

Negative complement-fixation tests with rheumatoid factor positive sera

A simple method for selective removal of rheumatoid factor

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Sera from patients with rheumatoid arthritis regularly contain inhibitors of complement fixation (CF) to antigen-antibody complexes (Heimer and Levin, 1965). Removal of rheumatoid factor (RF) by absorption of sera with heat-aggregated human γ -globulin resulted in a higher number of positives and a rise in CF antibody titre against antigens from herpes simplex, mumps, and morbilli viruses (Stanford, 1972). Penttinen, Wager, Pyrhönen, Leinikki, and Teppo (1974) examined about 4,000 sera for CF with 25 viral antigens. They found that 1.4% were CF nonreactors though antibodies against some viral antigens could be shown by other methods. Most of the CF nonreactor sera showed high RF titres. The authors therefore recommend that a latex type RF test be carried out when a falsely low or negative CF is suspected. If a raised RF titre is found, the RF should be removed or destroyed before titrating CF antibodies.

Absorption of sera with aggregated γ -globulin is an expensive and time-consuming procedure. We have earlier shown that kaolin strongly adsorbs RF under conditions when antibodies to viral or bacterial antigens are not bound (Haukenes and Aasen, 1972). The efficiency of this method in unmasking CF antibodies in rheumatoid sera was therefore examined.

Materials and methods

SERA

RF-positive sera with Waaler-Rose titres of 320 or above were obtained from specimens sent to the serology laboratory. Control sera, matched for sex and age, were collected from blood donors. All sera were stored at -20°C .

SEROLOGICAL METHODS

The RF was titrated by the Waaler-Rose test. The sheep cells were sensitized with 1/3 of the basic agglutinin titre of rabbit antiserum. The titrations were performed in tubes, and the agglutination was read by the resuspension method, because the pattern reading by the

microtitre method was unreliable after kaolin treatment. The agglutination was graduated from 1 to 3. The latex RA test was performed with a reagent from Hyland (Travenol Lab. S.A., Brussels).

The CF tests were performed by the microplate technique. The herpes simplex and rubella antigens were supplied by Behringwerke, Marburg-Lahn, West Germany, and the mumps antigen was a combined S and V antigen from Orion Oy, Helsinki. The rubella antigen, which was a Tween/Ether extract of rubella-infected BHK/21 cells intended for use in the HI test, contained sufficient amounts of CF-V antigen to be used in the CF test at a dilution of 1:2. This antigen was preferred to the CF-S antigen, because the latter gives positive reactions for only a few years after clinical infection, while rubella CF-V antibodies may be found throughout life. The morbilli antigen was prepared in our laboratory from infected Vero cells as described by Katz and Enders (1969).

Before titration all sera were heat inactivated at 56°C for 30 minutes. Antigen and serum dilutions were mixed for about 5 minutes before complement was added.

KAOLIN TREATMENT

The sera were treated with kaolin (25% in borate saline, pH 9.0, Flow Labs., Irvine, Scotland) essentially as described by Halonen, Ryan, and Stewart (1967). Serum was diluted 1 to 4 in saline with Mg^{++} and Ca^{++} (Auletta, Gitnick, Whitmire, and Sever, 1968) and mixed with an equal volume of 25% kaolin yielding a serum dilution of approximately 1 in 8. The final pH of the mixture was about 8.5. After 20 minutes at room temperature with repeated stirring, the kaolin was centrifuged and the supernatant serum titrated.

The probability of significance in difference was obtained by the χ^2 test. When the geometric means of the total groups were calculated, the negatives were set to 1/2 of the lowest detectable titre.

Results

(A) RF TITRES BEFORE AND AFTER TREATMENT WITH KAOLIN

Forty-seven sera with Waaler-Rose titres of 320 or above were treated with kaolin. It is seen from Table I that almost all RF was removed by this treatment as

Table I Kaolin treatment of Waaler-Rose positive (titre ≥ 320) sera

No. of sera	Waaler-Rose titre (GM)	
	Untreated	Kaolin treatment
47	935	<10*

* Total group. GM = geometric mean titre of 18 positives: 16.

measured by the Waaler-Rose test. Kaolin treatment also removed primate RF as the latex test became negative or only weakly positive.

(B) RECOVERY OF RF FROM KAOLIN AFTER ADSORPTION

To make sure that the RF actually is held back by kaolin, attempts were made to elute the RF from kaolin. This could easily be accomplished by raising the pH to 11 and the temperature to 37°C for 20 minutes. It is seen from Table II that the recovery of RF was almost complete.

