THE APPLICATION OF A HAEMAGGLUTINATION TECHNIQUE TO THE STUDY OF *TRICHOMONAS VAGINALIS* INFECTIONS

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Although there is an extensive literature on the subject of trichomoniasis, reports on the serological investigation of *Trichomonas vaginalis* infections are scanty and the results inconclusive, the emphasis in serological work having been on bovine infections with *Trichomonas foetus* rather than on human infections with *T. vaginalis*.

Riedmüller in 1932 (quoted by Trussell, 1947) tested the sera of a number of women for complement-fixing antibody using *T. foetus* as antigen. His results were uniformly negative irrespective of the presence or absence of trichomonas infection. Wendlberger (1936) carried out complement fixation tests with an alcoholic extract of a culture of *T. vaginalis* as antigen. The culture employed for the production of this antigen contained bacterial contaminants. Positive results were observed in 22 out of 32 sera taken from known cases of trichomoniasis, while 16 sera from apparently healthy women were negative. A series of 400 sera was examined by Trussell, Wilson, Longwell, and Laughlin (1942) by a complement fixation technique with a washed suspension of *T. vaginalis* as antigen. The test was positive with 52 out of 110 sera from known cases of trichomoniasis (47%). Of 290 " normal " female sera tested, 48 were positive (16%).

The results recorded in the latter two papers provide the only evidence for the occurrence of serum antibodies in human trichomoniasis at present known to the author.

A micro-agglutination technique was introduced in 1941 by Robertson working with *T. foetus*. This test was shown to give valuable information in the investigation of *T. foetus* infections of cattle (Kerr and Robertson, 1941; Pierce, 1947). Trussell (1946) applied a similar technique to human infections with *T. vaginalis*. Having established that the method would give successful results in experimentally immunized rabbits, 200 human sera were examined, but over 90% of these were recorded as negative and the remainder gave only low titre positive reactions.

Both complement fixation and micro-agglutination methods for the detection of antibody to *T. vaginalis* were tested in this laboratory, but they gave only weak reactions with human serum.

A new approach to the problem was suggested by the observations of Muniz (1950), who used a haemagglutination technique similar to that employed by Middlebrook and Dubos (1948) in their studies on tubercle bacilli. He applied it to investigations on trypanosomiasis, sensitizing erythrocytes with a polysaccharide fraction of *Schizotrypanum cruzi*, and observing lysis rather than agglutination as evidence of positive reactions. An attempt was therefore made to apply the technique of agglutination of sensitized red cells for the detection of circulating antibodies in human trichomoniasis. The results are recorded below.

Methods

Cultivation of *T. vaginalis*.—The isolation of *T. vaginalis* and its cultivation in laboratory media without bacterial contaminants was formerly a difficult problem. Once antibiotics became available it was soon discovered that the addition of these substances to the medium allowed the protozoa to grow while suppressing the bacterial contaminants. Unfortunately the only media which would give an adequate growth of protozoa contained agar or some other solid constituent which made difficult the harvesting of the organisms after they had grown. This was overcome and satisfactory growth obtained in the absence of the solid component by incubating the cultures anaerobically.

The medium which was found to be most suitable for routine use was a modification of the cysteine-peptone-liver-maltose (C.P.L.M.) media described by Johnson and Trussell (1943), the agar being omitted and " difco " bacto-tryptose substituted for bacto-peptone; the methylene blue indicator was also omitted. Thus the final composition of the medium was as follows:

Bacto-tryptose	..	..	..	3·2 g.
Cysteine hydrochloride	..	..	0·24 g.
Maltose	..	..	..	..	0·16 g.
Ringer solution	..	..	..	96·0 ml.
Difco liver infusion	..	..	32·0 ml.

The Ringer solution for the C.P.L.M. medium was composed as follows:

NaCl	..	..	..	0·6%
NaHCO₃	..	..	..	0·01%
KCl	..	..	..	0·01%
CaCl₂	..	..	..	0·01%

No *p*H adjustment was necessary. As heat-sterilized batches were less successful than those which had been Seitz-filtered, the latter method of sterilization was adopted throughout the investigation. The original C.P.L.M. medium contained 10% of human serum, but as it was hoped to immunize rabbits with the cultures the same percentage of rabbit serum was substituted for human serum. The serum was inactivated at 56° C. for 30 minutes and added immediately before the medium was to be inoculated.

This medium does not keep well, and must be used within about a week of preparation.

