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Incomplete agglutinins against *Treponema pallidum*

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It is well known that both syphilitic infection and immunization with nonviable *T. pallidum* give rise to a variety of circulating antibodies, including antilipoidal reagin and specific treponemal antibodies. These antibodies can be detected by means of various serological procedures, some of which have been widely used in routine diagnostic practice.

The purpose of this communication is to record experiments in which evidence was obtained that syphilitic human sera contain incomplete treponemal agglutinins which can be demonstrated by the use of an antihuman globulin serum.

Material and methods

The rabbit adapted Nichols strain of *T. pallidum* was used throughout. The organisms were harvested from rabbit testicle syphilomas 8 days after inoculation by shaking minced slices of the infected tissue in a phosphate-buffered saline of pH 7·4 (PBS). The treponeme suspension was freed of testicular debris by differential centrifugation and adjusted to the density of approximately 150 organisms per high dry microscopic field (400×).

The sera examined were those submitted to this Department with a request for serological tests for syphilis; they were obtained from syphilitic individuals, patients with diseases other than syphilis, and from blood donors. The sera were kept at 4°C. and heated at 56°C. for 30 min. just before use.

The removal of Wassermann antibody was effected by absorbing the sera with Kahn antigen using the method of Király (1959).

Absorption of the sera by treponemes was carried out as described previously (Metzger and Podwińska, 1968).

2-mercaptoethanol treatment of the sera was performed by the method of Deutsch and Morton (1957).

The human globulin antiserum was prepared in rabbits according to the method of Proom (1943).

The VDRL slide flocculation test was performed as described in the 'Manual of Tests for Syphilis' (1969).

The TPI test was performed by the procedure of Nelson and Diesendruck (1951) with the following modifications: the basal medium contained double strength sodium thioglycollate, and bovine serum albumin was replaced by gelatin to give a final concentration of 100 mg. per cent.

The FTA–100 test was carried out as described by Cherry, Goldman, and Carski (1960).

The treponeme agglutination reaction was performed in the following manner. 2-fold dilutions of a serum examined starting from 1/20 were made in PBS. To 0·1 ml. of each serum dilution an equal volume of treponeme suspension was added; the mixtures were incubated in a water bath at 37°C. for 18 hrs. The presence or absence of agglutination was determined by examination of a drop of each mixture under darkfield microscopy. The degree of agglutination was read as 0,1+, 2+, 3+, and 4+ on the basis of the estimated percentage of treponemes in clumps. The treponeme agglutination titre of a serum was taken as the highest dilution which gave definite 1+ agglutination.

Results

It has been shown (Hardy and Nell, 1955, 1957; Metzger and Podwińska, 1965) that pathogenic *Treponema pallidum* when first recovered from syphilitic lesions in rabbits is not agglutinated or is very poorly agglutinated by syphilitic or immune sera. During storage, however, a spontaneous change of the treponemes occurs, which results in the development of complete agglutinability. It was found in initial experiments that treponemes which had not been agglutinated during incubation with a syphilitic human serum could be readily agglutinated by the addition of an antihuman globulin serum to the suspension of such organisms, which had previously been washed free of syphilitic serum. It was obvious from this result that the treponemes were coated during incubation with some antibodies of the syphilitic serum but these were unable *per se* to cause a visible reaction; as such they conformed to the definition of incomplete antibodies, and are hereafter referred to as incomplete agglutinins as opposed to the complete treponeme agglutinating antibodies.

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Further experiments were designed:

(1) To find the best conditions for the reaction between incomplete agglutinins and treponemes;

(2) To establish a technique for the performance of the antiglobulin treponeme agglutination test (Antiglob. Aggl.) on the basis of the results obtained in the experiments under **(1)**;

(3) To determine some of the characteristics of the incomplete treponeme agglutinins and their incidence in human sera.

