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1. INTRODUCTION

The **Complement Fixation Test (CFT)** is a test primarily designed to measure specific antibody levels in sera. It is one of the oldest serological methods for diagnosis of infectious diseases, looking back at 90 years of experience to draw upon.

Since the CFT is a rather complex antibody detection method the following information is especially designed for medical staff. This brochure will inform you about the theoretical background as well as about some practical aspects of the test to support you in your daily laboratory routine.

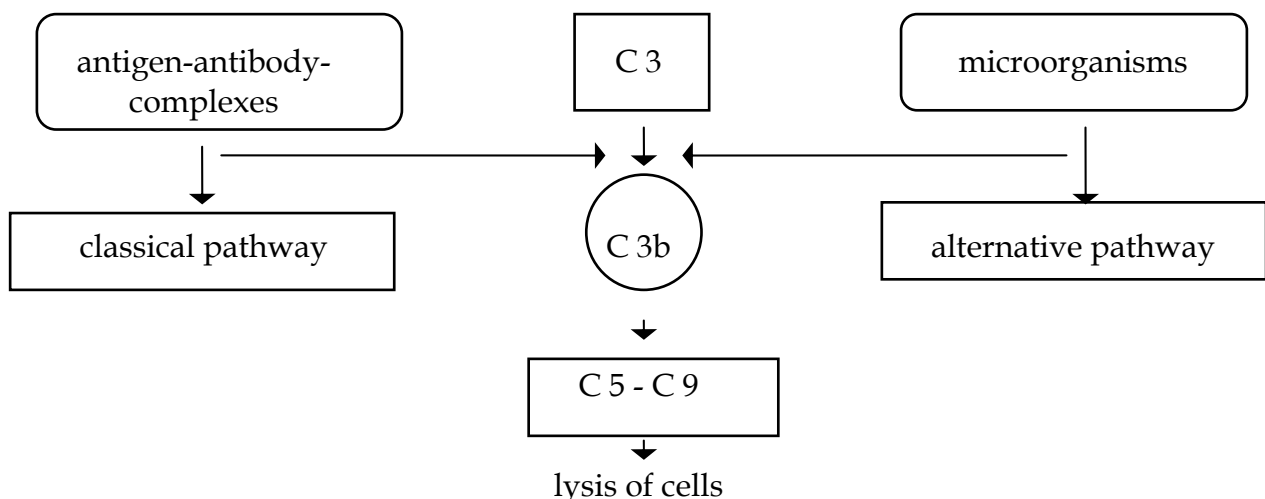
2. THE COMPLEMENT SYSTEM

At the end of the last century Paul Ehrlich created the term „complement system“ since he believed that these proteins are only supporting molecules („complementum“) of the immune system. Today it is well known that complement is an important and integral component of the non-specific immune response.

More than 20 proteins belong to the complement system and are divided into the component groups C1 to C9. The numbers of the components are given according to the date they were first identified. Similar to the enzyme system responsible for coagulation, the complement system is an enzyme cascade with intermediate and end products. An advantage of this cascade system is that the different products are able to fulfill different functions: Chemotaxis (chemically attraction of immune cells), opsonization (support of macrophage activity) or the induction of histamine release from mast cells.

The most important function of the complement system for the immune system as well as for the complement fixation reaction is the ability of the end product of the enzyme cascade to lyse membranes.

Activation of the enzyme cascade is induced by antigen-antibody complexes (classical pathway), particularly the Fc-region of antibodies which becomes activated when antibody binds to an antigen, or by microorganisms (alternative pathway). The following figure shows a simplified scheme of both pathways.



Activation of the enzyme cascade via the alternative pathway is less specific and the historically older pathway. Parts of microorganisms - such as LPS molecules - may also activate this pathway if present in large quantities, in the absence of any specific antibody production. This is very important since antibody production does not start immediately after infection. After a "delay period" specific antibodies appear. The alternative pathway provides a mechanism enabling the immune system to respond to the pathogen, immediately.

3. CFT - THE CLASSICAL COMPLEMENT PATHWAY *in vitro*

3.1 CFT - reagents

Serum: endogenous complement in sera has to be inactivated by heat (30 min, 56°C); material in which the ion balance was altered (e.g. EDTA blood) should be avoided;

Antigen: Serion Immundiagnostica GmbH offers more than 50 antigens, single specificities and pool antigens. Antigens are freeze-dried and have to be reconstituted in water. The working dilution has to be prepared with veronal buffer. The working dilution contains 2 units of antigen. The stability of reconstituted antigens is one week at 4°C and two months at -20°C (undiluted!). Repeated freezing and thawing of the antigen should be avoided, therefore it is recommended to freeze the antigen in smaller aliquots.

