16345

Hemolysis with Human Complement, Human Cells, and Tannic Acid: Application to Complement Fixation Test.*

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It has long been known that tannic acid has an effect on erythrocytes which is analogous to the action of amboceptor; small concentrations of this substance produce agglutination of the cells but no hemolysis, while the addition of complement results in hemolysis.[1,2] An investigation of this phenomenon was undertaken in this laboratory, as part of a study on the mechanism of action of complement. At first, considerable difficulty was encountered in demonstrating a consistent hemolytic effect with tannic acid and complement. It was found that the reaction was extremely sensitive to slight variations in the concentration of the sodium chloride solution employed as diluent for the reagents involved. When, however, the concentration of salt solution was fixed at 0.7%, rather than the usual 0.85%, it became possible to determine the optimal concentrations of tannic acid, complement and red cells for the reaction, with reproducible results. It was then learned that lysis of human erythrocytes could be brought about by fresh serum from the same or other individuals, in the presence of tannic acid. Moreover, accurate titrations of human complement could be made which yielded results comparable to those in the standard amboceptor-sheep cell system.

The present report deals with an application of the above findings to the complement fixation test, using human cells, human serum, and tannic acid as the indicator system. By this method, estimations of specific antibody for lymphocytic choriomeningitis virus, streptococcus MG polysaccharide, and Wassermann antigen were performed, with results similar to those obtained by the standard complement fixation test.

Materials and Methods. Diluent. Sodium chloride dissolved in distilled water to make a 0.7% solution was employed as diluent for all reagents used in the test, in place of the usual physiological saline. The importance of using this diluent will be demonstrated below.

Cells. Blood obtained from a normal human subject was added to oxalate crystals. The cells so obtained were washed 3 times in physiological saline solution, and then suspended in sufficient 0.7% saline to make a cell concentration of 1%.

Complement. Whole human blood was allowed to clot at room temperature and then centrifuged at 2,000 r.p.m. for 10 minutes. The serum so obtained was kept in an ice bath. Fresh lots of cells and complement were prepared each day.

Tannic Acid. A single lot of tannic acid‡ was used in all the tests to be described. A 1% solution of tannic acid in 0.7% saline was made each day as a stock solution, and 2-fold dilutions from this were tested for the optimal tannic acid concentration by the method described below. In practice, it was found that a dilution of 1:16, or a 0.06% solution, was invariably suitable for producing hemolysis with complement.

Antigen-Antibody Systems. Three systems were employed in the tests to be reported. These were: (1) *Lymphocytic choriomeningitis virus antigen and antibody.* The antigen

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1 Reiner, von L., and Fischer, O., *Z. f. Immunitätsforsch.*, 1929, **61**, 317.

2 Landsteiner, K., *The Specificity of Serological Reactions*, Harvard University Press, 1946.

‡ C. P. Crystalline Tannic Acid, lot No. 1, 1945, J. T. Baker Co., Phillipsburg, N.J.

consisted of guinea pig spleen soluble antigen, prepared by the method of Smadel, Baird and Wall.[3] The antiserum was hyperimmune guinea pig serum, prepared after the method of these authors. (2) *Streptococcus MG polysaccharide antigen and antibody.*§ The antigen consisted of a solution of purified polysaccharide from streptococcus MG.[4] diluted 1/50,000 in 0.7% saline. Rabbit antiserum against this material was used as antibody. (3) *Wassermann antigen and antibody.*‖ A standard preparation of Eagle beef-heart Wassermann antigen and several specimens of human luetic sera were used.

Each of the sera was heated for 30 minutes at 56°C before the test. Appropriate controls for the anticomplementary effect of both sera and antigens were included in each test.

Sensitization of Cells with Tannic Acid. Two parts of a 1% suspension of washed human red cells were mixed with one part of 0.06% tannic acid, in 0.7% saline. This mixture was prepared at least 15 minutes before being used, for reasons which will be described below.

