January 1971

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W.E. Engelhard
University of Nebraska - Lincoln

B.W. McCashland
University of Nebraska - Lincoln

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W. E. Engelhard and B. W. McCashland

Department of Bacteriology, and Department of Physiology and Institute for Cellular Research, University of Nebraska, Lincoln, Nebraska

Received for publication July 14, 1958

Tetrahymena, along with other genera of the family *Frontoniidae*, have been the subject of many scientific investigations concerning problems in protozoan physiology. Unfortunately in many instances, difficulty arises when attempting to identify properly the form that was used in the particular study. Until rather recently, relatively little had been accomplished by way of a comprehensive study of the interrelated genera, and of these very few have dealt with taxonomic problems serologically.

For example, Corliss, (1953a) has compiled histories of many of the available strains. Twenty-one separate strains of *Tetrahymena pyriformis* were recognized and synonomous identities included in descriptions of each strain. In addition, three strains of *Tetrahymena vorax*, one strain of *Tetrahymena patula*, and two strains of *Glaucoma scintillans* were given proper recognition.

Individual strains, however, vary in size, morphological characteristics (Corliss, 1953b), and physiological behavior (for example, growth rate, nutritional requirements, oxidative metabolism).

Since strains were gathered from many types of environments, physiological differences of the types described above could be inherent within the strains employed. Therefore, we chose to study certain members within the group to determine if the strains may be related antigenically.

Although immunological testing has been somewhat neglected in protozoan taxonomy, antigenic responses of protozoa have been used in diagnostic procedures. Craig (1937) applied the complement fixation test in diagnosis of amoebiasis (*Entamoeba histolytica*). Dourine (*Trypanosoma equiperdum*) has also been detected using the same principle (Mahler, 1913). Demonstration of antigenic properties of free living protozoa (*Glaucoma*) was described by Robertson (1939). Tanzer (1941) prepared antisera to two species of *Colpidium* and one of *Glaucoma* (all actually *Tetrahymena* species). These cells and corresponding antisera were cross-tested. Antisera were also tested against several flagellated species. The ciliates share antigenic properties. However, they are not related to any great extent to the flagellates on the basis of antiserum reaction.

Kidder *et al.* (1945) have studied antigenic determinants of five strains of *Tetrahymena geleii* (*T. pyriformis*) and two strains of *T. vorax*. The strains of *T. geleii* were antigenically related to one another but not to strains of *T. vorax*.

The following describes our studies of the antigenic properties of several strains of *Tetrahymena* and presents a method for the preparation of antisera for investigating these antigen-antibody relationships.

Material and Methods

Media. All protozoa were grown at 24 C in a normal saline medium containing 1.0 per cent proteose peptone and 1.0 per cent glucose as nutrients.

Strains. The protozoa used in the study were obtained from the Osborn Botanical Laboratory, Yale University. Six strains of *T. pyriformis* and one of *T. vorax* were used. *T. vorax* was selected since this species should show less antigenic relation to any of the strains of *T. pyriformis* than would be shown between strains within the species. The strains selected and origin of each are as follows: *T. pyriformis*, strain CHS, isolated by E. Chatton in 1925 at Strasbourg, France; L I and L II, isolated by J. B. Loefer in 1948 in Texas; (use of these two strains will enable us to compare the relative importance of geographical origin in strain variation); E, isolated by A. M. Elliott in 1932 in New York City; HS, isolated by A. Phelps from a thermal well (39.5 C) in Texas; W, isolated by C. L. Claff in 1939 at Woods Hole, Massachusetts; *T. vorax* strain V_1, isolated by D. M. Lilly in 1940 at Providence, Rhode Island.

Preparation of antigens. Two types of antigens were used: (a) cells in the living, motile phase, and (b) merthiolate killed cells. In both instances, the cells were washed free of nutrient, then resuspended in normal saline (living) or normal saline and merthiolate (killed).

In preparation of cells to be used as "killed antigens," 1 L of culture solution was inoculated with 10 ml of a 72-hr culture and incubated for 72 hr at 24 C. This growth period provided the maximal number of living cells: cells of each strain were separately siphoned from the bottom of the container and concentrated by centrifugation. Cells were washed 3 times in saline and resuspended in a 100-ml aliquot and 4 ml of merthiolate added (1:5000 merthiolate in normal saline). These suspensions were refrigerated until

used for injection, at which time they were diluted to give a reading of 60 on a Klett-Summerson[1] colorimeter. This would furnish a mass of cellular substance, equivalent to 50,000 to 75,000 cells per ml.