Isolation of Protozoa from Vaginal Swabs.—Strains of *T. vaginalis* employed in this investigation were isolated from vaginal swabs taken from patients reporting to a local gynaecological out-patient department. Specimens were only taken for culture when direct examination of the vaginal discharge showed numerous active flagellates. Antibiotics were added to the freshly prepared medium to give the following final concentrations:

Penicillin and streptomycin	..	1,000 u./ml.
Chloromycetin	..	..	500 „

The vaginal swabs taken from patients were placed in a tube containing about 1 ml. of the above medium. They were then sent directly to the laboratory, or, if this was not convenient, could be incubated at 37° C. overnight without ill effect. On arrival in the laboratory the culture medium was examined for active trichomonads and subcultured to 10 ml. volumes of the same medium; these tubes of media were then incubated anaerobically. Cultures were examined for active flagellates every two to three days and subcultured to fresh medium. After a few passages it was generally possible to obtain a bacteria-free culture of the protozoa. The absence of bacterial contaminants was as a rule evident from the macroscopic appearance of the culture, the protozoa after three days' incubation giving a fine granularity to the medium quite unlike the turbidity produced when bacteria were present.

When a new strain was isolated in " pure " culture, subcultures were incubated aerobically and anaerobically to establish that it was in fact free from bacterial contaminants. It was found that if antibiotics were now omitted from the culture medium, poor growth was obtained, and this seemed to be mainly the result of the absence of streptomycin; consequently, antibiotics were incorporated in the medium throughout the investigation.

Preparation of Immune Rabbit Serum.—The trichomonads from 100 ml. of a four-day culture in modified C.P.L.M. medium were deposited by centrifugation and washed three times in saline before being re-suspended in 10 ml. of saline. The average number of organisms now present was estimated by counting in a haemocytometer and found to be approximately 10^7 per ml.

This suspension was used to immunize a rabbit which had previously been bled to obtain a control sample of serum. Two injections were given intravenously each week for three weeks, beginning with 0.5 ml. in the first week, 1.0 ml. in the second week, and 1.5 ml. in the third week. Four days after the final injection the rabbit was bled and the serum separated.

As a preliminary test both pre- and post-immunization sera were tested for complement-fixing antibodies using as antigen the suspension of trichomonads with which the rabbit had been immunized. This test demonstrated the presence of some antibody in the immune serum, though the titre (1/10) was very much lower than that obtained by Trussell in 1946.

Preparation of Antigen and Sensitization of Erythrocytes.—In order to obtain an extract of the trichomonads suitable for sensitizing red cells the standard laboratory methods employed in the grouping of streptococci were tried.

The deposited protozoa from 30 ml. volumes of a three-day culture were washed three times in saline and treated according to the methods described by Lancefield (1933), Fuller (1938), and Maxted (1948). The resulting extracts in a volume of 1.0 ml. were then tested undiluted for their ability to sensitize sheep erythrocytes as demonstrated by the subsequent agglutination of the treated cells by immune rabbit serum.

The attempt to destroy the enzyme in the antigen extract prepared by Maxted's method was unsuccessful, as the erythrocytes were lysed. Serial dilutions of this antigen were tested, and although lysis was eliminated by the dilution of the extract beyond 1/20 no agglutination of treated red cells could be observed at any dilution. The extract prepared by the formamide method (Fuller, 1938) gave the most promising results, agglutination of treated red cells being observed up to a dilution of 1/100 of the immune rabbit serum. There was no agglutination of sensitized cells with the control sample of serum from the same rabbit, or of untreated erythrocytes with the immune rabbit serum.

Having established that the antigen prepared by formamide extraction of the trichomonads would sensitize red cells, titrations were made to determine the highest dilution of the antigen which would produce sensitization.

In the preliminary test against immune rabbit serum it was found that 1/50 was the highest dilution of antigen capable of producing sensitization when mixed with an equal volume of a 2% suspension of washed sheep cells. In all subsequent experiments the sensitizing capacity of each antigen was titrated against the immune rabbit serum before being used to sensitize cells for the testing of other sera. Equal parts of antigen dilutions were mixed with 2% sheep red cell suspension and held for one hour at 37° C.; the mixtures were then tested with the immune rabbit serum. The highest antigen dilution, which still produced maximum agglutination, as determined by the titration of the immune rabbit serum, was taken as the optimum and used to sensitize cells in subsequent tests. A typical titration of antigen in this way is shown in Table I.