Titration of Complement. Complement was titrated in the presence of the antigen used for the test. It was usually sufficient to set up 6 different amounts of complement, *e.g.* 0.07, 0.06, 0.05, 0.04, 0.03, and 0.02 cc of serum each contained in 0.2 cc of 0.7% saline. To each amount of complement were added 0.2 cc of the antigen, and 0.2 cc of 0.7% saline. These mixtures were then incubated at 37°C for 30 minutes, since this was the duration of fixation in all tests to be described. Following this period, 0.6 cc of the mixture of cells and tannic acid were added to each tube. The results were read after 30 minutes further incubation at 37°C. The endpoint, or the last tube in which complete lysis occurred, was selected as one unit. For the complement fixation test, 1.5 units were employed.

The Test. The following amounts of the various reagents were used: Complement—0.2 cc (containing 1.5 units). Antigen—0.2 cc. Serum—0.2 cc. Sensitized cells—0.6 cc, consisting of 0.4 cc of 1% cells and 0.2 cc of 0.06% tannic acid.

Antiserum, antigen, and complement were added to the tubes in the order named. The tubes were then placed in a 37°C water bath and incubated for 30 minutes. At this time, 0.6 cc of sensitized cells were added to each tube, and the tubes reincubated for 30 minutes. Readings were then made of the degree of hemolysis. Fixation of complement was interpreted as occurring in those tubes showing no lysis, or one-plus lysis on a scale of 4+.

Results. The effect of small variations in the concentration of sodium chloride on the hemolytic reaction with human cells, human complement, and tannic acid is shown in Table I. In the experiment illustrated here, titrations of a sample of human complement were made in the presence of 0.06% tannic acid and 1% red cells. In each titration a different concentration of sodium chloride was used as diluent for the reagents involved. It will be seen that the greatest complement activity was evident in the row in which 0.7% sodium chloride was used as diluent, while the reaction was almost completely inhibited in concentrations of 0.85 and 0.9% sodium chloride.

The optimal concentration of tannic acid was found to be between 0.06 and 0.03%. When stronger solutions of tannic acid were used, for example a 0.1% solution, hemolysis did not occur. Solutions of less than 0.01% were inactive.

One percent suspensions of red cells yielded more consistent and reproducible results than 2% suspensions. With higher concentrations of red cells, incomplete hemolysis usually occurred regardless of the amounts of tannic acid or complement.

It was of great importance to sensitize the red cells with tannic acid at least 5 minutes before adding them to complement. If cells

§ Streptococcus MG polysaccharide and antiserum were kindly supplied by Dr. Frank L. Horsfall, Jr.

‖ Wassermann antigen and luetic sera were kindly supplied by Dr. Thomas Farmer.

[3] Smadel, J. E., Baird, R. D., and Wall, M. J., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 71.

[4] Thomas, L., Mirick, G. S., Curnen, E. C., Ziegler, J. E., and Horsfall. F. L., Jr., *J. Clin. Inv.*, 1945, **24**, 227.

TABLE I.
Effect of Saline Concentration on Lysis of Human Red Cells by Human Complement and 0.06% Tannic Acid.

% of NaCl	Volume of Complement in 0.2 cc						Controls	
	.06	.05	.04	.03	.02	.01	.06 cc complement	.06% tannic acid
.60	++++*	++++	+++-	+++	0	0	0	0
.65	++++	++++	++++	++++	0	0	0	0
.70	++++	++++	++++	++++	+++	0	0	0
.75	++++	++++	+++	++	0	0	0	0
.80	++++	++++	++	0	0	0	0	0
.85	++	+	0	0	0	0	0	0
.90	+	+	0	0	0	0	0	0

* ++++ Complete hemolysis.
\+ Slight hemolysis.
0 No hemolysis.

TABLE II.
Complement Fixation with Lymphocytic Choriomeningitis, Streptococcus MG Polysaccharide, and Wassermann Antigens and Antibodies, Using Human Complement, Human Red Cells, and Tannic Acid.