DEVELOPMENT OF ANTIGLOBULIN TREPONEMAL AGGLUTINATION TEST

(i) Titration of antiglobulin serum

Several portions of a heavy suspension of freshly isolated treponemes were incubated with an equal volume of 2-fold dilutions of a syphilitic human serum pool in a 37°C. water bath for 18 hrs. The treponemes from each test tube were then sedimented by centrifugation, washed free of serum, and re-suspended in PBS to the original density. The suspension from each test tube was then divided into several 0·4 ml. aliquots, and to each of these 0·4 ml. of 2-fold increasing dilutions of a heat-inactivated antihuman-globulin serum were added. The presence or absence of agglutination was read after incubating the reaction mixtures at 37°C. for 18 hrs. The results are recorded in Table I. The degree of agglutination of the sensitized treponemes diminished when the dilution of the syphilitic serum and dilution of the antiglobulin serum were increased. The highest dilution of either serum at which a positive reaction was observed was 1/320. However, a dilution of 1/40 was selected as a working titre of the antiglobulin serum for further studies because it gave the strongest agglutination of the treponemes sensitized with even highly diluted syphilitic serum. Table I also shows that the antiglobulin serum did not agglutinate treponemes suspended in PBS alone. On the other hand, some sera of nonsyphilitic subjects were found to give weakly positive results when they were tested at dilutions not exceeding 1/20 using antiglobulin serum at low dilutions.

(ii) Length of sensitization period

Table II shows that the sensitization process was already completed after 20 min. and prolongation of the incubation beyond this time did not enhance the degree of agglutination. In further experiments, however, the sensitization time was prolonged to 1 hr.

TABLE II *Effect of duration of sensitization and agglutination upon treponeme agglutination*

Treponemes incubated with pooled syphilitic human serum		*Sensitization (min.)*	*Degree of agglutination of sensitized treponemes incubated with antiglobulin serum diluted* 1/40 *(hrs)*				
			1	2	3	7	18
Dilution 1/20		5	1+	1+	2+	2+	2+
		10	1+	2+	3+	3+	3+
		20	1+	3+	4+	4+	4+
		60	1+	3+	4+	4+	4+
		18 hrs	1+	3+	4+	4+	4+
	1/160	5	0	1+	1+	1+	1+
		10	0	1+	2+	2+	2+
		20	1+	1+	3+	3+	3+
		60	1+	1+	3+	3+	3+
		18 hrs	1+	1+	3+	3+	3+

(iii) Length of agglutination period

The data in Table II show also that 3 hrs' incubation was required for the sensitized treponemes to be most completely agglutinated by the antiglobulin serum.

(iv) Effect of incubation temperature

Table III shows that the best temperature for sensitizing treponemes by incomplete agglutinins of the syphilitic serum is 37°C. No effect of temperature was noted upon the agglutination of treponemes already sensitized by the antiglobulin serum; the degree of agglutination was the same at each of the temperatures applied.

TABLE I *Titration of antihuman globulin serum*

Treponemes sensitized by serum	*Dilution*	*Degree of agglutination of sensitized treponemes by antiglobulin serum diluted*						
		1/10	1/20	1/40	1/80	1/160	1/320	1/640
Pooled syphilitic human	1/10	4+	4+	4+	4+	4+	2+	0
	1/20	4+	4+	4+	4+	3+	2+	0
	1/40	4+	4+	4+	3+	3+	2+	0
	1/80	4+	3+	3+	3+	1+	1+	0
	1/160	2+	2+	1+	1+	1+	0	0
	1/320	2+	1+	1+	0	0	0	0
	1/640	0	0	0	0	0	0	0
Pooled nonsyphilitic human	1/10	1+	1+	0	0	0	0	0
	1/20	1+	0	0	0	0	0	0
Treponemes not sensitized		0	0	0	0	0	0	0

TABLE III *Effect of incubation temperature upon sensitization and agglutination of treponemes*

Sensitization temperature (1) (°C.)	*Degree of agglutination of sensitized treponemes by antiglobulin serum after incubation (2) (temperatures °C.)*			
	4	18	37	45
4	0	0	0	0
18	1+	1+	1+	1+
37	4+	4+	4+	4+
45	2+	2+	2+	2+

Notes: (1) Treponemes were incubated with pooled syphilitic human serum diluted 1/20 for 1 hr
(2) Sensitized treponemes were incubated with antihuman globulin serum diluted 1/40 for 3 hrs

(v) Technique of antiglobulin treponemal agglutination test

On the basis of the results obtained in the above experiments, the following technique was applied in further experiments designed to determine some of the characteristics of the incomplete treponeme agglutinins.

