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Microagglutination Test for Early and Specific Serodiagnosis of Tularemia

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A microagglutination test with safranin-stained *Francisella tularensis* antigen was compared with a conventional tube agglutination test for the serodiagnosis of tularemia. The microagglutination test was performed in round-bottom microtiter plates by using 0.025 ml of the antisera and of the antigen. The antibody titers obtained by using the microagglutination test were 8 to 64 times higher than those seen with the tube agglutination. By the microagglutination test, the serum agglutinins were detected 3 days earlier in rabbits and 9 days earlier in humans than by the tube agglutination test. The microagglutination test also detected residual circulating antibodies in humans more than 20 years after recovery from infection. These early agglutinins were shown to be in the immunoglobulin M class because of their sensitivities to 2-mercaptoethanol. No significant group agglutination reaction with *Brucella abortus* was observed. These observations indicate that the microagglutination test is a useful tool for the early and specific serodiagnosis of tularemia.

Since the agglutination reaction of *Francisella tularensis* was initially reported by Francis and Evans (2), it has been a useful tool for the diagnosis of tularemia. Although a conventional tube agglutination (TA) test has been preferentially used to detect and measure serum agglutinins of tularemia patients, certain disadvantages have been reported (1, 4). On the other hand, Gaultney et al. (3) reported some advantages of a microagglutination (MA) test in which safranin-stained *F. tularensis* was used as an antigen (i.e., less antigen was required and higher titers were obtained). In addition, Brown et al. (1) and Massey and Mangiafico (4) proposed the use of the MA test instead of the TA test for serodiagnosis of tularemia. They emphasized several advantages, such as greater ease, rapidity, economy of reagents, and easier interpretation of the results. These authors, however, did not refer to the increased sensitivity of the MA test because they adjusted the concentration of antigen in order to obtain the same titer as the TA test. By adjusting the antigen to an appropriate concentration, the present investigation has raised the sensitivity of the MA test, resulting in the possibility that the MA test could be a useful tool for an early and specific serodiagnosis of tularemia.

Since the concentration of antigen is critical to get accurate and reproducible results in the MA test (1, 4), a box titration was undertaken to determine the minimum concentration of the antigen at first. The Ootake strain of *F. tularensis* (a virulent strain isolated from a tick, *Haemaphysalis flava*) was harvested after cultivation on pig liver hemoglobin agar (5) at 37°C for 24 h. A stock antigen solution containing 3×10^{10} CFU of the whole cell per ml was made in 0.85% saline–0.5% Formalin. The safranin-stained antigen for the MA test was prepared from the above stock antigen as follows. After centrifugation at 2,000 × *g* for 10 min, the pellet was suspended in the original volume with 0.85% saline–0.005% safranin–0.5% Formalin. These antigens were stored at 4°C until used. Formalin added as a preservative did not change the antigen titer as reported previously (1).

Portions (0.025 ml) of serial twofold dilutions of the antisera (human sera with a TA titer of 1:160 for *F. tularensis*) were made in 96-well round-bottom microtiter plates (FUJIREBIO Inc., Tokyo, Japan) with a microdiluter, starting at 1:5 and ending at 1:5,120; the last row contained only the 0.85% saline used as the diluent for the serum-free control. To each well, an equal volume of an appropriate dilution of the safranin-stained antigen was added. As a result, the final concentrations of the serum samples ranged from 1:10 to 1:10,240. The plates were gently agitated for 20 s and incubated overnight at 37°C after being sealed with a plastic cover. The results were read visually according to the pattern described by Gaultney et al. (3). The negative reactions are easily seen as a well-defined buttonlike pattern of the stained cells in the wells in which higher concentrations of the antigen and higher dilutions of the antisera were used. The optimal concentration of antigen was determined to be 1:32 (the final concentration is 1:64 in the plate), because beyond that point the clear negative pattern described above was difficult to detect. When lower antigen concentrations were used, higher antibody titers were obtained. For example, the undiluted antigen yielded an antibody titer of 1:80, whereas the antigen diluted 1:32 had a titer of 1:2,560 for the same sample of antiserum (Fig. 1). For the TA test, serum samples (initially diluted to 1:10) were serially diluted with 0.85% saline in test tubes (12 by 105 mm). A sample (0.03 ml) of the stock antigen was added to 0.5 ml of the diluted serum samples and mixed. The results were read after an overnight incubation at 37°C. The agglutinin titers of sera of 10 tularemia patients previously determined by the conventional TA test were compared with the titers made by using the MA test. The MA test showed 8 to 64 times higher titers than the TA test for all serum samples (Table 1). Three independent, blind titration experiments clearly showed that the antibody titers of each individual were reproducible among the independent tests and that the variation in the antibody titers between MA and TA tests was due not to technical difficulties but to the differences in the serum lots used (data not shown). The sera from five patients who had recovered from natural infection with *F.*