TABLE 1

PRELIMINARY TITRATION OF ANTIGEN AGAINST IMMUNE RABBIT SERUM

Antigen Dilution	Immune Rabbit Serum Dilutions						Con-trols
	1/5	1/10	1/25	1/50	1/100	1/250	
1/5	+ + + +	+ + + +	+ + + +	+ + + +	+ +	−	−
1/10	+ + + +	+ + + +	+ + + +	+ + + +	+ +	−	−
1/25	+ + + +	+ + + +	+ + + +	+ + + +	∓	−	−
1/50	+ + + +	+ + + +	+ + +	±	−	−	−
1/100	+ + + +	+ + +	+	−	−	−	−
Normal cells	−	−	−	−	−	−	−

+ + + + = maximum agglutination; − = no agglutination.

When human sera were tested against unsensitized sheep cells it was found that the majority showed the presence of agglutinins to low titre. In order to avoid having to absorb the sera to remove these heterophile agglutinins before testing with sensitized cells, human group O cells were tested in the hope that they could be used in place of sheep cells. This was unsuccessful. Similar attempts to sensitize rabbit erythrocytes or suspensions of bacterial cells were equally unsuccessful. It was decided, therefore, to continue using sheep cells and to absorb all sera before test with cells from the same sheep. The method of absorption is described later.

Six absorbed human sera from known cases of trichomcniasis and an equal number of control normal sera were tested for their ability to agglutinate sensitized sheep cells. Three of the sera from cases gave a positive result to a dilution of 1/100; the control sera were negative. The test was repeated with the same sera, but this time guinea-pig complement was added before placing the test in the water bath. It seemed possible that the results would be more easily read and perhaps the test made more sensitive if lysis rather than agglutination was taken as the evidence of the antibody–antigen reaction. Lysis occurred in the presence of complement to the same dilution as agglutination had occurred in its absence. As there was no striking increase in sensitivity it was decided to continue the investigation, taking the occurrence of agglutination as evidence of the presence of serum antibody. Once the serum had been absorbed it was possible to obtain a quick qualitative result by slide agglutination, but this gave less information than did a full test with a series of dilutions of the absorbed serum in small tubes.

There was no suggestion in titrations such as that shown in Table I that an excess of free antigen might inhibit the agglutinating action of the serum on sensitized cells. None the less, it was felt that the possibility of such an inhibitory effect had to be carefully excluded before using the technique in the examination of any extended series of human sera.

Five rows of tubes were set out, each row containing the same series of dilutions of immune rabbit serum.

An equal volume of an antigen dilution was added to each row. Thus to each tube in the first row was added 1/5 antigen, to each in the second row 1/10, and so on for 1/25 and 1/50 dilutions of the antigen; the fifth row served as a control and to this an equal volume of normal saline was added. These serum-antigen mixtures were placed in the water bath at 37° C. for 30 minutes and then tested in the usual way for their ability to agglutinate sensitized cells. There was a slight reduction in the agglutinating titre in the presence of antigen dilutions 1/5 and 1/10, the end-point being about one serum dilution lower than in the control series (Table II). It was, however, unlikely that any excess of antigen in the cell suspensions employed would significantly reduce the agglutinating titre of the serum under test.

In order to test this under working conditions one further experiment was performed. A 2% suspension of sheep erythrocytes was mixed with an equal volume of a 1/10 dilution of antigen and incubated at 37° C. for one hour. After incubation, half of the suspension was removed and centrifuged, the deposited cells washed and re-suspended in saline to the original volume. This suspension of washed sensitized cells and the

TABLE II

AGGLUTINATION OF SENSITIZED ERYTHROCYTES BY IMMUNE RABBIT SERUM IN EXCESS ANTIGEN

Dilution of Antigen	Immune Serum Dilutions					Control Saline Only
	1/5	1/10	1/25	1/50	1/100	
1/5	+ + + +	+ + + +	+ +	+ +	−	−
1/10	+ + + +	+ + + +	+ +	+ +	±	−
1/25	+ + + +	+ + + +	+ + +	+ +	+ +	−
1/50	+ + + +	+ + + +	+ + +	+ +	+ +	−
Control, no antigen ..	+ + + +	+ + + +	+ + + +	+ + +	+ +	−

+ + + + = maximal agglutination; − = no agglutination.

original suspension were then tested against immune rabbit serum. There was only a slight difference observed in the degree of agglutination at the end-point, the same end-point being obtained with the two suspensions. It was thus concluded that it would be reasonable to omit the washing and re-suspension of the red cells to be used in routine agglutination tests.

Technique for Testing Human Sera.—The following method was employed in all routine tests.

