	95
	Cooke	PA 2					4	41	15			181 (142-214)	92-100	93
	Linbro	Fresh					1	13	46			269 (226-302)	92-100	98
D	Cooke	Fresh					2	37	21			200 (180-214)	92-100	97
	Linbro	PA 1				35	22	3				55 (45-76)	83-100	95
	Cooke	PA 1	1			38	21					50 (45-53)	92-100	98
	Linbro	PA 2				60						40 (40)	100	100
	Cooke	PA 2	1	3	13	42	1					31 (24-40)	67-100	92
E	Linbro	Fresh				42	18					49 (45-56)	100	100
	Cooke	Fresh				57	9					44 (40-53)	100	100
	Linbro	PA 1				2	30	26	1	1		111 (83-151)	83-100	93
	Cooke	PA 1				2	29	27	2			111 (71-142)	92-100	93
	Linbro	PA 2				14	45	1				69 (60-80)	100	98
	Cooke	PA 2			1	23	27	8	1			66 (45-113)	83-100	93
	Linbro	Fresh				1	45	14				93 (76-107)	100	98
	Cooke	Fresh			9	42	9					80 (64-95)	75-100	100

ment occurred between the results produced by the PA lot 1 antigen in the Cooke microtitration plates and the results produced by the fresh antigen in the Linbro plates. These results were also in the closest agreement with the GMTs and the actual end point titers established for the pools in the STT.

DISCUSSION

Our comparison of the IHA for the detection of antibodies to *P. pseudomallei* in the STT with the IMHA in the AMT with fresh antigen confirmed the findings of Alexander et al. (1) and established that the IMHA method could be expanded to include the AMT method.

The consistently higher titers observed with the AMT method with pool C (our high pool) may reflect the phenomenon noted by Hirata and associates (4), in that the titer varied with

the initial serum concentration in the microtitration technique. That is, the lower the initial serum dilution, the higher the titer. Hirata and his associates (4) attributed these exaggerated titers to an apparent excess carry-over of antibody by the diluting loops. The increased titers we noted were less dramatic than those observed by Hirata et al. (4), but did account for a significant statistical difference in the GMTs of the two methods. We did, however, presoak the diluting loops with the PBS-BSA diluent, which may have minimized the carry-over. Since the status of the patient's disease cannot be related to the degree of antibody titer (1), a variation in a titer as high as 1,280 would appear to be unimportant.

Perhaps the lower AMT titers for pool D (our low pool) and the individual sera of the patients were due to a decreased ratio of antibody to serum, and we were seeing the phenomenon

reported by Hirata et al. (4) in reverse. Alexander et al. (1) presented convincing data to support the use of the 40 titer as indicative of infection, as did Clayton et al. (2). Kishimoto and his associates (7), on the other hand, selected an IHA titer of 80 as diagnostic. Our AMT results indicate that there would be fewer false negatives if a titer of 40 was considered the diagnostic titer. Only with additional testing of sera from documented cases of melioidosis with the stabilized antigen can a significant titer be established. Our preliminary findings indicate that the 40 titer appears to be the correct level with the stabilized antigens.

Although no change in the reactivity of the PA-stabilized antigen was noted during storage, the reactivity of the two lots of antigen differed. Whether this difference was due to the lot of PA reagent used to stabilize the antigens or to inherent differences in the initial sensitization of the sheep cells is unknown. Hirata and Brandriss (3) also noted a batch-to-batch variation in aldehyde-fixed cells. The lot variation that we observed was most obvious in the Cooke polyvinyl microtitration plates and would have been undetected if only the Linbro plates had been used.

In general, the AMT with either PA-fixed antigen or fresh antigen used would provide a laboratory with special testing capabilities for the study of patients with fevers of unknown origin. The sensitizing antigen (11) is stable for a number of years (1) and can be prepared without special equipment or unusual difficulty. The PA-stabilized antigen offers the advantage of eliminating the need to sensitize cells whenever the test is requested.

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