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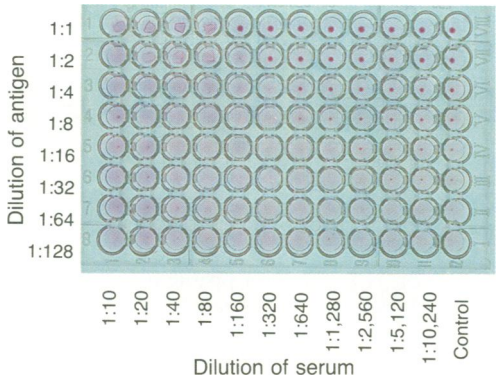

FIG. 1. Box titration of a serum specimen from a patient with tularemia by the MA test. Saline (0.85%) instead of the serum was added in the control row in which the wells with higher concentrations of the antigen than 1:32 show a buttonlike negative pattern. In the wells with lower concentrations of the antigen, on the contrary, the buttonlike pattern is not visible. Prozone is seen on the left lower corner of the plate, and postzone is seen on the right upper corner.

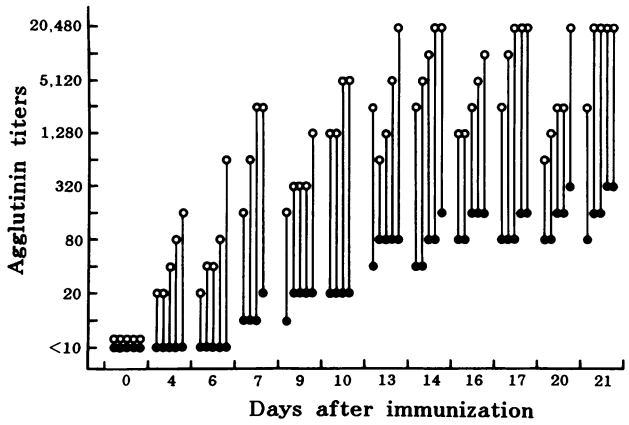

FIG. 2. Time course of the antibodies in rabbit antisera against *F. tularensis*. The serum samples were taken at indicated days after intravenous administration of the antigens from five rabbits, and each specimen was measured for antibody by TA (●) and MA (○) tests.

tularensis 24 to 31 years earlier were examined both by MA and by TA tests. The MA test detected the antibodies (1:40 to 1:80) in all serum samples. In contrast, a low titer (1:10) was detected in only one serum sample by the TA test (data not shown). The studies described above indicate that the MA test is definitely more sensitive than the TA test.

The early detection of agglutinin was evaluated next. At first, the development of agglutinins in rabbit sera immunized intravenously with *F. tularensis* (6) was examined by both TA and MA tests (Fig. 2). Agglutinins at titers of 1:20 to 1:160 were readily detectable as early as day 4 in all serum samples by the MA test. On the other hand, agglutinins at titers of 1:10 to 1:20 were first seen on day 7 by the TA test. In addition, a total of 71 serum specimens obtained from 40 patients with tularemia in Ohara General Hospital were examined for time course of the agglutinins both by TA and MA tests (Fig. 3). The MA test detected agglutinins as early as day 4 in 1 of 4 serum samples, on day 5 in 3 of 6 serum samples, and after day 7 in all the serum samples collected. On the other hand, the TA test could not detect the agglutinins before day 10. There were three exceptional serum samples in which the agglutinin was detected on days 6, 7, and 10. It was as late as day 16 that the TA test detected agglutinins in all serum samples.

The time course of the immunoglobulin classes of the agglutinin was also determined by the MA test. A total of 29 serum specimens were obtained from 19 tularemia patients on different days within 60 days after the onset of the illness. The samples were treated with 2-mercaptoethanol (2-ME). An equal volume of 0.2 M 2-ME was added to a well containing 0.025 ml of a 1:2.5 dilution of serum and incubated at 37°C for 2 h after being mixed; the serial twofold dilution was then added (Fig. 4). The 2-ME-sensitive agglutinins (immunoglobulin M) were detected as early as day 4, and by day 20 their titers were often into the thousands. The 2-ME-resistant agglutinins (immunoglobulin G), however, did not appear until several days later, and their titers did not exceed 100 until after day 20. The 2-ME treatment of the serum samples determined that the antibodies detected in the early stages of illness were in the immunoglobulin M class. This suggests that the early diagnosis of tularemia can readily be done by utilizing a system that is sensitive to the immunoglobulin M fraction of the antiserum. This is especially important when only a single serum specimen from the acute stage of the infection is available.

