EXPERIMENTAL INVESTIGATIONS INTO THE HAEMAGGLUTINATING FACTOR IN BLOOD AND JOINT FLUID IN RHEUMATOID ARTHRITIS*

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Shortly after Rose, Ragan, Pearce, and Lipman (1948) had published their paper on the agglutination of sensitized sheep cells in rheumatoid arthritis, investigations were started at the King Gustaf V Research Institute into the haemagglutinating factor that induces the reaction. The first reports of our experiments, presented by Svartz and Schlossmann (1949) and Svartz (1951a, b; 1952), were mainly concerned with the importance of removing the heterophil haemagglutinins which almost invariably exist in human serum, before carrying out the principal reaction. Similar observations were published by Sulkin, Pike, and Coggeshall (1952). The first attempts to absorb heterophil haemagglutinins had been made by Meyer (1922). Schlossmann and I have obtained positive results with our type of modification of the sheep cell test in 85 to 90 per cent. of cases of rheumatoid arthritis (Schlossmann and Svartz, 1950). The test was considered positive when the titre was 1 : 64 or higher. The frequency of positive reactions varied somewhat from one period to another depending on the nature of the cases tested. The more cases of active rheumatoid arthritis are included in a series the higher will be the percentage of positive reactions. But purely technical factors also influence the results. The sheep cells must be fresh and the amboceptor suitable, and the results should be read on two occasions: first when the

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tubes are taken from the refrigerator and then after they have stood for 2 hours at room temperature.

Sensitization.—Like other investigators, we have tried *inter alia* to elucidate the significance of the sensitizing process. The most important question is whether it is necessary—as is generally done—to let the amboceptor serum exert its influence on the blood corpuscles for some time before the final reaction is carried out.

Table I shows that the order in which the ingredients are mixed makes no difference. The erythrocytes need not be subjected to a sensitizing procedure before being used for the reaction. The rheumatoid factor may be added to the blood corpuscles first and the anti-erythrocyte serum (amboceptor-serum) may be added last, or all three ingredients may be mixed simultaneously. This opens up a new view of the problem of sensitization, though it is still not clear what conclusions may be drawn from these observations.

Differentiation from Other Diseases.—After rheumatoid arthritis, disseminated lupus erythematosus yields the highest percentage of positive reactions (about 30 per cent. clearly positive as well as a number of slightly positive tests). Scleroderma apparently comes third in regard to the percentage of positive reactions obtained.

TABLE I
THREE MAIN CONSTITUENTS OF HAEMAGGLUTINATION TEST MIXED TOGETHER IN DIFFERENT ORDER

Method	Order of Mixing			Titre
Routine	(1) Erythrocytes	(2) Amboceptor 2 hrs	(3) Patient Serum containing Rheumatoid Factor	1 : 2,048
Amboceptor added 2 hrs after Patient Serum	(1) Erythrocytes	(2) Rheumatoid Factor 2 hrs	(3) Amboceptor	1 : 2,048
Three Constituents Mixed Together Simultaneously	(1) Erythrocytes	(2) Amboceptor + Rheumatoid Factor		1 : 2,048

We then questioned whether the haemagglutinating factors in rheumatoid arthritis and in these other diseases were identical or could be differentiated. If the latter were the case, more specific reactions for the various diseases could probably be obtained. The investigations of Waaler (1940) showed that the haemagglutinating factor accompanies the serum gamma-globulins; Schlossmann and I therefore tested various globulin fractionation methods, and we obtained illuminating results by fractionating with ammonium sulphate and also with CO₂. The latter method demonstrated that the haemagglutinating substance invariably found in the blood of guinea-pigs behaves differently from the rheumatoid factor.

Precipitation in Cold.—This method brought the problem of specificity a good way nearer solution. Serum was diluted fourteen times with distilled water and then allowed to stand for 48 hrs at +4° C. The remarkable fact was then demonstrated that the haemagglutinating factor in rheumatoid arthritis could be precipitated in cold, whereas the haemagglutinating factor in other diseases remained as a rule in the supernatant fluid (Svartz and Schlossmann, 1953a, b; 1955; 1957). Cold precipitation thus proved to be an excellent means of avoiding "non-specific" reactions in rheumatoid arthritis, and this method has now been adopted for testing in various other laboratories. The method has also been modified so that, instead of our procedure of precipitation, centrifugation, and washing, dialysis has been used at the same temperature as in our experiments (Lospalluto and Ziff, 1956; Ziff, Brown, Lospalluto, Badin, and McEwen, 1956).