Control antigen: made of uninfected culture material (e.g. cells, egg material). Control antigen is used for the detection of non-specific reactions. Control antigen is used in a similar way as the antigen, both reactions should be carried out in parallel. If serum gives positive results with antigen and control antigen, unspecific reactions may be the reason. If the obtained antigen-titer is at least two dilutions higher than the corresponding control antigen titer, the serum evaluation is positive, since not only unspecific antibodies but also specific antibodies are detected.

Veronal buffer (CF buffer): provides optimal pH-value (7.2) and ideal divalent ion composition which is essential for the complement reaction.

Complement: made from guinea pig serum; supplied freeze-dried. Reconstituted in water the complement is stable for 3-4 weeks at 4°C. Once diluted with veronal buffer, complement is unstable and should be kept cool and used immediately. The working dilution (please see indication on the label) may vary from lot to lot.

Erythrocytes: made of sheep blood; supplied in Alsever solution. Concentration in use: 1 %.

Hemolytic ready-to-use system: The hemolytic system is a ready-to-use reagent which can be used for the complement fixation test without preparation.

Information regarding the performance:

If the ready-to-use hemolytic system for SERION CFT is used, a higher complement concentration is necessary. For example if a 1:55 dilution is indicated on the label of the complement, a 1:40 dilution should be used instead.

Mix the hemolytic system thoroughly and incubate the quantity required for the test run at 37°C for 30 minutes. Pipette 50µl per cavity, then follow the general CFT instruction manual.

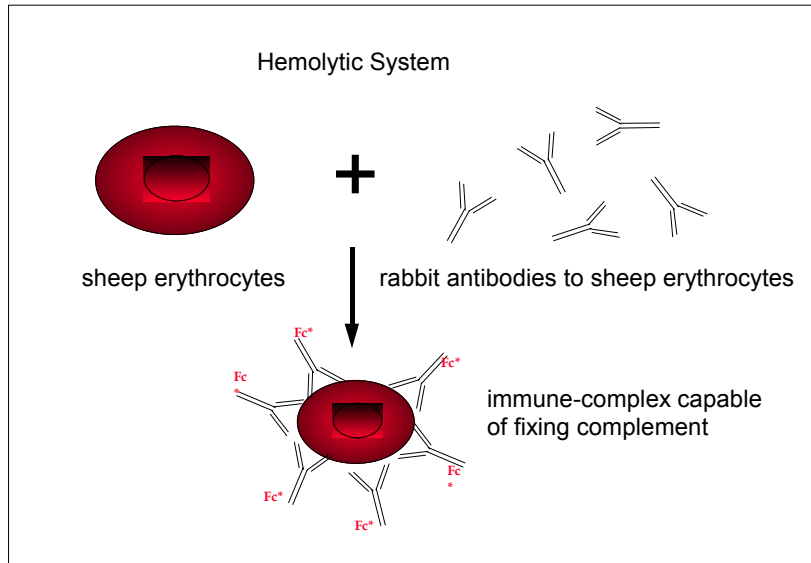
Amboceptor: rabbit serum that was immunized with sheep erythrocytes. When erythrocytes and amboceptor are mixed, an antigen-antibody complex forms, representing the hemolytic system (HS) or indicator system. Since the complex formation is a biological reaction, precise incubation of the HS, regarding time and temperature, is necessary.

Control sera: positive and negative control sera provided to reinsure the correct performance of the test.

3.2. CFT - reaction

Complement will be activated if mixed with sera and antigen (also referred to as **fixation** of complement). if antibody-antigen complexes are formed but there is no substrate to act on, the activity will decline continuously.