To evaluate the specificity of the MA test for tularemia, 50 serum samples (from tularemia patients) which had agglutinin titers to the homologous antigen ranging from 1:10 to ≥1:10,240 were titrated against *Brucella abortus* antigen, a

TABLE 1. Comparison of titers in the sera of tularemia patients by MA and TA

Serum sample (code no.)	Agglutinin titer		Ratio of MA to TA titers
	MA	TA	
1	320	20	16
2	320	20	16
3	640	40	16
4	640	80	8
5	1,280	80	16
6	2,560	80	32
7	5,120	80	64
8	10,240	160	64
9	10,240	320	32
10	20,480	640	32

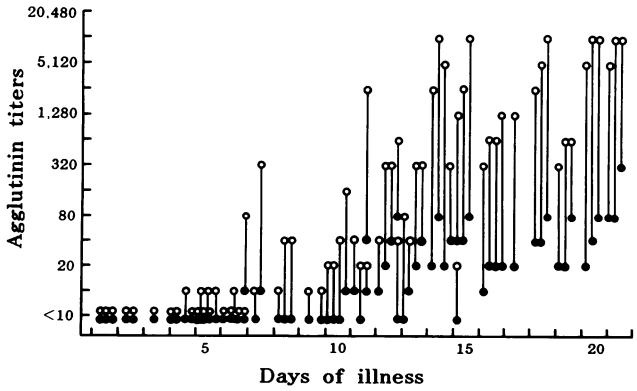

FIG. 3. Detection of agglutinins in the sera of tularemia patients. The serum samples taken on different days of the illness were examined for agglutinins by both TA (●) and MA (○) tests.

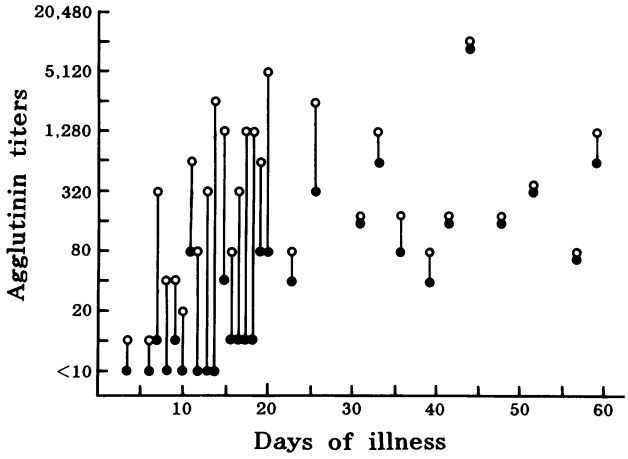

FIG. 4. Time course of the immunoglobulin classes of the agglutinins in the sera of tularemia patients. The agglutinins were determined by the MA test before (○) and after (●) 2-ME treatment.

smooth-type clone of Nagashima strain prepared in the same manner as *F. tularensis* (Table 2). The optimal concentration of *B. abortus* antigen was also determined to be 1:32 by the box titration with anti-*B. abortus* rabbit antisera (data not shown). All serum samples with homologous agglutinins at less than 1:1,280 were negative to *B. abortus*. Although 6 of 15 samples with a homologous titer of more than 1:2,560 also agglutinated *B. abortus* antigen, the titers against *B. abortus* were very low, the highest being 1:80, which was 128 times lower than the homologous titer. The specificity of the MA test was also evaluated by examining the sera of individuals with no history of tularemia. A total of 80 serum samples from people of different ages were examined by the MA test against *F. tularensis* antigen, and only 7 agglutinated at a titer as high as 1:10 (Table 3). The reaction of the tularemia serum samples with *B. abortus* antigen was negligible. This

TABLE 2. Agglutinin titers of sera from tularemia patients to *F. tularensis* and *B. abortus* measured by the MA test

Agglutinin titer to *F. tularensis* (no. of specimens tested)	No. of serum specimens at agglutinin titer to *B. abortus* of:				
	<10	10	20	40	80
10 (5)	5				
20 (4)	4				
40 (4)	4				
80 (3)	3				
160 (3)	3				
320 (6)	6				
640 (6)	6				
1,280 (4)	4				
2,560 (5)	4		1		
5,120 (3)	2	1			
≥10,240 (7)	3		2	1	1

TABLE 3. Detection of agglutinin to *F. tularensis* by the MA test in the sera of humans with no history of tularemia

Agglutinin titer	No. of serum specimens at age (yr):				
	1–19	20–39	40–59	≥60	Total (%)
<10	19	18	20	16	73 (91.25)
10	1	2	0	4	7 (8.75)

group reaction is a common problem with the TA test and has frequently led to misdiagnosis. Although low agglutinin titers were detected by the MA test in the serum samples of a few individuals with no history of tularemia, we are not certain whether these were specific reactions.

In the present study, the MA test was compared with a conventional TA test by using antisera from rabbits immunized against *F. tularensis* and sera of patients with tularemia. The use of relatively low concentrations of the antigen stained by safranin raised the sensitivity, resulting in the early detection of agglutinin. Recently, enzyme-linked immunosorbent assay for *F. tularensis* was reported to be a specific and sensitive assay (7). It is hard to compare those results with our results, since the detailed chronological data are not presented in the paper. The MA test could be as sensitive as the enzyme-linked immunosorbent assay, because 34 of 50 (68%) specimens were positive within the first 2 weeks (Fig. 3).

These observations clearly demonstrate that the MA test for tularemia is sensitive, specific, and useful for early serodiagnosis of tularemia.

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