We then tried precipitation in cold with disseminated lupus erythematosus and scleroderma (Svartz and Schlossmann, 1957). Only one out of thirty cases of lupus erythematosus showed a positive cold precipitate reaction (Table II). The diagnosis in this case is not yet quite clear, as L.E. cells have been found to be present; since Kievits, Goslings, and Schuit (1956), Goslings and Kievits (1957), and Olhagen (personal communication) found L.E. cells in rheumatoid arthritis, this may be a case of severe rheumatoid arthritis resembling lupus erythematosus.

Only two cases of scleroderma showed a positive haemagglutination reaction, but one of them also showed a positive cold precipitate reaction (Table III). Olhagen has recently found that this patient had severe arthritis resembling rheumatoid arthritis, and an x-ray examination of the joints also showed changes typical of rheumatoid arthritis. This case therefore seems to show a combination of rheumatoid arthritis with scleroderma.

TABLE II
AGGLUTINATION BY WHOLE SERUM AND COLD PRECIPITABLE FRACTIONS IN PATIENTS WITH DISSEMINATED LUPUS ERYTHEMATOSUS

Number of Controlled Samples	Agglutination Titre	
	Whole Serum	Cold Precipitable Fraction
5	1 : 16	0
7	1 : 32	0
4	1 : 64	0
2	1 : 64	1 : 4
2	1 : 128	0
2	1 : 128	1 : 4
2	1 : 256	0
1	1 : 256	1 : 4
1	1 : 256	1 : 8
1	1 : 256	1 : 256
1	1 : 512	1 : 4
1	1 : 512	1 : 8
1	1 : 1,024	0

TABLE III
HAEMAGGLUTINATION TEST IN SCLERODERMA

Number of Patients	Whole Serum	Cold Precipitable Fraction
7	Negative	—
1	1 : 8,192	1 : 2,048
1	1 : 128	Negative
1	1 : 256	*
1	1 : 512	*
1	1 : 1,024	*

* The test was performed before 1952 when the cold precipitate reaction was not yet used.

The fact that the rheumatoid factor is precipitated in cold, whereas the haemagglutinating substances in lupus erythematosus, scleroderma, and other diseases are not, indicates that different substances are involved. The difference may not be great, yet it is wide enough for these substances to behave differently in cold fractionation.

Just as other biological reactions are not entirely specific for one disease only, cold precipitation is not entirely specific for rheumatoid arthritis. The diagnosis was not quite clear in the one case designated as lupus erythematosus and in the one case of scleroderma which gave a positive cold precipitation reaction. We have also observed weakly positive reactions in two cases of hepatitis, one case of myeloma, and a few cases of psoriasis.

Fractionation in cold is thus a first step towards isolating the rheumatoid factor.

Zone Electrophoresis.—These experiments were conducted by the method of Carlson (1954) in a special type of starch column; between 20 and 25 fractions were obtained. Fig. 1 (opposite) shows that only very small quantities of protein were found in the cold precipitate except in the gamma area.

Fig. 1.—Zone electrophoresis on redissolved cold precipitate from rheumatoid arthritis serum (8-fold concentrated).

Thus, the haemagglutination factor was demonstrable in the gamma-globulin fraction and in that part of the beta area bordering on the gamma area. Minor quantities of globulins outside these fractions did not give rise to haemagglutination.

Ultra-Centrifugation.—Prof. Hugo Theorell gave permission to conduct ultra-centrifugation experiments at his Institute, and this process has been carried out on a number of cold precipitates from rheumatoid arthritis serum since January, 1957, with the assistance of A. Ehrenberg, an engineer at the Institute (Svartz, 1957). A top and a bottom fraction were obtained by analytical ultra-centrifugation. The results are shown in Table IV. The top fractions had a sedimentation constant of 6 to 6.4 Svedberg, the bottom fractions 19 to 20 S. Thus, the bottom fraction contained macroglobulins. The molecular size was apparently about 1 million.

