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Quantitative Micro-Complement Fixation Test

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A quantitative micro-complement fixation test capable of detecting nanogram quantities of antigen is described. The test is simple to perform and is highly reproducible. Typical results from three antigen-antibody systems are given.

In the past decade various immunological micro-analyses of increased sensitivity have been introduced (1, 3, 11). These have aided in the elucidation of problems in microbiology, virology, biochemistry, and medicine in which only minute quantities of antigen are available. By modifying basic complement fixation techniques worked out by Mayer and his co-workers (6), Wasserman and Levine (10) developed a quantitative micro-complement fixation test (MCFT). With sufficiently potent antisera, this method is capable of detecting nanogram quantities of antigen. Moore and Perez (7), by further altering conditions, developed an MCFT able to detect picogram quantities of antigen. This sensitive test requires special equipment and skill.

We have been using an MCFT which is simple to perform, can be set up rapidly, is highly reproducible, uses only small quantities of reagents, requires no special equipment or skills, and can detect nanogram quantities of antigen. This paper describes the technique in detail and gives typical results obtained with three different antigen-antibody systems.

MATERIALS AND METHODS

Antigens. Crystalline horse liver alcohol dehydrogenase (H-L-ADH; EC 1.1.1.1) was prepared by a slight modification of the method described by Dalziel (2). The L-asparaginase (Asp;¡ EC 2.5.1.1) was kindly supplied by A. Philips. Ferritin (Fe; Pentex Corp., Kankakee, Ill.) was recrystallized three times in this laboratory.

Antisera. Detailed immunization schedules for preparation of the rabbit antisera are given in Table 1. Sera from the last bleedings from each rabbit were used in this study. All sera were heated at 56 C for 30 min.

Preparation and standardization of reagents. (i) Buffer. Five-times concentrated buffer is prepared in a 2-liter volumetric flask by dissolving 83.0 g of NaCl and 10.19 g of sodium-5,5-diethylbarbiturate in about 1,500 ml of distilled water. To this are added 34.6 ml of 1 N HCl, 5.0 ml of 1 M $MgCl_2 \cdot 6H_2O$, and 5.0 ml of 0.3 M $CaCl_2$. The volume is then taken up to the 2-liter mark with distilled water. For use in the MCFT, the concentrated buffer is diluted fivefold and sufficient gelatin is added to give 0.1% gelatin. The *p*H of the isotonic buffer should be 7.3. The buffer is stored at 2 to 5 C. (ii) Sheep erythrocytes. The sheep erythrocytes were purchased from a local supplier in sterile modified Alsever's solution and were kept at 2 to 5 C for 5 to 7 days after collection before being used. For preparation, a sample is removed by aseptic technique and is washed at least three times with a 10-fold volume of isotonic buffer. A 5% suspension of the packed cells is prepared in isotonic gelatin buffer. One milliliter of this suspension is lysed with 14 ml of 0.1% Na_2CO_3 and the optical density (OD) of the hemolysate is determined in a Beckman DU spectrophotometer with 1-cm cuvettes at 541 nm. The OD corresponding to 10^9 cells/ml in the 5% suspension has been found to be 0.700 by direct cell counting. This suspension is then adjusted to contain 10^9 cells/ml by use of the following formula: OD of lysate/0.700 = total volume to contain 10^9 cells per ml/volume of 5% suspension.

This equation can be solved to give the amount of buffer (in milliliters) to be added to any volume of 5% cell suspension of known OD to yield a suspension containing 10^9 cells/ml. (iii) Hemolytic antibody. Hemolysin was prepared by immunization of rabbits with thoroughly washed, boiled sheep erythrocyte stroma. Sera obtained were standardized as described previously (6). (iv) Sensitized erythrocytes. Erythrocytes (10^9 cells/ml) are sensitized by addition of an equal volume of standardized hemolysin. After incubation at 37 C for 10 min, they are ready for use in the MCFT. We found that sensitized erythrocytes could be stored at 2 to 5 C overnight and used the next day with no change in susceptibility to immune lysis. (v) Complement. Complement represents pooled sera from 16 to 20 adult guinea pigs. Blood was taken by cardiac puncture and allowed to clot in petri dishes for 2 hr at room temperature. The serum was removed, centrifuged for 30 min at 700 × *g* at 0 C, and then pipetted into small screw-cap vials in 0.6-ml portions. These were immediately frozen in a dry ice-alcohol bath and stored at −60 C in an electric deep freeze.