Table II An RF positive serum: adsorption to and elution from kaolin

Treatment of serum*	Agglutination (Waaler-Rose) in dilution 1:							
	10	20	40	80	160	320	640	1280
Untreated	3	3	3	3	3	1	1	0
Kaolin treated	1	0	0	0	0	0	0	0
Kaolin eluate	3	3	3	3	2	2	0	0

* Serum was treated with kaolin at pH 8.5 and room temperature for 20 minutes, and kaolin was then subjected to pH 11 and 37°C for 20 minutes.

(C) COMPARISON OF CF ANTIBODY TITRES IN RF POSITIVE AND CONTROL SERA

Seventeen to 25 sera with an RF titre (Waaler-Rose) of 320 or above and matched controls were tested for CF antibodies against herpes simplex, rubella, morbilli, and mumps virus antigens. The comparisons were always made from parallel titrations of rheuma-

toid sera and controls with each antigen. Some sera had to be excluded in each set-up because of anti-complementarity.

The difference in number of positives was very marked and highly significant with the herpes simplex antigen (Table III), while the geometric mean titres of the positives were the same. Almost identical results were obtained with the morbilli virus antigen.

Also with the other two antigens the RF positive sera had fewer CF antibody positives, but the comparison is here less valid because of the generally low titres (Table IV).

Table IV CF antibodies to mumps and rubella virus antigens in rheumatoid sera and controls

Serum	Mumps		Rubella	
	Positive (%)	GM log ₂	Positive (%)	GM log ₂
RF+	6	0	0	—
RF-	60	3	35	2.4

GM = geometric mean titre of total group.

(D) CF ANTIBODY TITRES AFTER ABSORPTION WITH KAOLIN

After treatment with kaolin the number of CF positives and the geometric mean titres in the RF+ group increased (Table V) and no longer differed from the RF-negative control sera. Treatment with kaolin had no significant influence on the CF titres of the RF-negative control sera.

(E) RISE IN TITRE AFTER KAOLIN TREATMENT

The rise in CF titres with herpes simplex and morbilli antigens after kaolin treatment, expressed in dilution steps, was recorded (Figure). The values given in the Figure are likely to be too low for those sera which were negative (8) before treatment and positive after. It appears that a 2- or 3-step rise is most frequently encountered. In no instance was a decreased titre obtained. Similar results were obtained with the

Table III CF antibodies to herpes simplex and morbilli virus in rheumatoid sera and controls

Serum	Positive (no.)	Negative (no.)	Positive (%)	GM titre of positives, log ₂	GM titre of total group, log ₂
Herpes simplex					
RF+	9	16	36	5.1	3.1
RF-	23	2	92	5.5	5.2
Morbilli					
RF+	8	15	35	5.5	2.9
RF-	22	1	96	6.0	5.8

GM = geometric mean.

Table V CF antibody titres after removal of RF by kaolin treatment

Kaolin-treated serum	Herpes simplex		Parotitis		Rubella		Morbilli	
	Positive (%)	GM titre	Positive (%)	GM titre	Positive (%)	GM titre	Positive (%)	GM titre
RF+	88	5.1	58	2.9	44	2.6	91	5.7
RF-	92	5.2	62	3.0	35	2.6	96	5.5

GM = geometric mean titre of total group.

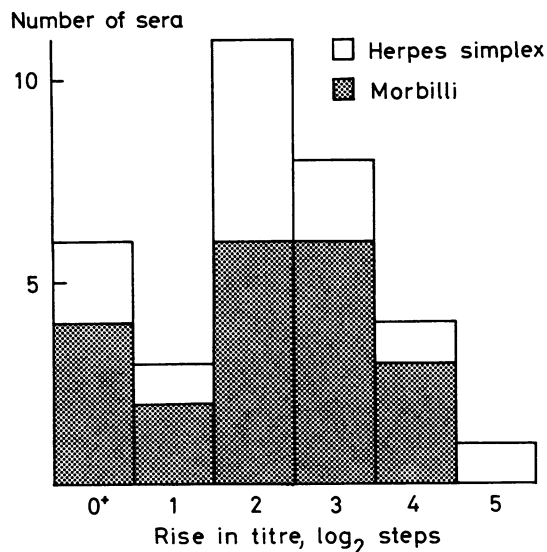


FIGURE Rise in CF titre after kaolin treatment of RF positive sera. * Sera which were CF positive before kaolin treatment

morbilli virus antigen. There was no correlation between the original Waaler-Rose titre and the degree of inhibition of CF.