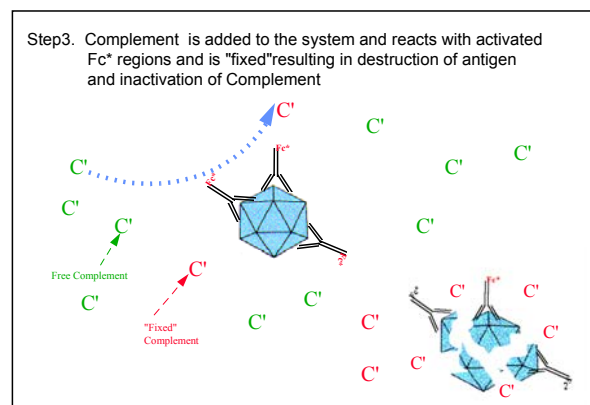
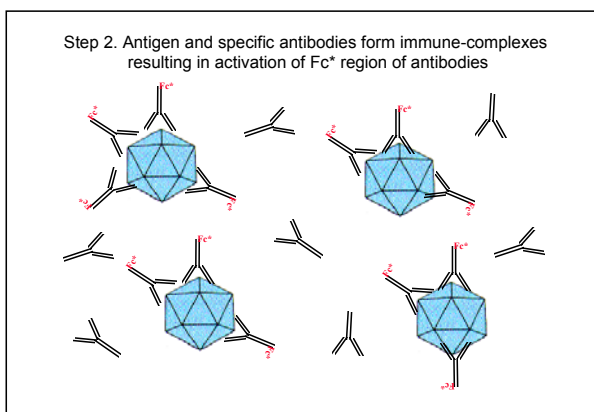
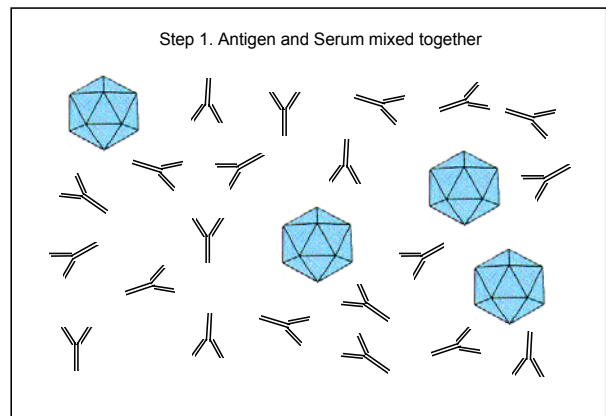
This reaction is not visible and a further indicator system, the **hemolytic system** (HS), is required. The hemolytic system consists of sheep erythrocytes which have been sensitized by addition of anti-sheep erythrocyte antibodies, grown in rabbits (**Amboceptor**), forming an immune complex of antibodies and erythrocytes.



The HS is added to the mixture of serum, antigen and complement. Two reactions are possible:

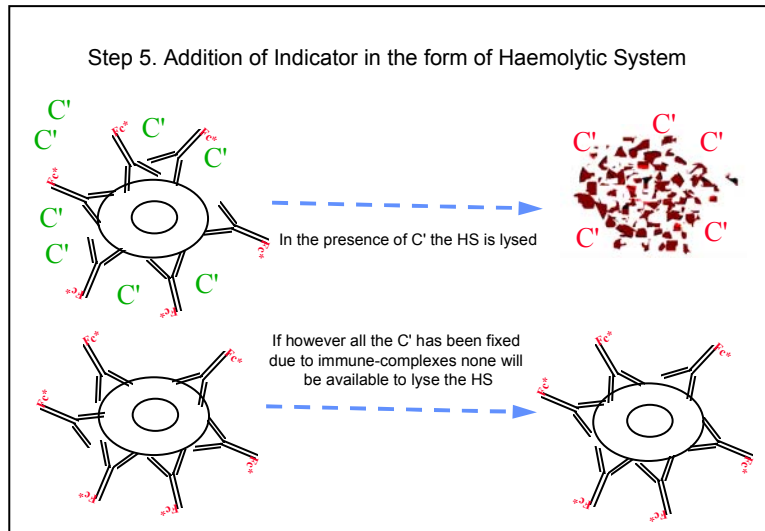
a.)

If serum contains specific antibodies a specific **antibody-antigen complex** will be formed. Complement will be fixed by this immune complex and is not available anymore for other antigen-antibody complexes like the HS. Therefore, the **erythrocytes** will not be affected by the complement and **remain unlysed**.



b.) If serum does not contain specific antibodies **no antibody-antigen complexes** are formed with the specific antigen. Complement will not be activated and remains **unfixed** in the reaction. It is now able to react with another antibody-antigen complex that will be introduced by addition of the HS (erythrocytes and anti-erythrocyte antibodies). Complement will be fixed by the HS resulting in the destruction of the erythrocyte membrane and in **lysis of the erythrocytes**.

