Identification of the Substance Active in Sheep Cell Agglutination Test for Rheumatoid Arthritis. (20768)

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Rose *et al.*(1) proposed a sheep erythrocyte agglutination test for rheumatoid arthritis in which the serum of arthritic patients, previously treated to remove a non-specific agglutinating factor, specifically agglutinates sheep red cells sensitized with sheep cell antibody. Freund and Stulberg(2), and others (3,4) have introduced modifications to increase the sensitivity of this test and have made studies of its reliability. The electrophoretic pattern of plasma from arthritic patients shows the presence of a large amount of mucoprotein(5,6) and gamma globulin (7-9), and a characteristic peak in the descending boundary pattern(10). The factor responsible for the agglutination was reported to be a globulin(1). We have attempted to concentrate the plasma factor responsible for this test and to determine its chemical nature.

Methods and materials. The sheep cell agglutination test(1) as modified by others (3,4) was further modified and used as follows: A. Preparation of Red Blood Cells. Sheep red blood cells, preserved with Alsever's solution, were washed 3 times with physiological saline, and used as packed cells, and as 1% and 0.5% suspensions in saline. B. Preparation of Sensitized Red Blood Cells. Commercial anti-sheep red cell hemolysin was diluted in serial 2-fold dilution, and 0.5 ml of each dilution was mixed with 0.5 ml of a 0.5% suspension of sheep cells. The mixtures were incubated for one hour at 37°C, allowed to stand overnight at 4°C, and then examined for agglutination of the red cells. The agglutinating titer of hemolysin was designated as the highest dilution which produced agglutination. Hemolysin solution containing ½ agglutinating titer was incubated with an equal volume of a 1% suspension of sheep red cells for 30 minutes at room temperature. The sheep cells were now designated "sensitized". C. Preparation of Patients' Plasma and Observations of Hemagglutination Titers. The normal agglutinating component in plasma was removed by heating each plasma specimen at 56°C for 30 minutes and then adsorbing the factor on sheep red cells added in the ratio of one volume of packed cells to 4 volumes of plasma. After 40 minutes the red cells were removed by centrifugation and the plasma treated a second time with sheep cells. The plasma was then diluted in serial 2-fold dilution to a final concentration of 1/5120, and 0.5 ml of sensitized sheep cells added to 0.5 ml of each dilution. Control tubes were prepared by diluting plasma 1/10, 1/20, and 1/40 and treating 0.5 ml of each dilution with 0.5 ml of a 0.5% suspension of unsensitized red cells. All tubes were incubated at 37°C for one hour, allowed to stand overnight at 4°C, and the degree of agglutination read as 1+, 2+, 3+ or 4+. The number of agglutination units present in 100 ml of undiluted plasma was defined as the number of dilutions required to give a 1+ agglutination test under the conditions of the test. The electrophoretic patterns were obtained (11,12) with the Longsworth modification of the Tiselius instrument using an acetate buffer pH 4.5 for mucoprotein, and a barbital buffer pH 8.5 for the other plasma proteins.

Results. A comparison of the amount of the agglutinating factor with the amount of mucoprotein in the plasma of normal and of diseased patients indicates that the agglutinating factor is not mucoprotein (Table I). High levels of agglutinating factor were always associated with high levels of mucoprotein. However, low levels of agglutinating factor, particularly in cancer, were also associated with high levels of mucoprotein. In addition, it was found that mucoprotein prepared from arthritic blood having a high agglutination titer, by the method of Mehl *et al.*(13) caused no agglutination in the absence of other serum proteins. In order to concentrate the agglutination factor, pooled

TABLE I. Relation of Amount of Mucoprotein to Amount of Agglutinin in Plasma.

Patient	Diagnosis	Total protein, g/100 ml	Mucoprotein, relative amt	Agglutination, units*
R	Normal	7.28	1	20
W		6.89	1	10
S		7.31	1	20
N	R.A.†	8.10	3	5120
Wo		9.59	3	80
D		8.88	3	5120
O		8.00	2	1280
P		9.53	1.5	640
F		7.60	2.5	80
C	Cancer	6.03	3	20
C		6.99	2	40
Sh	Still's disease	7.53	4	160

* For definition, see text.
† R.A.—Rheumatoid arthritis.

samples of arthritic plasma were fractionated by the Cohn ethanol method(14), by the use of sodium sulfate(15) and by dialysis and precipitation at low ionic strength. The addition of alcohol to arthritic sera caused the loss of all agglutinating activity. This was unexpected in view of a report by others(16) that lipid extraction with an alcohol-ether mixture does not destroy this factor.