Preparation of the "living antigens" was accomplished in the same manner except that cells were grown in test tubes containing 30 ml of culture medium. The cells were washed and resuspended in normal saline to give a reading of 30 on the Klett-Summerson colorimeter. In instances where larger numbers of cells were required, cells were taken from 1 L cultures.

Preparation of antisera. Pure Dutch strain male rabbits weighing approximately 1.5 to 1.7 kg were used. Control sera were obtained by intracardiac puncture. Pairs of animals were then injected intravenously (i.v.) with each of the 7 species of *Tetrahymena* into the lateral marginal ear vein. Two rabbits were used for each living and merthiolate-killed strains. The protocol for the preparation of the antiserum for both types of cells was as follows. The initial day 0.1 ml of antigen was introduced i.v.; this amount was then doubled on 4 consecutive days, that is, 0.2 ml on the 2nd day, 0.4 ml on the 3rd day, 0.8 ml on the 4th day, and 1.6 ml on the 5th day. Following a 1-week rest period, 2.0 ml were again injected i.v. A similar amount was injected after another 7-day rest period. All animals were bled by intracardiac puncture 5 days after the last injection. Several animals were also injected with 2.0 ml 3 weeks after the last injection and bled 5 days later. Titers observed on sera thus obtained were essentially similar to the other immune sera. All sera were separated after 12 hr refrigeration and stored at −10 C until used. Aseptic procedure was followed throughout. No evidence of an anaphylactic-like response occurred. The animals exhibited no loss of weight.

Antigenic Studies

Immobilization tests. Cells of a 72-hr strain culture were washed 3 times in normal saline by gentle centrifugation (600-RCF) and then resuspended in normal saline so that a final cell concentration of 30 to 40 cells per 0.01 ml was obtained. Cells treated in this manner displayed normal motility. These cell suspensions were tested against inactivated (heated at 56 C for 30 min), undiluted, and 1:10, 1:100, 1:500, 1:1000, and 1:10,000 saline dilutions of normal and immune sera.

Tests were accomplished in depression slides which were kept in Petri dishes containing a small amount of water to prevent evaporation of the serum-cell mixture. For each test conducted in triplicate, 0.01 ml of cell suspension and either 0.01 ml of normal serum, undiluted antiserum, or antiserum dilution were mixed in a depression slide. This furnished a final cell count of 30 to 40 cells in antiserum dilutions of 1:20, 1:200, 1:1000, 1:2000, and 1:20,000. The mixtures were maintained at 24 C and were observed after 10 min and again after 60 min. The number of cells in a depression could be counted as well as the number of cells that were immobilized. From these values, the average percentage of immobilization was calculated at each serum concentration. In this fashion, cells of the 7 strains were tested against homologous and heterologous antisera. The antiserum titer giving 50 per cent immobilization of cells was calculated by Behren's integration.

Agglutination tests. The tube agglutination test was employed. Duplicate, 2-fold dilutions (1:20 to 1:320) of inactivated control and immune sera in 0.5-ml amounts were prepared. To each above serum dilution was added 0.5 ml of the killed antigen suspension diluted 1:10. The mixture was incubated at −4 C overnight and prior to reading incubated at 37 C for 30 min. The titers were taken as the smallest amount of serum which effected minimal agglutination. In each case the appearance of the control was used as the standard.

Complement fixation tests. This test was essentially that described by Smadel (1948). Duplicate 2-fold dilutions (1:4 to 1:1024) of inactivated control and immune sera were prepared in 0.5-ml amounts. The optimal antigenic dose (0.5 ml of a 1:20 dilution) and 2 units of guinea pig complement contained in 0.5 ml were added to one series. The same amount of complement without antigen was added to the other series to determine whether the rabbit sera were anti-complementary. All tubes were incubated at −4 C overnight and prior to adding indicator system, reincubated at 37 C for 30 min. Two units of sensitizer (antibody prepared against sheep red blood cell (RBC)) in 0.5 ml volume and 0.5 ml of a 1.5 per cent sheep RBC suspension were then added. The tubes were again incubated at 37 C for 30 min and read.

Results and Discussion

With all test procedures used, the highest titers were obtained, without exception, with antigen and homologous antiserum. In no instance was an appreciable titer observed with normal serum.

Results of the immobilization tests are summerized in table 1. It should be added that all cells were immediately immobilized and killed in undiluted homologous or heterologous antiserum. Lysis was not observed to occur. Death resulted as the cells became immobile and eventually assumed a spherical shape. In no instance did immobilized cells regain motility.