MCFT. The vital part of this method is the use

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of pipettes calibrated to deliver 0.025 ml per drop. The delivery of uniform-sized drops was assured by coating the detachable needle tips on disposable pipettes (Linbro Chemical Co., New Haven, Conn.) with a thin film of silicone grease immediately before use. Care was taken that the drops were delivered at a uniform rate, less than 90 drops per min, and with the pipette held in a vertical position. Tubes (13 by 100 mm) were coated with a 1% silicone solution (Siliclad, Clay Adams, New York, N.Y.). This was found to be essential for obtaining consistently reproducible results.

A preliminary checkerboard complement fixation analysis was done to bracket roughly the concentrations of antibody and antigen for use in the MCFT. The MCFT reaction mixtures contained 4 drops (0.10 ml) of an appropriate antiserum dilution, 5 drops (0.125 ml) of complement dilution, and 4 drops (0.10 ml) of various antigen dilutions. The complement dilution was adjusted from the results of a complement titration so that 5 drops yielded about 80% hemolysis, i.e., 1.32 50% hemolytic units. Reactions were set up in duplicate in a crushed-ice bath. Care was taken to insure that, in addition of the reagents, the drops did not hit and remain on the sides of the tubes. (This is the reason for use of these relatively wide tubes.) With each experiment, antigen and antiserum controls were included and a complement titration was performed.

After 16 to 18 hr at 2 to 5 C, 2 drops (0.05 ml) of optimally sensitized sheep erythrocytes (5×10^8 cells/ml) were added to each tube. The tubes were incubated for 60 min at 37 C with occasional thorough mixing. Then, with a Cornwall syringe, 3.0 ml of 0.15 M NaCl was added to each tube. The unlysed cells were sedimented by centrifuging at $700 \times g$ for 15 min. Oxyhemoglobin was determined in the supernatant fluids by its absorption at 414 nm in a Beckman DU spectrophotometer by using standard Pyrex cuvettes of 1-cm light path. Per cent lysis and per cent complement fixation were computed as shown in Tables 2 and 3.

TABLE 1. *Preparation of antisera*

Antigen	Amt of antigen[a] (mg)													
H-L-AHD	**1** 3 (F)	**7** 3 (F)	**12** 3 (F)	**19** 3 (F)	**27–28** (B)	**36** 3 (F)	**40** 3 (F)	**43** 3 (F)	**47–49** (B)	**132** 4 (F)	**139** 4 (F)	**143–144** (B)		
L-Asparaginase	**1** 0.75 (A)	**5** 0.75 (A)	**7** 0.75 (A)	**9** 0.75 (A)	**12** 1.5 (A)	**14** 1.5 (A)	**16** 2.25 (A)	**19** 2.25 (A)	**21** 3.0 (A)	**26–27** (B)	**185** 1.0 (F)	**194** 1.0 (F)	**201** 1.0 (F)	**208–210** (B)
Ferritin	**1** 0.75 (A)	**7** 0.75 (A)	**8** 1.5 (A)	**11** 1.5 (A)	**12** 3.0 (A)	**13** 4.5 (A)	**14** 4.5 (A)	**19** (B)						

[a] Numbers in boldface indicate day of inoculation and day of bleeding. (F) Antigen in complete Freund's adjuvant administered in several sites, intramuscularly; (A) alum-precipitated antigen (1.5 mg/ml) administered intravenously; (B) bleedings taken by cardiac puncture.