Sera to be examined were heated at 56°C. for 30 min. before use. 2-fold dilutions of the sera starting from 1/20 were made in phosphate buffered saline at pH 7·4 in 13 × 95 mm. centrifuge test tubes. To 0·4 ml. of each serum dilution was added an equal volume of a suspension of freshly isolated *T. pallidum*, except when otherwise indicated. The density of the suspension was approximately 150 organisms per high dry field (400 ×). The mixtures were incubated in a water bath at 37°C. for 1 hr. After this time, the treponemes in each test tube were sedimented by centrifugation at 5,000 G. for 30 min., washed free of serum, and re-suspended in 0·4 ml. PBS. An equal volume of heat-inactivated (at 56°C. for 30 min.) human globulin antiserum, diluted 1/40, was then added to each treponeme suspension, and the mixtures were again incubated at 37°C. for 3 hrs. Agglutination was estimated microscopically as described under "Material and Methods".

CHARACTERISTICS OF INCOMPLETE TREPONEMAL AGGLUTININS

(i) Antigenic receptors for incomplete agglutinins

It has been demonstrated (Hardy and Nell, 1957; Metzger and Podwińska, 1967) that *T. pallidum* possesses a heat labile and a heat stable antigen each of which participates in the agglutination reaction. The possibility existed that one of these antigenic components, or both, might be receptors for the incomplete agglutinins present in syphilitic human sera. The results of experiments designed to show this are recorded in Table IV. It can be seen that both

TABLE IV *Effect of 2-mercaptoethanol treatment and absorption with Kahn antigen and unheated and heated treponemes upon syphilitic human serum titres in agglutination and antiglobulin agglutination tests*

Pooled syphilitic human serum	*Serum titres with treponemes*			
	Freshly isolated unheated		*Stored at 4°C. for 20 days and heated at 100°C. for 1 hr before use*	
	Aggl. (1)	*Antiglob. Aggl.* (2)	*Aggl.* (1)	*Antiglob. Aggl.* (2)
Unabsorbed	< 20	320 (3)	2560	160
Absorbed with Kahn antigen	n.t. (4)	320	1280	160
Treponemes—freshly isolated unheated	n.t.	< 20	1280	< 20
Treponemes—stored at 4°C. for 20 days and heated at 100°C. for 1 hr before use	n.t.	80	< 20	< 20
Treated with 2-mercaptoethanol	n.t.	320	320	160

Notes: (1) Treponeme agglutination test
(2) Treponeme antiglobulin agglutination test
(3) Reciprocals of highest serum dilution that gave definite 1+ agglutination
(4) Not tested

freshly isolated unheated treponemes and those in which the heat labile portion had been destroyed by heating were coated with incomplete agglutinins. This indicates that the heat stable antigen certainly participates in the sensitizing reaction. Evidence that a heat labile treponeme component is also involved has come from absorption experiments. A syphilitic serum, from which incomplete agglutinins reacting with the heat-stable antigen had been removed by absorption with heated treponemes, still retained the capacity to sensitize freshly isolated unheated treponemes having either antigenic component.

(ii) Distinction from Wassermann antibody

To demonstrate this, the antiglobulin treponemal agglutination test was performed on a group of syphilitic human sera from which the Wassermann antibody had been removed by absorption with Kahn antigen. The results of a typical experiment are recorded in Table IV, which shows that removal of the Wassermann reagin from the serum did not affect the incomplete agglutinin titres against either unheated or heated treponemes.