In summary, the following reactions are possible with CFT:



3.3. Standardization of the test

Before performing the test several components have to be standardized in order to obtain consistent results. Antigen may show anti-complementary activity and induce the complement cascade in a non-specific way, leading to a reduction of active complement within the mixture. Therefore, each antigen should be tested with a range of complement dilutions in order to determine the correct complement concentration. To simplify the handling of the test run we require a common complement dilution and amboceptor dilution for every antigen. Therefore, time consuming pre-testing is necessary to find the optimal reaction conditions or concentrations.

Exclusive use of SERION CFT reagents makes pre-testing unnecessary!

All pre-testing for antigen, complement and amboceptor dilution is carried out in the manufacturer's laboratories. Recommended working dilutions are indicated on the labels of the reagents.

4. TEST RUN

4.1. First day of CFT

Patient's sera and control sera are diluted 1:10 with veronal buffer. To inactivate the endogenous complement sera are incubated at 56°C for 30 minutes. Using a round bottom microtiter plate a dilution series is carried out with all sera from 1:10 to 1:160 (see pipetting scheme).

Serum control (SC) at 1:10 is also included to detect any anticomplementary activity in serum itself. Serum may contain pre-existing antibody-antigen complexes which can lead

to false positive results. Serum with anticomplementary activities must be treated with complement (see below) before running the test.

Antigen is added to each well except for the serum controls.

Complement is added to each well.

Incubation: at 2 - 8°C overnight

4.2 Second day of CFT

Addition of the preheated **hemolytic system** (erythrocytes mixed with amboceptor)

Incubation: at 37°C for 30 min.

The test is stopped by removing the microtiter plate from the incubator and centrifugation of the microtiter plate.

4.3 Complement control

Complement control should be performed for each antigen. Information about the correct function of the HS is given. Additionally, it informs about the different incubation times for each antigen: usually the reaction is stopped, when the first two wells (containing 2 and 1 units of complement) show complete hemolysis (see pipetting scheme).

4.4 Practical considerations

Prepare diluted complement with cold reagents immediately before use.

Preheat microtiter plates evenly on the second day prior to addition of Hemolytic System. Do not stack the plates when you put them into the incubator. This would lead to temperature gradients on the inside of the plates (warm edges and cold centers).

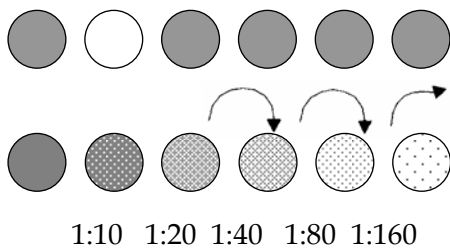
Incubation temperature: keep constant temperature by opening the incubator not more than absolutely necessary.

If possible preheat the veronal buffer when preparing the HS. Always use the waterbath, not the incubator for incubation of HS-components to reensure that the correct temperature is reached.

Agitate the suspension of the HS immediately prior to dispensing into plates to prevent sedimentation and varying erythrocyte concentration.

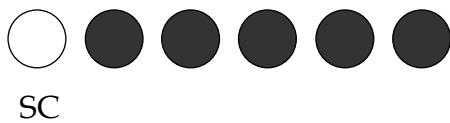
5. PIPETTING SCHEME

Dilute sera 1:10 in veronal buffer (VB), incubate sera at 56° C for 30 min.

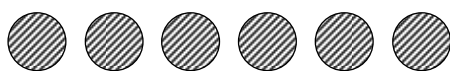


pipette 25 µl of **veronal buffer** in each well, except for the second well

add 25 µl of **diluted serum (1:10, s.a.)** to well 1 - 3: start dilution as indicated (25 µl steps)

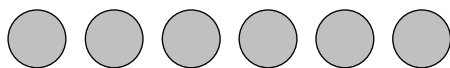


add 25µl of **antigen**-working dilution to each well, except for the first well (SC = serum control)



add 25µl of **complement**-working dilution to each well

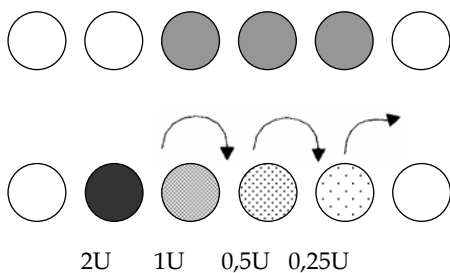
mix plate carefully, incubate for 16-20 h at 4° C



add 50 µl of **hemolytic system** to each well, incubate for 30 min. at 37° C
(hemolytic system: 1 part **amboceptor**-working dilution (1:2500); 1 part **erythrocyte-suspension** (1-2%))

mix plate carefully, incubate at 37° C, until lysis of the complement control

complement control:

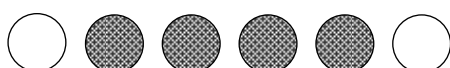


pipette 25µl of **veronal buffer** as indicated

add 25µl of **complement**-working dilution as indicated, start dilution in 25µl steps

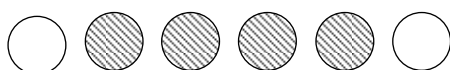


add 25µl of **antigen**-working dilution as indicated



add another 25µl of **veronal buffer** (to fill up volume to 100 µl)

mix plate carefully, incubate for 16-20 h at 4° C



add 50µl of **hemolytic system**

mix plate carefully, incubate at 37° C, stop reaction, when lysis is observed in the first

two wells.

6. INTERPRETATION OF CFT TITERS

6.1. CFT Titers

Determination of complement-fixing antibodies allows an interpretation of the infection status of the patient (e.g. acute, convalescent, absent). In general complement-fixing antibodies appear almost instantly after onset of symptoms (3 to 6 days after infection). After reaching a maximum they are detectable for up to 6 months. Low basic titers may last for several years.

Interpretation of results is as follows:

100%	inhibition of hemolysis records a value of	4 = positive
75%	inhibition of hemolysis records a value of	3 = positive
50%	inhibition of hemolysis records a value of	2 = negative
25%	inhibition of hemolysis records a value of	1 = negative
Traces of	inhibition of hemolysis records a value of	+/- = negative
	Complete hemolysis records a value of	0 = negative

Day to day variations of the test procedure may lead to variations in titers. Patient's sera as well as control sera allows a tolerance range of +/- one dilution. Especially for borderline results, analysis of paired sera is absolutely recommended.

Borderline values are given by the manufacturer of the CFT reagents and are determined by statistically calculated values during test evaluation. Therefore, the clinical findings as well as other data (age of the patient etc.) must be taken into account for interpretation of CFT titers.

Negative Titers:

No indication of an infectious with the homologous agents is evident.

Borderline Titers:

Borderline CFT titers may represent either remaining antibodies of a previous infection or low titers due to an acute infection in the early stage of the disease. For discrimination it is important to obtain information about the time span from onset of symptoms and about the time of collection of the serum sample. Due to the inherent importance the physician should provide this information. Dependent on the clinical findings analysis of a second serum sample taken 10-14 days later, may be recommended.

Control Titers:

Generally borderline titers are regarded as basic titers. If antibody titers of a given serum sample are up to 2-fold higher than the basic titers the result is expressed as a so-called 'control titer'.

Positive Titers:

If a single serum shows a CFT titer 2 - 3 steps higher than the borderline titer the result is interpreted as a positive titer. Generally these titers indicate an acute infection. A recent vaccination may result in high (positive) CFT titers. The diagnostic value of CFT antibody titers is considerably higher if titer dynamics (raise of titer up to 4 dilutions) can be detected, that is the testing of paired sera.

Titers shown in the table below are obtained by test series carried out with reagents from Serion Immundiagnostica GmbH. The specifications can not automatically be transferred to reagents of other distributors as CF-reaction strongly depends on antigen preparation and calibration of the test.

Interpretation of titers with SERION CFT reagents

Pathogen	borderline titers	control titers	positive titers
Adenovirus	20	40	80
Aspergillus fumigatus metabolic; somatic			> 10
Bordetella pertussis	10	20	40
Brucella	10	20	40
Campylobacter intestinalis	10	40	80
Campylobacter jejuni	10	40	80
Chlamydia species	10	20-40	80
Coxiella burnetii phase I	10	20	40
phase II	10	20	40
Coxsackievirus A9, B1-6	20	40	80
Cytomegalovirus	infant: child: adult:	20 40 80	40 80 160
Echinococcus granulosus	10	20	40
Echovirus	20	40	80
Entamoeba histolytica	10	20	20
Epstein-Barr Virus	20	40	80
TBE Virus	10	20	40
Helicobacter pylori	10	40	80
Herpes Simplex Virus 1 and 2	40	80	80/160
Influenza A/B Virus	40	40	80
Legionella pneumophila	10	40	80
Leptospira species	10	40	80
LCM Virus	child: adult:	>10 >20	
Listeria monocytogenes	10	40	80
Measles Virus	child: adult:	20/40 40	80 80
Mycoplasma pneumoniae	10	40	80
Neisseria	10	40	80
Parainfluenza Virus Pool (1,2,3)	40	80	160
Parotitis Virus	10	40	80
Picornavirus Pool	20	40	80
Poliovirus	10	10	20