TABLE 2. *Complement titration*[a]

Tube	Amt of Complement (1/891) (drops)	Amt of complement (1/11) (drops)	Amt of buffer (drops)	Amt of EA[b] (drops)	OD_{414}			OD avg[c]	OD'[d]	Lysis[e] (%)
					A	B	C			
1	5		8	2	.128	.128	.130	.129	.126	19.8
2	6		7	2	.245	.242	.230	.239	.236	37.1
3	7		6	2	.365	.338	.360	.353	.350	55.0
4	8		5	2	.420	.415	.422	.419	.416	65.4
5	9		4	2	.481	.480	.484	.482	.479	75.2
6	10		3	2	.520	.520	.526	.522	.519	81.6
7	11		2	2	.560	.560	.562	.561	.558	87.8
8			13	2	.003	.002	.004	.003		
9		3	10	2	.638	.632	.648	.639	.639	

[a] The von Krogh plot of this data gave $1/n = 0.22$, $K = 6.9$ drops.
[b] Sensitized sheep erythrocytes (5×10^8 cells/ml) added after overnight storage at 2 to 5 C.
[c] Average of triplicate determinations.
[d] Average OD minus average OD of cell control (tube 8).
[e] OD'/OD' of complete control (tube 9) $\times$ 100.

TABLE 3. *Quantitative micro-complement fixation with H-L-ADH and rabbit no. 169-3 anti-H-L-ADH*[a]

Expt	Component				Determination					
	Dilution of H-L-ADH	Amt of H-L-ADH (drops)	Amt of buffer (drops)	Amt of H-L-ADH nitrogen (ng)	OD_{414} A	OD_{414} B	OD_{avg}[b]	OD'[c]	Lysis[d] (%)	Complement fixed[e] (%)
Tube no.										
1	1/90,000	2	2	0.216	0.455	0.454	0.455	0.448	81.9	18.1
2	1/90,000	3	1	0.326	0.360	0.350	0.355	0.348	63.6	36.4
3	1/90,000	4		0.434	0.261	0.269	0.265	0.258	47.2	52.8
4	1/30,000	2	2	0.725	0.169	0.168	0.169	0.162	29.2	70.8
5	1/30,000	3	1	1.080	0.116	0.106	0.111	0.104	19.0	81.0
6	1/30,000	4		1.452	0.146	0.150	0.148	0.141	25.8	74.2
7	1/7,500	2	2	2.891	0.475	0.480	0.478	0.471	86.0	14.0
Controls										
Ag	1/7,500	2	6	2.891	0.550	0.550	0.550	0.543		
Ab			4		0.556	0.552	0.554	0.547		
C			8		0.556	0.559	0.558	0.551		
Cell			13		0.007	0.006	0.007	0.000		
Complete[f]					0.696	0.695	0.696	0.689		

[a] Four drops of a 1/11,000 dilution of antiserum (i.e., 3.9 ng of Ab N) added to tubes 1 to 7 and to the Ab control.
[b] Average of duplicates.
[c] OD_{avg} of reactants minus OD_{avg} of cell control.
[d] OD'/OD' of antibody control × 100.
[e] One hundred minus per cent lysis.
[f] Thirteen drops of C added.

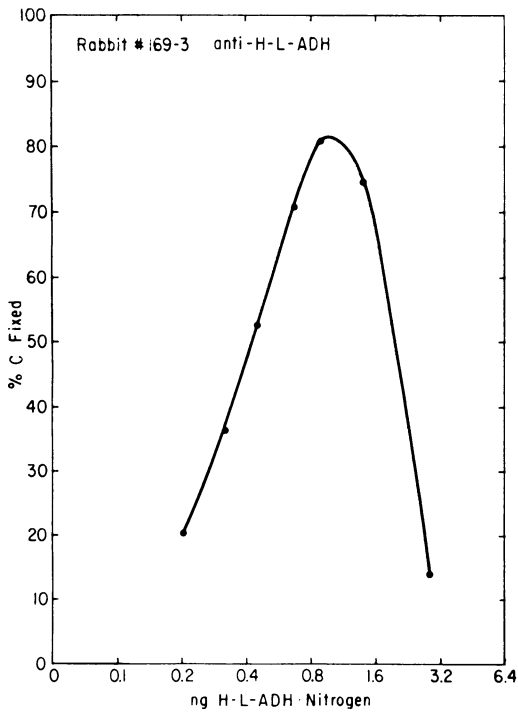

FIG. 1. *MCF curve obtained by reaction of various concentrations of H-L-ADH with a constant amount of rabbit no. 169-3 anti-H-L-ADH (1/11,000).*

RESULTS

Complement titration. To determine accurately the quantity of complement for use in the MCFT, titrations were performed under the same conditions as the actual fixation, i.e., overnight incubation of the various complement dilutions at 2 to 4 C. The experimental design and results of one such titration are given in Table 2.