TABLE IV
ULTRA-CENTRIFUGATION (60,000 r.p.m. and 33 min.)
OF COLD PRECIPITATE FROM RHEUMATOID
ARTHRITIS SERUM

Cold Precipitate	Sedimentation Coefficient	Haemagglutination
Fraction I (top)	6.4 S	Negative
Fraction II (bottom)	19 S	Positive
Fraction I (top)	6.6 S	Negative
Fraction II (bottom)	19 S	Positive
Fraction I (top)	about 6 S	Negative
Fraction II (bottom)	about 20 S	Positive

Haemagglutination tests were conducted on both the top and the bottom fractions, and the reaction was negative on the top fraction and strongly positive on the bottom fraction. This proved that the haemagglutinating factor was present in a fraction containing macroglobulins.

Table V shows ultra-centrifugation performed twice on cold precipitate from the same rheumatoid arthritis serum, and the quantities of low and high molecular globulins. The percentage of macroglobulins in the bottom fractions was 42 to 44 per cent.

TABLE V

ULTRA-CENTRIFUGATION OF SAME COLD PRECIPITATE ON TWO DIFFERENT OCCASIONS

Date	Cold Precipitate	c : a 6.6 S (mg./ml.)	c : a 22 S (mg./ml.)	Haemagglutination Reaction
29.1.57	Bottom ..	2.3	1.4	Positive
	Top ..	4.3	3.4	Positive
		0.8	0	Negative
5.2.57	Bottom ..	c : a 6.4 S (mg./ml.)	c : a 20 S (mg./ml.)	Haemagglutination Reaction
	Top ..	2.3	1.4	Positive
		4.1	2.9	Positive
		0.7	0	Negative

Zone-electrophoresis was then carried out on the macroglobulin fraction (Fig. 2). The haemagglutinating factor was found exclusively in the gamma fraction; it appeared that the factor was still not isolated—which indeed was hardly to be expected.

Preparative ultra-centrifugation was also tried (Table VI). The bottom fractions were afterwards run in separation cells. Sedimentation constants from 19 to 25 S could be observed in these fractions.

It usually happened that the bottom fractions were somewhat turbid. The turbidity was not dissolved by dilution, but had to be removed by ordinary centrifugation.

TABLE VI

PREPARATIVE ULTRA-CENTRIFUGATION OF COLD PRECIPITATE FROM RHEUMATOID ARTHRITIS SERUM (497)

Fractions Numbered from Bottom	Haemagglutination Titre
1	1 : 512
2	1 : 1,024
3	1 : 1,024
4	1 : 1,024
5 (top)	1 : 16

Number 1 after Run in Separation Cell			
Cold Precipitate	Sedimentation Constant	mg./ml.	Haemagglutination Titre
Bottom ..	19 S + 25 S	0.58	1 : 256
Top ..	6.4 S	0.85	1 : 16

Muller-Eberhard, Kunkel, and Franklin (1956) have recently conducted experiments of a similar nature to those just described.

By means of serial ultra-centrifugation, experiments were made further to eliminate the low molecular globulins (Table VII).

TABLE VII

REPEATED ANALYTICAL ULTRA-CENTRIFUGATION OF REDISSOLVED COLD PRECIPITATE FROM RHEUMATOID ARTHRITIS SERUM (513)

Centrifugation	c : a 7 S (mg./ml.)	c : a 20 S (mg./ml.)	C20/C7	Haemagglutination Titre Bottom Fractions
I	3.6	3.2	0.9	1 : 512
II	1.7	2.1	1.2	1 : 256
III	1.3	1.0	0.8	1 : 64
IV	0.65	1.15	1.8	(precipitated) 1 : 128
V	0.4	0.8	2	1 : 64
Mixed Top Fractions				1 : 32

The fraction from the third centrifugation showed a sudden decrease in haemagglutination titre. There were signs of precipitation which could not be dissolved on the walls of the separation cell. It is obvious that a great deal of the haemagglutinating factor had followed the precipitate. We also noted

another difference between the second and third bottom fractions; there were two peaks at the level of 19 to 25 S in Fraction II, but in Fraction III the globulins with the higher sedimentation constant had disappeared.

As to the evaluation of the different sedimentation constants in human sera, the experience of Wallenius, Trautman, Kunkel, and Franklin (1957) has been very useful.