(iii) Distinction from specific treponeme agglutinins

Table IV also shows that, when syphilitic human serum was absorbed with freshly prepared unheated treponeme suspension, the agglutinin titre of the serum remained unaltered after absorption whilst its reactivity in the antiglobulin agglutination re-

action with both unheated and heated organisms was completely abolished. On the other hand, absorbing the serum with stored heated treponemes removed both complete and incomplete agglutinins against the homologous suspension; such absorbed serum, however, retained the ability to sensitize freshly isolated unheated treponemes though at the lower titre. This was understandable because absorption with heated treponemes, *i.e.* those deprived of the heat labile component, removed from the serum only antibodies against the heat-stable portion leaving antibodies reacting with the heat labile antigens of the treponemes.

(iv) Effect of 2-mercaptoethanol

Table IV also shows that treating the syphilitic serum with 2-mercaptoethanol lowered its agglutinin titre but did not affect the serum titres in the antiglobulin agglutination test.

(v) Heat sensitivity

The effect of time and temperature of heating upon incomplete and complete agglutinins is illustrated by Table V. The incomplete agglutinins were more thermostable than the complete agglutinins. The former resisted heating at 70°C. for 30 min., but the latter lost their ability to agglutinate treponemes after being exposed to this temperature for 15 min.

TABLE V *Heat sensitivity of complete and incomplete treponemal agglutinins*

Heat treatment of sensitizing serum		*Serum titres in tests*	
Temperature (°C.)	*Time (min.)*	*Agglutination*	*Anti-globulin agglutination*
Unheated	—	1,280	320
56	30	1,280	320
60	15	640	160
60	30	320	80
70	15	< 20	80
70	30	< 20	40
80	15	< 20	< 20

(vi) Incidence in human sera

Three groups of sera were obtained:

(1) From patients in various stages of syphilitic infection, treated and untreated;

(2) From blood donors and persons suffering from diseases other than syphilis;

(3) From biological false positive reactors.

These were tested by VDRL, TPI, FTA–100, and antiglobulin treponemal agglutination tests. The results are summarized in Table VI, which shows that the antiglobulin agglutination test was positive only in syphilitic sera exhibiting a complete agreement with the TPI test. The incomplete agglutinin titres of the sera ranged from 1/20 to 1/640; the value found most frequently was 1/160. In the group of 14 sera from patients with primary syphilis, both the TPI and Antiglob. Aggl. tests were negative and VDRL and FTA–100 tests gave positive results. Incomplete treponemal agglutinins were not found in sera of persons with no history of syphilis and diagnosed as biological false positive reactors on the basis of positive standard serological tests and negative TPI and FTA–100 tests.

TABLE VI *Comparison of results of Antiglob. Aggl., TPI, FTA–100, and VDRL tests on 524 sera of syphilitic and non-syphilitic individuals*

Test				*No. of sera showing indicated reactivity*
Antiglob. Aggl.	*TPI*	*FTA–100*	*VDRL*	
+	+	+	+	251
0	0	+	+	14
0	0	0	+	32
0	0	0	0	227

Discussion

The present studies have shown that there exist in the sera of syphilitic individuals two incomplete antibodies, one of which reacts with the thermolabile and the other with the thermostable antigenic component of *T. pallidum*. Their presence can be revealed by the use of an antihuman globulin serum which causes a rapid agglutination of treponemes that were previously in contact with a syphilitic human serum. For this reason, the term incomplete agglutinins has been introduced for the designation of these antibodies, and, correspondingly, the term antiglobulin treponeme agglutination test has been proposed for the reaction used to detect their presence in syphilitic sera.

A suspension of freshly isolated treponemes not treated by heat or chemicals has been found to be the most satisfactory antigen for the antiglobulin agglutination test, because such treponemes are, as a rule, not agglutinated by the complete agglutinins. In addition, such organisms have both a thermolabile and a thermostable antigen and these permit the detection of the incomplete agglutinins directed to either antigenic component. Although inagglutinable are preferable to agglutinable treponemes, the latter can be also used as the antigen in the antiglobulin agglutination test. It should be borne in mind that the agglutination of treponemes by complete antibodies occurs after several hours' incubation, and that even treponemes with fully developed agglutinability are not clumped by a syphilitic serum during the brief 1-hr incubation period used in the antiglobulin

agglutination reaction. However, the use of agglutinable treponemes has some limitations. The development of agglutinability which occurs in ageing suspensions of treponemes has been shown to be accompanied by the loss of their heat labile antigen (Metzger and Podwińska, 1967). In consequence, such treponemes will allow the detection of the incomplete agglutinin only against the thermostable portion of the organisms. The same proviso is valid for the use of treponemes heated or treated by a variety of chemicals, because such procedures have been shown to damage or destroy the heat labile protein antigen of *T. pallidum* (Metzger and Podwińska, 1968).