Preparation of Sensitized Cell Suspension.—A suitable volume of a 2% suspension of sheep cells was prepared and half of this added to an equal volume of normal saline to provide a 1% control suspension. The other half was added to an equal volume of a suitable dilution of antigen in saline (usually 1/10) and thoroughly mixed. Both suspensions of cells were then placed in the 37° C. incubator for one hour.

Absorption of Sera.—Serum for the test was diluted 1/5 (0.5 ml. to 2.0 ml. of saline) and inactivated at 56° C. for 30 minutes. After inactivation 0.1 ml. of packed sheep cells was added to the serum and allowed to act for 20 minutes in the 37° C. incubator; the tubes containing the serum red cell mixture were then centrifuged to deposit the cells and the procedure repeated without removing the supernatant from the deposited cells. After the second period of absorption the samples were again centrifuged, the supernatant serum removed, and serial dilutions prepared for testing.

Performance of the Test.—Serial dilutions of the adsorbed serum were distributed in 0.2 ml. volumes in 3 in. × ½ in. tubes. The first three dilutions were duplicated for testing with normal sheep cells in order to exclude heterophile agglutination. Then 0.2 ml. of sensitized red cells was added to each tube in the series and 0.2 ml. of normal cells to the three control dilutions. Two further controls were included, each containing 0.2 ml. of saline to exclude non-specific agglutination of the two cell suspensions. The contents of the tubes were mixed and the whole test placed in the water bath at 37° C. for 30 minutes.

Although gross agglutination could be detected immediately after the tubes were removed from the water bath, it was necessary to allow the red cells to sediment, completely before the results were finally read. Reading of results was based on the appearance of the plaque of red cells appearing at the bottom of the tube and checked by gentle agitation to re-suspend the agglutinated cells.

An arbitrary system of noting the results was employed, the agglutination being recorded as + + + +, + + +, etc., according to the degree observed. The agglutinating titre was taken as the last tube showing at least + + + agglutination.

Results

Altogether 10 " strains " of *T. vaginalis* have been isolated from patients, but only six of these have been used for the preparation of antigen. Table III, abstracted from the individual titrations, shows the type of results obtained.

Four of these six antigens were tested against two bovine sera known to contain antibody against

TABLE III
COMPARATIVE TITRES OF SIX ANTIGENS TESTED AGAINST IMMUNE RABBIT SERUM

Antigen 1/10	Immune Rabbit Serum Dilutions						
	1/5	1/10	1/25	1/50	1/100	1/250	1/500
Dodd ..	+ + + +	+ + + +	+ + + +	+ + + +	+ + +	−	−
Dean ..	+ + + +	+ + + +	+ + +	+ + +	+ +	−	−
Gibson ..	+ + + +	+ + + +	+ + +	+ +	±	−	−
Beardmore	+ + + +	+ + + +	+ + + +	+ + +	+	±	−
Griffith..	+ + + +	+ + + +	+ +	+	±	−	−
Macieson	+ + + +	+ + + +	+ + + +	+ + + +	+ +	+	−

+ + + + = maximum agglutination; − = no agglutination.

the serologically distinct Manley and Belfast strains of *T. foetus* and against the standard vaginalis rabbit serum. The results obtained are shown in Table IV.

It will be seen that agglutination of red cells sensitized with *T. vaginalis* extract is produced by *T. foetus* antisera with all antigens tested, but that the titre in each case is lower than those obtained with the immune rabbit serum. The converse reaction, i.e., testing *T. vaginalis* immune sera against cells sensitized with *T. foetus* extracts, has not so far been investigated.

One hundred samples of " normal " human serum were obtained from the Regional Transfusion

TABLE IV
ANTIGENS PREPARED FROM DIFFERENT " STRAINS " OF *T. vaginalis* TESTED AGAINST IMMUNE BOVINE SERA AND IMMUNE RABBIT SERUM

Antigen	Serum*	Serum Dilutions						Saline Control
		1/5	1/10	1/25	1/50	1/100	1/250	
Gibson ..	Manley	+ + + +	+ + + +	+ +	−	−	−	−
	Belfast	+ + + +	+ + + +	+	−	−	−	
	Rabbit vaginalis	+ + + +	+ + + +	+ + +	+ +	±	−	
Beardmore ..	Manley	+ + + +	+ + + +	+	−	−	−	−
	Belfast	+ + + +	+ + + +	−	−	−	−	
	Rabbit vaginalis	+ + + +	+ + + +	+ + + +	+ + +	+	∓	
Griffith ..	Manley	+ + + +	+ + + +	±	−	−	−	−
	Belfast	+ + +	−	−	−	−	−	
	Rabbit vaginalis	+ + + +	+ + + +	+ +	+	∓	−	
Macieson ..	Manley	+ + + +	+ + + +	+ +	−	−	−	−
	Belfast	+ + + +	+ + + +	−	−	−	---	
	Rabbit vaginalis	+ + + +	+ + + +	+ + + +	+ + + +	+ +	+	
Normal cells ..	Manley	−	--	−				−
	Belfast	−	−	−				
	Rabbit vaginalis	−	−	−				

* All sera adsorbed with sheep cells before testing.   + + + + = maximum agglutination; − = no agglutination.