Generally, immobilization was effected at higher titers by antiserum prepared against living antigens than against killed antigens. This is especially indicated in reactions of CHS cells against immune sera

[1] Klett Manufacturing Company, New York, New York.

and to a lesser degree with L I, L II, and HS cells and immune sera. However, cells of strains W, E, and V_1 were most strongly affected by antiserum induced by killed cells.

Comparing titers of each antigen with the seven antisera prepared with living cells, it is apparent that L II and HS are immobilized most effectively (1:2000 or above); with E, CHS, and W least; and V_1 and L I sharing an intermediate position.

Agglutination reactions (table 2) also exhibited best specificity with homologous antisera. Although titers are lower than in immobilization tests, antigenic comparisons are possible. For example, it is evident that V_1 and W share an antigenic pattern common to the other protozoa. (V_1 exhibited high titers, 1:160 or above, with all antisera except W and E, whereas W shared with all except L I, E, and V_1). The converse is true for HS and CHS (HS strain effects high titers only with the homologous (HS) and V_1 antiserum, whereas CHS, the most antigenically distinct of this series, exhibits avidity only in the presence of homologous antiserum). The L I, L II, and E antigens share for the most part the intermediate position (L I, L II, and E antigens gave low titers with HS, CHS, E, V_1—W, HS, CHS, E—and L II, HS, CHS, V_1 antisera, respectively). However, in contrast to the complement-fixing antibodies, as will be seen in table 3, none of the antigens shares determinates with all of the antisera (agglutinins). With the complement fixation test, essentially similar titers were observed when either living or killed cells were used. However, this serological procedure is the one least to be desired for detecting antigenic differences. Although highest titers were observed in the presence of antigen and homologous antiserum, cross reactions are more apparent with this test. Nevertheless, it is apparent that strains W and CHS are closely related to other members of this seven-series group (titers of 1:128 or higher with heterologous antisera), whereas HS and E are most distantly related (titers of 1:64 or less with heterologous antisera), with L I, V_1, and L II sharing an intermediate role.

By arranging the strains in the order of decreasing antigenic similarity (expressed as titer differences in the ability to combine with antisera), and by comparing the three serological tests used, some degree of identification of the antigenic relationships and dissimilarities can be realized (figure 1).

It is apparent that the complement fixation test is the least desirable for differentiating these *Tetrahymena* species (very few open spaces observed). With the exception of the E and HS antigens, the other five cross react with three or more of the antisera prepared with these seven strains. All antisera with the exception of CHS cross react with at least four of the seven antigens.

TABLE 1

*Immobilization of seven strains of Tetrahymena by homologous and heterologous antiserum**

Antigen	Antiserum						
	W	L I	L II	HS	CHS	E	V_1
W	5/4	2/3	0/3	2/3	1/2	2/3	1/2
L I	2/1	5/3	5/0	3/1	3/2	2/0	5/3
L II	4/2	6/4	6/5	4/0	3/2	4/2	5/6
HS	4/6	3/3	4/3	6/4	5/3	3/3	3/1
CHS	1/0	2/0	2/0	1/0	6/4	1/0	2/0
E	4/6	2/5	1/5	1/4	2/5	4/6	1/5
V_1	2/4	5/5	6/5	2/6	1/4	2/4	6/6

* Titer causing 50 per cent immobilization is represented by the numbers: 0 = <1:20; 1 = 1:20; 2 = 1:200; 3 = 1:1000; 4 = 1:2000; 5 = 1:20,000; 6 = >1:20,000. Numbers above the diagonal represent titer to antiserum prepared against the living antigen; below the diagonal the titer to antiserum prepared against the merthiolate-killed antigen.

TABLE 2

*Tube agglutination titers of seven Tetrahymena strains, killed cells being used as antigens**

Antigen	Antiserum						
	W	L I	L II	HS	CHS	E	V_1
W	5	2	4	4	5	2	1
L I	4	5	4	1	0	2	3
L II	3	5	5	1	2	1	4
HS	3	1	1	5	3	2	4
CHS	0	0	1	3	4	3	0
E	5	5	1	1	2	5	3
V_1	2	5	4	4	5	2	5

* Highest titers causing agglutination are indicated by the numbers: 0 = <1:20; 1 = 1:20; 2 = 1:40; 3 = 1:80; 4 = 1:160; 5 = 1:320.