Chromatography.—Paper chromatography on re-dissolved cold precipitate proved unsuitable, and other types of chromatography were tried, in collaboration with Dr. L. Carlson.

Chromatography with a phosphate column yielded no results of importance.

Chromatography with carboxy-methyl cellulose by the method of Peterson and Sober (1956) proved very valuable. A glass cylinder was filled with carboxy-methyl cellulose, which is a cation exchanger, as in other forms of column chromatography. Cold precipitate from serum containing the rheumatoid factor was dissolved in phosphate buffers with different pH and allowed to filter through the carboxy-methyl cellulose (Fig. 3). In this way, 30 fractions were obtained, and in three of these the existence of a strong haemagglutinating factor could be demonstrated. In another peak, traces of haemagglutination factor were found.

Fig. 4 shows another example of chromatography with carboxy-methyl cellulose. Chromatography was also carried out on the bottom fraction from ultra-centrifugation with similar results. Repeated chromatography proved capable of isolating the rheumatoid factor to a certain extent.

Fig. 3.—Chromatography of cold precipitate from rheumatoid arthritis serum on carboxy-methyl cellulose.

Fig. 4.—Chromatography of cold precipitate on carboxy-methyl cellulose.

Inhibition.—Research on the inhibition of the haemagglutination reaction is being carried on by Ragan, Heller, Ziff, and many other workers. The question has been very intensely studied by Ziff and his colleagues, who have investigated inhibiting substances in the euglobulin fraction of human serum. We have tried his method but we have not obtained the same high percentage of inhibiting substances in sera from healthy subjects.

Winblad (1952) and Svartz and Schlossmann (1953a, b, 1956, 1957) have demonstrated the presence of inhibitors in normal rabbit serum and pointed out that such serum often

normally contains an inhibitor. By injecting rabbits with rheumatoid arthritis serum from which heterophil agglutinins had been removed, we were able to produce a factor which neutralized the rheumatoid factor at a high titre. Also the anti-rabbit serum was previously absorbed with normal red cells. These experiments show that the rheumatoid factor may act as an antigen.

We have also carried out experiments designed to inhibit the haemagglutination reaction by means of various drugs, including cortisone, gold, aspirin, and penicillin. They did not cause any inhibition, but the sulpha drugs were found to have the strongest effect *in vitro* (Table VIII). This should also be tried *in vivo*, but so far it has only been possible to show the effect with a concentration that can never be induced in a human being.

TABLE VIII
SULPHONAMIDE COMPOUNDS PRODUCING COMPLETE INHIBITION OF HAEMAGGLUTINATION REACTION

Types of Compounds	Lowest Concentration showing Inhibition
<i>Elkosin</i> : 6-sulphanilamido-2,4-dimethylpyrimidine sodium (20%)	1:20 : 100
<i>Dimezathine</i> : 2-p-aminobenzolsulphonamid-4,6-dimethylpyrimidine (33%)	0:52 : 100
<i>Sulphadimine</i> : 2-sulphanilamido-4-methylpyrimidine (20%)	1:20 : 100
<i>Sulphapyridine</i> : Sulphanilamidopyridine (20%)	1:20 : 100
<i>Sulphonazole</i> : 2-sulphanilamidothiazole (20%)	0:62 : 100
<i>Diamox</i> : 2-acetyl-amino-1,3,4-thiadiazole-5-sulphonamide sodium (10%)	0:31 : 100
<i>Sulphanilamide</i>	0:63 : 100
<i>Acetylsulphanilamide</i>	No Inhibition

In collaboration with L. O. Plantin and the Pharmacia Drug Company, attempts have been made to discover which radicals are necessary for the inhibiting activity of the sulpha drugs. Table VIII shows that sulphanilamide exerts a pronounced inhibiting effect on the haemagglutination reaction. If a hydrogen of the amino group is replaced by acetyl, the inhibitory power is lost. Our investigations in this field are being continued.

Summary

The haemagglutination of pre-treated red cells can be produced by serum from different diseases. These haemagglutinating substances are not identical—that in lupus erythematosus differing, for example, from that in rheumatoid arthritis.

Svartz and Schlossmann (1953a, b; 1955) showed that the haemagglutination factor was precipitable in cold in rheumatoid arthritis, but not—or only very seldom—in other diseases, such as disseminated lupus erythematosus and scleroderma.

