may conceivably have been due to accidental or spontaneous infection of this animal with *S. moniliformis*, since this microorganism is extremely common in rat colonies and considerable vigilance is required to eliminate it completely.

It will be of interest to determine whether the sensitized sheep cell agglutinins of the rat are gamma globulins with a sedimentation constant of approximately 19S, as in the human serum studies of Franklin *et al.*, and whether these tend to complex with 7S gamma globulins to form complexes of 22S or greater in the same manner as in human rheumatoid arthritis. Since the current series of rats have developed low titres, and Franklin *et al.*(24) found that only high titre sera showed demonstrable complexes of 22S or greater, it may be necessary to maintain chronic or recurrent infections in order to achieve high concentrations of the high molecular weight complexes in rat sera. On the other hand, it may be necessary to investigate this phenomenon in other species.

Summary. 1) Rats infected with a strain of *Streptobacillus moniliformis* developed an inflammatory reaction in joint regions in 109 of 116 animals. 2) Sensitized sheep cell agglutination titres of the serum euglobulin tested from 55 infected rats were significantly higher than those of 53 uninfected controls.

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Observations on Mechanism and Prevention of Non-Specific Agglutination of Leukocytes.* (24313)

THOMAS E. BRITTINGHAM† (Introduced by Carl V. Moore)

Dept. of Internal Medicine, Washington University School of Medicine, St. Louis, Mo.

Leukocytes have a striking tendency to agglutinate non-specifically. Thus, leukocytes obtained by sedimentation from normal whole blood and incubated with any normal un-

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† Public Health Research Fellow of Nat. Heart Inst.

treated serum promptly and invariably agglutinate. The present study suggests that such non-specific clumping is caused by polymerization of fibrinogen adsorbed by leukocytes, and demonstrates that the clumping may be avoided either by decalcifying test sera with disodium ethylene tetraacetate or by using leukocytes obtained from defibrinated blood rather than from whole blood. A study of the mechanism and prevention of non-specific clumping of white blood cells is relevant to the construction of a satisfactory leuko-agglutinin test.

Methods. Preparation of suspension of leukocytes: 16 ml of blood were obtained from normal human donors by clean venipuncture; the blood was allowed to flow directly into a 12 × 125 mm siliconed glass tube containing 0.4 ml of a 5% solution in distilled water of disodium ethylene diamine tetraacetate (this 5% solution is hereafter referred to as EDTA) for anti-coagulation. After being inverted 10 times for mixing, the tube was tilted to a 45° angle from the horizontal, placed in the refrigerator, and the blood allowed to sediment for 2½ hours. The entire supernatant (usually 4-5 ml in volume), including the grossly visible layer of white cells immediately on top of the erythrocytes, was then removed with a capillary pipet and transferred to a 10 × 100 mm siliconed glass tube, tube B. Tube B was centrifuged at only 200 RPM for 15 minutes in a centrifuge of radius 12 cm (radius measured to top of centrifuge cup). After centrifugation, a small button of cells was present at the bottom of Tube B. After removal of the supernatant as completely as possible by capillary pipet, the residual cell button was suspended in approximately 2.5 ml of autologous (*i.e.* obtained from the donor of the particular white cells being used) serum and incubated until reading. The autologous test serum was untreated except where otherwise specified. Typical cell counts of the incubation mixture were leukocytes 18,000 per cu mm, erythrocytes 9,000 per cu mm, and platelets 120,000 per cu mm. When anticoagulants other than EDTA were used, leukocytes were obtained in an identical manner. For 16 ml of blood the following quantities of anticoagulant were used: (1) 1.6 ml 3.2% sodium citrate; (2) 1.6 ml mixed oxalate solution (2.4 g ammonium oxalate and 1.6 g potassium oxalate dissolved in 100 ml distilled water); (3) 0.12 ml heparin solution (1000 International Units per ml); (4) 1.8 ml acid-citrate-dextrose solution (formula A, USP). The procedure for obtaining leukocytes from defibrinated blood was also essentially the same, except that such blood was initially sedimented for only 2 hours, then was centrifuged for 30 minutes at 500 rpm in order to increase the initial yield of supernatant. Blood was defibrinated by taking 16 ml of blood into a glass test tube containing 6 glass beads, inverting the tube regularly for 5 minutes, then removing the resultant clot with a wooden applicator stick, leaving about 12-13 ml of defibrinated blood in the tube. The incubation mixtures were examined for agglutination by gently shaking the incubation tube to suspend the leukocytes, transferring about 0.15 ml of the leukocyte suspension to a micro test slide, depressed cell type, and reading microscopically at 100 × magnification. In an unagglutinated suspension each leukocyte lay about equidistant from its neighbor, as if there were spheres of mutual repulsion about each cell. In an agglutinated suspension, on the other hand, there were in each low power field, 15 or more tight clumps, each containing 5 or more leukocytes.

Results. Agglutination of leukocytes in normal untreated serum. Leukocytes taken from whole blood anticoagulated with EDTA were invariably clumped when suspended in autologous serum not containing an anticoagulant. Clumping was visible microscopically within 60 seconds after suspension of the leukocyte button in the serum. The results were the same whether the serum was fresh, or whether thrombin had been inactivated either by storage of the serum for several days at 4°C., or by heating to 57°C for 30 minutes. The results were also the same if isologous (from normal humans other than the leukocyte donor) sera were used (Table I). About half of the leukocytes participated in the numerous small (5-20 cells) tight clumps seen, while the other half remained unagglutinated. After several hours of incu-

TABLE I. Effect of Normal Sera and Plasmas on Normal Leukocyte Suspensions. Mixtures incubated at 37°C for one hour.