MCF with H-L-ADH, Asp and Fe. Detailed results of the MCFT with H-L-ADH and anti-H-L-ADH are given (Table 3). The plot of this data is given in Fig. 1. With this antiserum, 1 ng of H-L-ADH nitrogen gave maximum per cent complement fixation.

In Fig. 2 are shown the results of MCF analyses with Asp and Fe reacting with their respective antisera. One nanogram of Asp gave maximal complement fixation. With Fe, 80 ng was necessary for maximum complement fixation.

DISCUSSION

In recent years, as a result of improved methods of isolation, small quantities of valuable biologically active molecules have become available in pure form. This has led to increased interest and activity in the identification of subunits of these molecules and in the study of the relatedness of similar proteins isolated from different sources. The MCFT developed by Wasserman

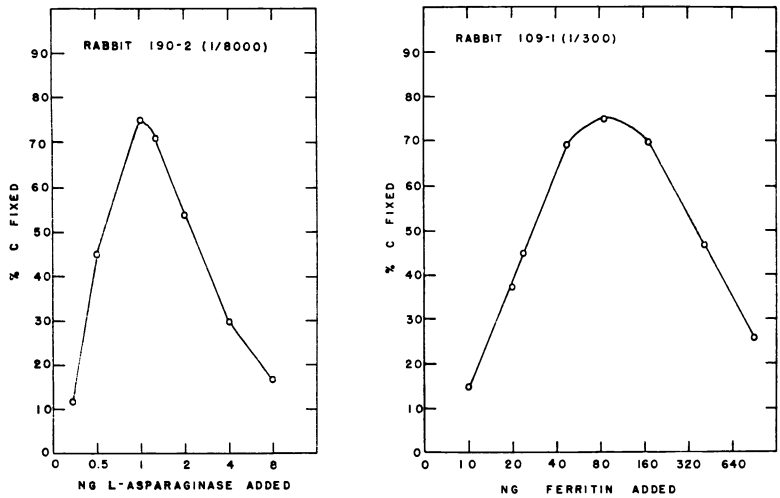

FIG. 2. *MCF curves obtained by reactions of various concentrations of L-asparaginase and ferritin with their respective rabbit antisera.*

and Levine (10) has been widely applied to such studies, e.g., the effect of urea on the antigenicity of pepsinogen (5), the antigenic analysis of variant and attenuated type I poliovirus (4), the immunologic comparison of mutant and wild-type subunits of tryptophane synthetase (8) and the immunologic comparison of different primate albumins (9).

The MCFT described in this paper is much easier to perform yet is equally sensitive and reproducible. After dilutions of the reactants are prepared, the addition of proper amounts to the respective tubes is accomplished quickly and with accuracy. Other quantitative MCF tests use volume delivery with serological pipettes or λ pipettes. This requires some skill and experience. When used by different technicians, rather large differences in volume delivered can result. The use of pipettes calibrated to deliver a specified volume per drop eliminates this possible source of error, provided the simple precautions described previously are taken. All the reagents are so highly dilute in the buffer that the surface tension is essentially that of the buffer which means that the drops of all reagents should be of uniform size.

This technique employs minute quantities of reagents, so it is most useful when only small quantities of antigen or antibody are available. For example, with 1 ml of anti-H-L-ADH it is possible to set up 110,000 MCF tests.

It should be emphasized that use of silicone-coated tubes is essential for the proper performance of this test. We adopted this step from Moore and Perez (7). Our duplicates were hopelessly erratic when tests were done with noncoated tubes. This may be due to variable adsorption of the highly dilute reagents on the glass surface of the tubes. The duplication is excellent when silicone-treated tubes are used (Table 2, 3).

Though the plots of per cent complement fixation versus antigen added resemble curves obtained with pure antigen-antibody systems, in this MCFT, as in all MCF tests, this cannot be used as a criterion of purity of the original antigen solution. The reason for this relates to the high sensitivity of these tests. In preparing a proper dilution of the major antigen, an impurity present in small amount in the original antigen solution will be so greatly reduced that its concentration will be below the threshold concentration sufficient for discernible complement fixation. The result is measurement of only the major antigen in a mixture.

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