Various attempts were made to isolate the cold precipitable factor in rheumatoid arthritis, by zone-

electrophoresis, ultra-centrifugation, and chromatography with carboxy-methyl cellulose.

By using zone-electrophoresis of cold precipitate, 20-25 protein fractions were obtained. The rheumatoid factor was found to be present mainly in the gamma-globulin area.

Ultra-centrifugation of cold precipitate from rheumatoid arthritis serum demonstrated that the rheumatoid factor was associated with a macroglobulin fraction showing a sedimentation constant of 19-25 Svedberg. The fractions containing low-molecular globulins did not evoke haemagglutination. Serial centrifugation in ultra-centrifuge was also performed.

The best method of isolating the rheumatoid factor so far attained is precipitation in cold followed by alternating ultra-centrifugation and chromatography with carboxyl-methyl cellulose.

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Études expérimentales du facteur hémagglutinant dans le sang et le liquide synovial dans l'arthrite rhumatoïdale

RÉSUMÉ

Des sérums de différentes maladies peuvent produire l'hémagglutination des globules rouges préparés. Ces substances hémagglutinantes ne sont pas identiques—celles de lupus érythémateux étant différentes, par exemple, de celles de l'arthrite rhumatoïdale.

Svartz et Schlossmann (1953a, b; 1955) ont montré que le facteur hémagglutinant était précipitable par le froid dans l'arthrite rhumatoïdale, mais pas—ou seulement très rarement—dans les autres maladies, telles que le lupus érythémateux disséminé et la sclérodermie.

Des tentatives variées ont été faites pour isoler le facteur précipitable au froid dans l'arthrite rhumatismale, par zones d'électrophorèse, ultra-centrifugation et chromatographie à l'aide de la carboxy-méthyl-cellulose.

L'électrophorèse du précipité à froid donna de 20 à 25 fractions protéiques. Le facteur rhumatismal se révèle présent surtout dans la zone de globuline gamma.

L'ultra-centrifugation du précipité à froid de sérum provenant d'arthrite rhumatismale démontra que le facteur rhumatismal était associé à une fraction de macroglobuline ayant une constante de sédimentation de 19 à 25 Svedberg. Les fractions contenant des globulines à poids moléculaire bas ne provoquaient pas d'hémagglutination. La centrifugation en série en ultra-centrifugeuse fut aussi effectuée.

Jusqu'à présent, la meilleure méthode d'isoler le facteur rhumatismal est la précipitation à froid, suivie par l'ultra-centrifugation et la chromatographie à l'aide de la carboxy-méthyl-cellulose, alternantes.

Investigaciones experimentales del factor hemaglutinante en la sangre y el líquido articular en la artritis reumatoide

SUMARIO

Sueros de enfermedades diferentes pueden producir la hemaglutinación de eritrocitos preparados. Estas

substancias hemaglutinantes no son idénticas—las del lupus eritematoso siendo, por ejemplo, diferentes de las de la artritis reumatoide.

Svartz y Schlossmann (1953a, b; 1955) demostraron que el factor hemaglutinante fué precipitable por el frío en la artritis reumatoide, pero que no lo fué—o sólo muy raramente—en otras enfermedades, como el lupus eritematoso diseminado y esclerodermia.

Se hicieron varias tentativas para aislar el factor precipitable por el frío en la artritis reumatoide, por zonas de electroforesis, ultra-centrifugación y cromatografía con carboxi-metil-celulosa.

La electroforesis del precipitado frío dió de 20 a 25 fracciones proteínicas. El factor reumático fué presente principalmente en la zona de la globulina gama.

La ultra-centrifugación del precipitado frío del suero procedente de sujetos con artritis reumatoide mostró que el factor reumático estuvo asociado a una fracción de macroglobulina con una constante de sedimentación de 19-25 Svedberg. Las fracciones conteniendo globulinas de peso molecular bajo no provocaban hemaglutinación. Se procedió también a la centrifugación en serie en ultra-centrifuga.

El mejor método que se conoce hasta ahora para aislar el factor reumático es la precipitación por el frío, seguida de ultra-centrifugación y de cromatografía con carboxi-metil-celulosa, alternantes.