In the fractionation of the serum of arthritic patients by the addition of sodium sulfate (Table II), the precipitate obtained at a concentration of 9% contained only a small fraction of the agglutination factor present in the plasma. Further addition of sodium sulfate to a concentration of 14% gave an additional small amount of protein precipitate which contained the highest concentration of agglutination factor, per gram of protein, of any fraction obtained. Further addition of sodium sulfate to a concentration of 18.6% caused the precipitation of protein possessing little activity; the supernatant solution was almost devoid of activity. The activity present in the original arthritic pool was recovered quantitatively in the various fractions within the rather wide limits of error of the method of determination. The electrophoretic examination of the active precipitate after extensive washing with the precipitating salt solution revealed the presence of alpha, beta, and gamma globulin. Rose *et al.*(1) reported that the activity was present in electrophoretically separated beta-gamma globulin fraction.

For the separation of the agglutination factor by dialysis, 100 ml of pooled plasma was dialyzed in Visking tubing against successive 4000 ml volumes of phosphate buffer of pH 6.8 and ionic strength varying from 0.1 to 0.01 (Table III). The precipitate formed after each period of dialysis was removed by centrifugation and the serum then replaced in the Visking tubing and dialyzed against a buffer of lower ionic strength. Dialysis was carried out at 4°C. For each dialysis 2 days were required for the composition of the 2 solutions to reach equilibrium. High concentrations of the agglutination factor were obtained in the fractions precipitated at ionic strength 0.1 and at ionic strength 0.06 to 0.04. This might suggest that the substance is not a single factor. The precipitation of the agglutination factor at ionic strength 0.1, however, may well be attributed to adsorption on fibrinogen, which is a principal constituent of this fraction, rather than to precipitation as the free factor. The protein present in this fraction had an electrophoretic mobility lower

TABLE II. Sodium Sulfate Fractionation of Plasma of Arthritic Patients.

Fraction*	Protein, g/100 ml plasma	Agglutination, units†	Agglutination, units/g protein
1. Whole plasma	6.10	640	110
2. Defibrinated plasma	5.70	1230	220
3. Precipitate from 9% Na_2SO_4	.30	20	70
4. Supernatant from (3)	5.10	640	130
5. Precipitate from 9-14% Na_2SO_4	.36	640	1600
6. Precipitate from 14-18.6% Na_2SO_4	1.72	160	90
7. Supernatant from (6)	3.00	20	6

* Precipitates were formed by successive addition of sodium sulfate to indicated concentrations. Concentrations are expressed as g/100 ml of final solution.
† For definition, see text.

TABLE III. Fractionation of Arthritic Plasma by Reduction of Ionic Strength.

Fraction*		Protein,† g/100 ml	Mobilities of electrophoretic peaks, $cm^2 volt^{-1} sec^{-1} \times 10^{-5}$	Agglutination, units†	Agglutination, units/g protein
1. Whole plasma		8.0	6.1 5.1 4.0 2.9 2.1 1.2	640	80
2. Precipitate at ionic strength	.10	.08	2.0	40	500
3. " " " "	.08	.12	4.8 2.4 1.1	20	166
4. " " " "	.06	.40	6.1 3.0 2.2	160	400
5. " " " "	.04	.21	3.2 6.3	160	800
6. " " " "	.02	.54	4.0 3.2 2.7	320	590
7. " " " "	.01	.50	6.3 3.9 3.1 2.5 1.0	40	80

* Precipitates were formed by successive dialyses of plasma of arthritic patients against phosphate buffer solutions of pH 6.8 and varying ionic strengths.

† As g of protein or agglutination units in 100 ml of plasma or the precipitate from 100 ml of plasma.

than that of fibrinogen (2.0 compared with 2.4 x 10^{-5} cm^2 $volt^{-1}$ sec^{-1}), thus suggesting that the fibrinogen might be adsorbed to another protein. In addition, it was observed that in those experiments in which clotting occurred during dialysis, a considerable portion of the agglutination factor was adsorbed on the clot. This adsorbed factor could be removed from the clot only by prolonged washing with physiological saline. When the clot was lysed with fibrinolysin, the agglutination factor was also destroyed.

Those fractions precipitated at ionic strength between 0.08 and 0.02 contained relatively high concentrations of agglutination factor. The electrophoretic patterns of these fractions showed peaks corresponding to those of albumin, alpha and beta globulin, and fibrinogen, 6.0, 4.5, 2.9, and 2.4 x 10^{-5} cm^2 $volt^{-1}$ sec^{-1}, respectively. However, the only peak appearing in all the fractions exhibiting agglutinating activity is the beta peak (μ = 2.0-3.2 x 10^{-5} cm^2 $volt^{-1}$ sec^{-1}). The agglutination factor appears to be either precipitated as such or to be adsorbed on the beta globulin and then precipitated with this fraction. The pool of arthritic blood plasma exhibited an electrophoretic anomaly in the beta fraction similar to that reported by Reed *et al.*(10). This anomaly was not observed in the electrophoretic patterns of the concentrates.