Serum	Antigen	Initial serum dilution					Antigen Controls
		1/40	1/80	1/160	1/320	1/640	
Anti-MG	MG*	0‡	0	0	0	++	++++
	LCM†	++++	++++	++++	++++	++++	++++
	NaCl	++++	++++	++++	++++	++++	++++
Anti-LCM	MG	++++	++++	++++	++++	++++	
	LCM	0	+	++	+++	++++	
	NaCl	++++	++++	++++	++++	++++	
		1/8	1/16	1/32	1/64	1/128	
Luetic	Wassermann	0	0	0	0	+	++++
	NaCl	++++	++++	++++	++++	++++	
Normal	Wassermann	++++	++++	++++	++++	++++	
	NaCl	++++	++++	++++	++++	++++	

* Streptococcal MG polysaccharide 1/50,000.
† Lymphocytic choriomeningitis virus soluble antigen, from guinea pig spleen.
‡ Symbols as indicated in Table I.

and tannic acid were added separately to complement, little or no hemolysis occurred. If tannic acid and complement were allowed to stand together for a few minutes before the addition of cells, no hemolysis occurred. When cells and tannic acid had been together for 15 minutes, they could be used for the test for a period of at least 8 hours without deterioration.

The results of complement fixation tests with the three antigen-antibody systems studied are shown in Table II. It will be seen that the anti-streptococcus MG polysaccharide serum fixed complement in a dilution of 1:320 with the polysaccharide antigen, and showed no fixation with the unrelated lymphocytic-choriomeningitis antigen. The antiserum for the latter antigen, on the other hand, fixed complement in a dilution of 1:80 and showed no fixation with the MG antigen. Normal rabbit serum showed no fixation with either antigen. The human luetic serum fixed complement with Wassermann antigen in a dilution of 1:128, while a normal human serum showed no fixation. Consistent results in the Wassermann test were more difficult to obtain than in the other antigen-antibody reactions described, because of the frequent appearance of an anticomplementary effect in Wassermann antigen with tannic acid-sensitized cells.

Each of the above sera and antigens were tested in 30-minute complement fixation tests by the usual method, using guinea pig complement, sheep cells, and rabbit amboceptor. The serum titers which were obtained

were substantially the same as those shown in Table II.

Comment. The observation of hemolysis of human cells by complement from the same individual, and the various conditions under which this reaction occurs, are of theoretical interest and suggest possible new approaches to the problem of complement's role in hemolysis. The complement fixation test itself may prove to be of practical value under some circumstances. There are certain advantages in a serological test which involves an homologous hemolytic system, instead of the 3 or more mammalian species which are represented in the usual complement fixation test. Moreover, the method makes it possible to do complement fixation tests under conditions where one or another of the usual reagents cannot be obtained.

There are certain limitations to the use of this method which should be pointed out. In Wassermann tests, the frequent finding of an anticomplementary effect of antigen presents a technical disadvantage. Moreover, the test is not feasible with antigens for which many human sera possess antibodies in low titer, such as influenza virus in allantoic fluid, since the complement employed for the test may be partially fixed due to the presence of antibody in the same serum. For the same reason, it is probable that certain tissue antigens, with which human sera may react non-specifically in low dilution cannot be tested by this method. However, in those systems which lend themselves to the homologous test, the results appear to be clear-cut and are reproducible.

Summary. Human erythrocytes undergo lysis in the presence of tannic acid and human complement, even when cells and complement are obtained from the same blood sample. This reaction is dependent upon an optimal concentration of 0.7% sodium chloride, and an optimal concentration of between 0.06 and 0.03% tannic acid. When these substances are present in proper concentration, human complement can be titrated with reproducible results.

Specific fixation of human complement by three separate antigen-antibody systems has been demonstrated, employing homologous erythrocytes sensitized by tannic acid. Antibody titers determined by this method were comparable to those obtained in the standard complement fixation test.

16346

Effect of Chloromycetin on Experimental Infection with Psittacosis and Lymphogranuloma Venereum Viruses.

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The results of the search for chemical and antibiotic substances which might be effective in the treatment of infections of viral and rickettsial origin have been reviewed recently by several groups of workers.[1-5] The contribution of Hamre and Rake[5] is of especial interest as regards the current subject since it deals particularly with viruses of the psittacosis-lymphogranuloma group. The pres-

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[5] Hamre, D., and Rake, G., *J. Infect. Dis.*, 1947, **81**, 175.