TABLE V
RESULTS OF HAEMAGGLUTINATION TESTS

Serum	Total No.	Negative	Positive at Dilutions							Positive		Negative	
			1/5	1/10	1/20	1/40	1/80	1/160	1/250	No.	%	No.	%
Normal male ..	50	31	6	10	3	0	0	0	0	19	38	31	62
,, ' female ..	50	39	3	5	3	0	0	0	0	11	22	39	78
Trichomonas vaginalis in female discharge	50	1	2	5	15	12	10	2	3	49	98	1	2
Normal children (7 male, 6 female) ..	13	6	4	3	0	0	0	0	0	7	—	6	—

Service, 50 from males and 50 from females. These were absorbed with normal sheep cells and tested for the presence of agglutinin for sensitized cells in the usual way. The results are shown in Table V; also included in this table are the results of the examination of 50 sera from women attending the V.D. Department who were selected for the purpose of the investigation because trichomonads were present in the vaginal discharge with or without producing clinical evidence of vaginitis; the table also shows the results on a small number of sera from children of both sexes below the age of puberty.

The results set out in Table V make it difficult to decide what titre should be regarded as significant. As all the sera have been absorbed and subsequently shown to be unable to agglutinate normal sheep red cells, it seems unlikely that positive results were due to the presence of heterophile antibody. If only the positive results in serum dilutions above 1/10 are considered as significant the percentage of positives in the sera from the different groups of persons is as shown in Table VI.

TABLE VI
AGGLUTINATION BY HUMAN SERA OF SHEEP RED CELLS TREATED WITH EXTRACTS OF *Trichomonas vaginalis*

Human Sera	No. Examined	Positive (%)	Negative (%)
Trichomonas vaginalis in female discharge	50	84	16
" Normal " female blood donors	50	6	94
" Normal " male blood donors	50	6	94
Children (7 males, 6 females) ..	13	0	100

Titre: 1/10 recorded as positive.

Comment

Very little is at present known about the occurrence of serum antibodies in patients with *T. vaginalis* infections.

Various serological methods have been employed, but the number of investigations is small and the results conflicting. An agglutination reaction is observed when human sera are tested against sheep cells treated with a formamide extract of *T. vaginalis*.

The significance of this reaction is not easy to assess. Tokura (1935) observed that the three trichomonads which may occur in human beings, i.e., *T. hominis*, *T. buccalis*, and *T. vaginalis*, gave serologically distinct reactions when tested against immune rabbit serum, but this observation is unconfirmed; however, there remains the possibility that some of the low titre positive results recorded in Table V above may have been due to antibody to one of these strains other than vaginalis, particularly as some low titre positive reactions were obtained when bovine sera containing antibodies against *T. foetus* were tested against cells sensitized with *T. vaginalis* antigen.

Although the occurrence of positive results at low titre with the sera of children suggests some non-specific effect, it does not follow that all low titre positives are non-specific. Indeed, it is quite possible that some of the positive results excluded from Table VI are, in fact, weak specific positives. This is borne out by the low incidence of positives in normal women shown in this table, where the recorded 6% is a good deal lower than would be expected considering the general clinical opinion as to the number of women who are symptom-free carriers of *T. vaginalis*. This has recently been put as high as 24%. If the number of positives recorded in normal women is an underestimate, then it is possible that the same criticism can be applied to the results recorded for " normal " males. While this is an interesting possibility there are no reliable figures against which to test it. It is probably true that *T. vaginalis* can produce a symptomless infection in the male and that the treated female can be reinfected in this way. Most venereologists would, I think, agree that such conditions as urethritis and prostatitis occasionally result from infection with *T. vaginalis*.

Possibly improvements in the antigen employed in the test described above would lead to an increase in the titre obtained with positive sera and, if not entirely excluding non-specific agglutination, at least make the distinction between true and false positives easier.

Although it is too early to draw conclusions, the results obtained suggest that the method described merits further study in relation to the problem of human trichomonas infections.

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