A further attempt at purification of the agglutination factor consisted of an initial precipitation with sodium sulfate, added in the amount of 18.6 g per 100 ml of final solution, and subsequent fractionation by dialysis and lowering of the salt content. The highest titer of agglutinating factor was again found in the fraction precipitating between 0.06 and 0.04 ionic strength. The activity of the various fractions and the electrophoretic analyses of the precipitates were essentially the same as for the previous experiments.

When the concentrated fractions of agglutination factor were lyophilized, all activity was lost. This was unexpected as whole plasma containing the active fraction may be kept indefinitely when treated in this manner.

Summary. The active principle responsible for sheep cell agglutination test for arthritis was purified and concentrated by salt fractionation and by dialysis against solutions of low ionic strength. The most active fractions were precipitated as plasma was adjusted from a concentration of 9% to a concentration of 14% sodium sulfate, or from ionic strength 0.06 to ionic strength 0.04. The electrophoretic fraction most consistently present in the concentrate was beta globulin. The agglutination factor is adsorbed on fibrin and is inactivated during lyophilization of concentrates and by the addition of alcohol to arthritic plasma.

1. Rose, H. M., Ragan, C., Pearce, E., and Lipman, M. O., PROC. SOC. EXP. BIOL. AND MED., 1948, v68, 1.

2. Freund, H. A., and Stulberg, C. S., *J. Mich. Med. Soc.*, 1950, v49, 1084.

3. Pike, R. M., Sulkin, S. E., and Coggeshall, H. C., *J. Immunol.*, 1949, v63, 441.

4. Heller, G., Jacobson, A. S., and Kolodny, M. H., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 316.

5. Weimer, H. E., Mehl, J. W., and Winzler, R. J., *Fed. Proc.*, 1950, v9, 244.

6. Kelly, V. C., Good, R. A., and McQuarrie, *J. Pediatrics*, 1950, v5, 824.

7. Lovgren, O., *Acta Medica Scandinavica*, Supplementum CLXIII, 1945, 61.

8. Dole, V. P., and Rothbard, S., *J. Clin. Invest.*, 1947, v26, 87.

9. Perlman, G. E., and Kaufman, D., *ibid.*, 1946, v25, 931.

10. Reed, A. F., Pike, R. M., Sulkin, E., and Coggeshall, H. C., *J. Lab. Clin. Med.*, 1951, v37, 264.

11. Mehl, J. W., Golden, F., and Winzler, R. J., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 110.

12. Coryell, M. N., Beach, E. F., Robinson, A. R., Macy, I. G., and Mack, H. C., *J. Clin. Invest.*, 1950, v29, 1559.

13. Mehl, J. W., Humphrey, J., and Winzler, R. J., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 106.

14. Cohn, E. G., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J., Melin, M., and Taylor, H. L., *J. Am. Chem. Soc.*, 1946, v68, 459.

15. Majoor, C. L. H., *J. Biol. Chem.*, 1947, v169, 583.

16. Pike, R. M., Sulkin, S. E., and Coggeshall, H. C., *J. Immunol.*, 1949, v63, 447.

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Effects of Cortisone on Blood Cells after Thermal Stress.* (20769)

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In the course of experiments on inflammatory phenomena (to be published) rabbits were subjected to extensive thermal injuries, and data on erythrocytes, leukocytes, and temperature were recorded. It was considered worthwhile to make a parallel series of records on rabbits treated with cortisone. A comparison of these is the basis for this report.

Methods. Male New Zealand whites weighing 1800 g to 2300 g were used. They were given a routine diet of rabbit pellets, greens and water during a period of one to 3 weeks and frequent blood counts were made to establish the norm for each animal. None was used if significant variations in blood count, temperature, or behavior indicated that the animal's health was not normal. Experiments were made on 4 groups of 10 each. The fur over the back, shoulders, sides and hips was sheared closely with clippers. The rabbit was anesthetized deeply with ether and the areas mentioned were dipped for 5 seconds into water at a temperature of 88° to 95°C. Pain was prevented by frequent injections of morphine during the subsequent 24 hours. Blood counts and temperature were taken at intervals ranging from 30 minutes to 7 hours

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