TABLE 3

*Complement fixation reactions in seven strains of Tetrahymena**

Antigen	Antiserum						
	W	L I	L II	HS	CHS	E	V_1
W	6/6	4/5	4/5	5/5	5/5	5/4	5/5
L I	5/4	6/6	6/2	5/4	2/2	4/3	5/5
L II	3/4	6/5	6/5	4/4	3/2	1/1	5/4
HS	3/3	4/4	3/3	6/6	3/4	1/2	2/3
CHS	5/4	6/5	5/5	5/5	5/5	5/5	5/5
E	4/4	3/3	3/2	3/2	3/2	5/4	3/2
V_1	5/4	6/4	6/5	5/4	4/4	2/2	6/4

* Highest titer giving a positive test is indicated by the numbers: 1 = 1:16 or lower; 2 = 1:32; 3 = 1:64; 4 = 1:128; 5 = 1:256; 6 = 1:512; numbers above the diagonal represent titer to antiserum prepared against the living antigen; below the diagonal the titer to antiserum prepared against the merthiolate-killed antigen.

Of the three tests studied, the immobilization procedure appears to be the best (more open spaces). The CHS and W strains are nicely separated, that is, they are not immobilized with antisera from heterologous types. The E species shares W antigens, however, this antigen can be absorbed out with small reduction of E immobilization titer. The three species could therefore be easily identified utilizing known antisera.

On the other hand, L I and V_1 share antigens with L II, V_1, and L I, L II, respectively. We can remove the V_1 antigen without reduction of titer; however, removal of L II antigen from L I antisera leads to a marked drop in titer. In addition, V_1 antisera absorbed with L I and L II antigens only slightly reduces the immobilization titer. With the exception of L I, therefore, absorbed sera could be used to identify the V_1 species. Finally, the HS and L II strains are the two most difficult to identify. Absorption of HS antisera to remove the common antigens W, L II, and CHS, and L II antisera to remove W, L I, HS, E, and V_1 antigens results in almost a complete depletion of immobilization effect with the homologous absorbed sera. However, to identify these two species (HS and L I) we could resort to the agglutination test since in the case of HS antisera we would have only to absorb with V_1 antigen and L II antisera with L I and V_1, even though L I antigen would remove considerable L II antibody. With the immobilization test, the W and L II antigens are those which most frequently cross react.

Utilizing the agglutination procedure CHS, HS, and E and L II show a minimum of cross reaction with the seven antisera, with CHS the only species antigenically distinct, for example V_1 sharing components with HS; L I and W with E; and V_1, L I with L II. With the exception of the latter we can absorb out the heterologous antibodies without appreciable loss of titer. It should also be pointed out that antisera prepared against L I, W, and V_1 share antibodies for three or more of the seven antigens, and that when the agglutination test is used L I, L II, and V_1 are the antigens which most frequently cross react.

Thus, it is seen that the protozoa lend themselves to antigenic studies. Such identification, however, depends upon the serological procedure used. The immobilization test provides the best procedure for these seven strains and when used with the agglutination test can, after a minimum of absorptions, distinguish seven different strains of closely related protozoa. The complement fixation test, on the other hand, in our laboratory was found to be the least desirable.

SUMMARY

Rabbit antisera were prepared against living and merthiolate-killed cells of *Tetrahymena pyriformis* strains W, L I, L II, HS, CHS, and E and *Tetrahymena*

A. Immobilization

L II shares with	W	L I	(L II)	HS		E	V_1
HS	W		L II	(HS)	CHS		
V_1		L I	L II				(V_1)
L I		(L I)	L II				V_1
E	W					(E)	
CHS					(CHS)		
W	(W)						

B. Agglutination

V_1 shares with		L I	L II	HS	CHS		(V_1)
W	(W)		L II	HS	CHS		
L I	W	(L I)	L II				V_1
L II		L I	(L II)				V_1
E	W	L I				(E)	
HS				(HS)			V_1
CHS					(CHS)		

C. Complement Fixation

W shares with	(W)	L I	L II	HS	CHS	E	V_1
CHS	W	L I	L II	HS	(CHS)	E	V_1
L I	W	(L I)	L II	HS		E	V_1
V_1	W	L I	L II	HS	CHS		(V_1)
L II		L I	(L II)	HS			V_1
HS		L I		(HS)		E	
E	W					(E)	

Figure 1. Antigenic comparisons of seven *Tetrahymena* strains arranged in order of decreasing antigenic avidity, tested with the immobilization, agglutination, and complement fixation procedures.

vorax strain V_1. Serological relationships were determined by three procedures: immobilization, agglutination, and complement fixation. Antigenic relationships in general were dependent upon the method employed. The immobilization test provided the best method for uncovering these differences. The complement fixation test did not provide satisfactory identification of the different species.

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