The incomplete treponeme agglutinins have been found in this study to be distinct from the true treponeme agglutinins (Hardy and Nell, 1955, 1957) and from the antilipoidal Wassermann antibody which is also capable of inducing agglutination of treponemes (Hardy and Nell, 1955; Király, 1959; McLeod and Stokes, 1955; Metzger and Podwińska, 1967). Evidence for this has come from absorption studies and from experiments in which the effect of 2-mercaptoethanol and heat treatment upon the titres of syphilitic sera, in the agglutination and antiglobulin agglutination tests, were compared. A study is now in progress in this laboratory to localize the treponemal antibodies in the classes of immunoglobulins of syphilitic serum. The results so far appear to indicate that the incomplete agglutinins with their very low sedimentation coefficient (below 7S) differ from the antibodies participating in other serological tests for syphilis.

The antiglobulin treponeme agglutination test was carried out on 524 sera obtained from patients in various stages of syphilitic infection and from individuals with no history of syphilis; the results were compared with those of the TPI, FTA–100, and VDRL tests. Incomplete agglutinins were found only in the serum of syphilitic subjects. It was interesting to note a correlation between the results of the TPI test and those of the antiglobulin agglutination reaction in view of the commonly recognized specificity of the former. The results of this pilot study indicate that the antiglobulin agglutination test, as employed here, is specific for the detection of syphilitic infection in man. The occurrence of incomplete agglutinins in the course of syphilitic infection is not a unique phenomenon. Incomplete antibodies have also been found in the serum of patients suffering from various infectious diseases and have proved useful in diagnosis (Doleżal, 1963). Final evaluation of the antiglobulin treponeme agglutination test depends like that of other tests on a critical analysis of results with sera from large numbers of patients. Studies on these lines are now being conducted in this laboratory.

Summary

It has been shown that syphilitic human sera contain two incomplete agglutinins, one of which reacts with a thermolabile and the other with a thermostable antigenic component of *T. pallidum*. The best conditions for this reaction have been established, and utilized to devise a simple antiglobulin agglutination test for the detection of the incomplete agglutinins in human sera. Evidence has also been presented to show that the antibodies participating in this reaction are distinct from the complete treponeme agglutinins.

The antiglobulin treponeme agglutination test was carried out on 524 sera from syphilitic and non-syphilitic individuals, and the results were compared with those of the TPI, FTA-100, and VDRL tests. The antiglobulin agglutination test was found to be positive only in the syphilitic sera, and to show complete agreement with the results of the TPI test.

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Agglutinines incomplètes contre *Treponema pallidum*

SOMMAIRE

Il a été montré que les sérums syphilitiques humains contiennent deux agglutinines incomplètes, l'une qui réagit avec un composé thermolabile de *T. pallidum*,

l'autre avec un composé thermostable. On a déterminé les meilleures conditions pour cette réaction et mis au point un test simple d'agglutination antiglobulinique pour la mise en évidence des agglutinines incomplètes dans les sérums humains. Il fut également prouvé que les anticorps intervenant dans la réaction sont distincts des agglutinines complètes du tréponème.

L'épreuve d'agglutination à l'antiglobuline tréponémique a été effectuée sur 524 sérums individuels provenant de syphilitiques et de non syphilitiques et les résultats ont été comparés avec le TPI, le FTA–100 et le VDRL. L'épreuve d'agglutination antiglobulinique se montra positive seulement pour les sérums syphilitiques et en accord complet avec les résultats du TPI.