Pathogen		borderline titers	control titers	vaccination: 40 positive titers
Polyomavirus		10	20	40
RSV	child:	10	40	80
	adult:	40	80	160
Rotaviruses	child:	10	40	80
	adult:	40	80	160
Shigella species		10	20	40
Toxoplasma gondii		10/20	20/40	80/160
Varicella-Zoster Virus		20	40	80
Yersinia species		10	20-40	80

6.2. Cross reactions

Cross reactions may be observed with the following antigens:

Herpes Simplex Virus and Varicella-Zoster Virus
Parotitis Virus and Parainfluenza Virus
Neisseria gonorrhoeae and Neisseria meningitidis
Brucella species and Yersinia enterocolitica 09
among the Enterovirus group
among Shigella species
among Parainfluenza Virus types

Often reactions with the heterologous/cross-reacting antigen are weaker than reactions with the homologous antigen.

7. DISRUPTIVE ELEMENTS AND MAIN SOURCES OF ERROR

- **Contaminated sera** or reagents may lead to an activation of complement through the „alternative pathway“ triggered by bacterial endotoxins (cell wall lipopolysaccharide of gram-negative bacteria), polysaccharides (yeast cell walls) and aggregated immunoglobulins. False positive results or „smears“ of the buttons can occur. Keep samples and reagents refrigerated and minimize contamination as far as possible. Never use obviously contaminated material.
- An **excess of antigen** may lead to **false negative results** even in the presence of specific antibodies. The latter is due to the requirement of at least 2 activated Fc antibody regions in close proximity to each other for fixing the complement. Therefore, all reagents should be used in the working dilutions as indicated on the label of the corresponding vials.
- **Different immunoglobulin classes from different species fix complement with varying degrees of efficiency. Human IgG-1, IgG-2 IgG-3 and IgM classes are highly reactive.** However IgG-4, IgA and IgE are unable to bind to the first component of the complement system and consequently react only via the alternative pathway which is less efficient. The CFT works well with human sera, detecting both IgG and IgM, however results from animal sera may not be as easy to interpret and should be evaluated according to individual experience. For example guinea pig complement is only poorly fixed by many avian immunoglobulins while bovine immunoglobulins are ineffective.
- Although SERION CFT reagents have been evaluated only for the analysis of human sera, the test may be used also for analysis of **animal sera** (mammals). **Inactivation of endogenous complement** is different for animals and humans. The following table shows inactivation times and temperatures for sera from different animals. For evaluation of the CFT carried out with animal sera it has to be taken into account, that the borderlines given for human sera may also differ for animal sera.

Serum	Inactivation-time	temperature
cow	30 minutes	56 °C
pig	60 minutes	60 °C
sheep	30 minutes	60 °C
horse	30 minutes	56 °C
goat	30 minutes	56 °C

Information given by the Veterinäruntersuchungsamt Potsdam, Germany

- **Plasma** can not be used due to the effects of clotting factors. Likewise EDTA and citrate bloods are unsuitable (removal of Mg^{2+} and Ca^{2+} from the system)
- **samples with anticomplementary activity:** Possible causes are pre-existing immune complexes, aggregation of immunoglobulin-overheating during inactivation, bacterial contamination, excessive freezing and thawing, some pharmaceuticals. Sera which show anticomplementary activity must be pre-treated with complement as follows:

50 (100) μ l of serum
 + 50 (100) μ l of undiluted complement

 Incubation for 30 min. at 37°C / water incubator

 + 400 (800) μ l of veronal buffer

 Incubation for 30 min. at 56°C / water incubator

If the anticomplementary activity is still visible after pre-treatment with complement, results can not be evaluated. Draw fresh blood! Patients with repeated anticomplementary activity should be examined (autoimmune disease, paraproteins etc.).

- Sera showing **agglutination** of the HS may indicate EBV infection with Paul-Bunnell antibodies present.

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