Discussion

Several investigators have shown that sera exhibiting high RF titres interfere with the binding of complement to antigen-antibody complexes. The inhibitor(s) concerned can be removed by absorption with aggregated IgG, but there is no agreement among the investigators as to the nature of the inhibitor (Heimer and Levin, 1965; Stanford, 1972).

We have earlier reported that RF can be adsorbed to kaolin under conditions at which antibodies to viral and bacterial antigens are not bound (Haukenes and Aasen, 1972). The efficiency of this method with regard to removal of CF inhibitors was therefore examined. With antigens from herpes simplex, mumps, rubella, and morbilli viruses we obtained results which agree with those reported by Stanford (1972), i.e. low reactivity of RF-positive sera in the CF test. Treatment with kaolin restored the true CF

antibody titre of these sera, as compared with the matched RF-negative control group.

In two respects our results are in contrast to those of Stanford (1972). We found no inverse correlation between the RF titre value and CF antibody titres, and we did not obtain higher titres against herpes simplex virus in the kaolin-treated RF-positive group than in the control group. Furthermore, the degree of inhibition expressed by increase in titre on removal of RF did not correlate with the original RF titre. Thus, some sera with a Waaler-Rose titre of 320 showed a 2- to 3-step rise in CF titre, while some sera with a Waaler-Rose titre of 1,280 showed no rise in CF titre upon removal of RF. However, it should be pointed out that our material was not selected with the intention of elucidating the above questions, and there are several differences in the techniques used which do not allow a direct comparison with the results reported by Stanford (1972).

Previously reported results (Haukenes and Aasen, 1972) and unpublished results from column chromatography with a kaolin material have shown that most IgG is eluted at pH 7, and IgM at about 8, while mononucleosis antibodies and especially RF (Waaler-Rose and Latex) are strongly bound and elute at pH values above 9. Cold agglutinins, on the other hand, are eluted together with IgG. The choice of a pH of 8.5 to 9 did not result in a significant reduction of antibody titres, whereas the RF was held back by kaolin.

With rheumatoid or other RF-positive sera, or when a falsely negative CF test is suspected, we recommend pretreatment with kaolin before titration of CF antibodies. The procedure is simple and inexpensive, seems to give the same results as absorption with aggregated IgG, and is to be preferred to destruction of RF by for example heat or chemicals.

Summary

Sera with high titres of RF showed often falsely low CF antibody titres or a negative reaction with some viral antigens. Kaolin treatment of sera at pH 8.5 effectively removes RF but not antibodies to viral or bacterial antigens. This simple method proved to restore the 'true' CF antibody titre and is recommended as a pretreatment of sera when an RF-mediated CF interaction is suspected.

References

- AULETTA, A. E., GITNICK, G. L., WHITMIRE, C. E., AND SEVER, J. L. (1968) *Appl. Microbiol.*, **16**, 691 (An improved diluent for rubella hemagglutination and hemagglutination-inhibition tests)
- HALONEN, P. E., RYAN, J. M., AND STEWART, J. A. (1967) *Proc. Soc. exp. Biol. (N. Y.)*, **125**, 162 (Rubella hemagglutinin prepared with alkaline extraction of virus grown in suspension culture of BHK-21 cells)
- HAUKENES, G., AND AASEN, J. (1972) *Acta path. microbiol. scand.*, **80**, 251 (Heterogeneity in the reactivity of antibodies with kaolin)
- HEIMER, R., AND LEVIN, F. (1965) *Ann. N. Y. Acad. Sci.*, **124** (II), 879 (Inhibition of complement fixation by rheumatoid sera)
- KATZ, S. L., AND ENDERS, J. F. (1969) In 'Diagnostic Procedures for Viral and Rickettsial Infections', 4th ed., pp. 521-522, ed. E. H. Lennette and N. J. Schmidt. American Public Health Association, New York
- PENTHINEN, K., WAGER, O., PYRHÖNEN, S., LEINIKKI, P., AND TEPPÖ, A.-J. (1974) *Lancet* **1**, 367 (Negative complement-fixation tests as an immunopathological finding)
- STANFORD, F. (1972) *Ann. rheum. Dis.*, **31**, 330 (Comparison of complement-fixing antibody titres in patients with rheumatoid arthritis and matched controls)