Test serum	Leukocytes obtained from whole blood anticoagulated with EDTA		Leukocytes obtained from defibrinated blood	
	Donor 1	Donor 2	Donor 1	Donor 2
Autologous				
Fresh	+	+	0	0
Aged*	+	+	0	0
Heated†	+	+	0	0
Isologous				
Fresh	+	+	0	0
Aged*	+	+	0	0
Heated†	+	+	0	0
Autologous, EDTA added‡	0	0	0	0
Isologous, EDTA added‡	0	0	0	0
Autologous plasma (EDTA)	0	0	—	—
Isologous plasma (EDTA)	0	0	—	—

* Test serum stored for 14 days at 4°C prior to use.

† Test serum heated to 57°C for 30 min. prior to use.

‡ EDTA added to concentration of 0.4 ml EDTA in 8 ml test serum.

+ = agglutination present. 0 = agglutination absent.

bation, all of the clumped cells adhered tightly together in a single mass. The agglutinated white cells seemed to include a disproportionately large number of the neutrophils present, and correspondingly a disproportionately small number of the lymphocytes. Virtually all of the platelets in the incubation suspension became involved in the leukocyte clumps, but the erythrocytes remained entirely unclumped. The sera from all 26 normal persons tested have shown this same phenomenon of agglutinating autologous leukocytes, but both acute leukemic leukocytes and the lymphocytes from chronic lymphocytic leukemic blood showed much less tendency than normal cells to agglutinate when incubated in untreated autologous serum. White cells taken from whole blood anticoagulated with agents other than EDTA, *i.e.* sodium citrate, mixed oxalate solution, heparin, or acid-citrate-dextrose solution, behaved just as did cells taken from EDTA blood in being promptly clumped by autologous serum not containing an anticoagulant.

Prevention of the agglutination of leukocytes in normal serum. If leukocytes were simply prepared from defibrinated rather than whole blood, they were never agglutinated by normal serum, even after several hours of incubation. For technical reasons, however, it may be advantageous in leuko-agglutinin testing to use leukocytes prepared from whole blood rather than from defibrinated blood. It was then found that non-specific agglutination of white cells obtained from whole blood (anticoagulated with EDTA) could be prevented by incubating the cells with EDTA plasma instead of serum, or by adding EDTA in adequate amount to the test serum. EDTA must be added to a concentration (0.4 ml EDTA in 8 ml of test serum) equivalent to that used for anticoagulating whole blood. The results of a typical experiment are summarized in Table I. White blood cells could be incubated in serum containing an adequate concentration of EDTA for more than 8 hours (at 4°C or 20°C or 37°C) without showing any agglutination whatever, although eventually clumping did occur. While non-specific clumping was suppressed by adding EDTA to the serum being tested, the action of pathologic leuko-agglutinins remained readily demonstrable and apparently unaffected by presence of EDTA.

Miscellaneous observations on EDTA action in preventing non-specific agglutination. EDTA acts by preventing agglutinates, not by dispersing them, for the agglutinates persisted if the EDTA was added to the test serum only after they had formed. If too low a concentration of EDTA (*e.g.* less than 0.2 ml EDTA in 8 ml of test serum) was used, non-specific clumping slowly occurred within the first 2 hours of incubation. Even the usually adequate concentration of EDTA in test serum did not prevent non-specific clumping of leukocytes if an excess of calcium ion was added to the serum at any time. On the other hand, if the serum had already been partially decalcified (by adding oxalate and centrifuging), a much lower concentration of EDTA than the usual adequate one sufficed

TABLE II. Effect of Different Anticoagulants in Preventing Non-Specific Leukocyte Agglutination. Cells incubated at 20°C with autologous serum.

Hr of incubation	Anticoagulant used				
	EDTA	Oxalate	Citrate	ACD	Heparin
0	0	0	0	0	+
1	0	0	+	+	+
4	0	0	+	+	+
8	0	0	+	+	+
12	0	+	+	+	+
16	+	+	+	+	+

Same anticoagulant was used in both incubation serum and in the whole blood from which leukocytes were obtained.

+ = agglutination present. 0 = agglutination absent.

to prevent false agglutination. Thus, the amount of EDTA effective in preventing non-specific clumping of white cells appears to be that amount necessary for complete decalcification of the test serum.

Relative effects of different anticoagulants in inhibiting non-specific agglutination. Leukocytes were obtained from whole blood and were incubated with autologous sera containing exactly twice the concentration of the same anticoagulant used in the whole blood from which the leukocytes were obtained. White cells from 10 normal donors were tested with each of 5 different anticoagulants. EDTA was repeatedly found to be the most effective anticoagulant in preventing non-specific agglutination (*i.e.* the longest periods of incubation elapsed before cell clumping began), but the potassium and ammonium oxalate mixture was almost as effective. Both sodium citrate and the acid-citrate-dextrose solution were distinctly less effective, some white cell agglutination usually being present after only one hour of incubation. Heparin (several different commercial preparations were tried) was by far the least useful anticoagulant, leukocytes and platelets in heparinized blood usually agglutinating within a few minutes of beginning sedimentation and long prior to beginning incubation. The results of a typical experiment are summarized in Table II. The results were unchanged when autologous plasma was used instead of serum with an added anticoagulant. Of interest was the fact that when we tried to use the mixed oxalate solution in half the usual concentration, it was, like EDTA, much less effective in preventing leukocyte agglutination than when in its usual concentration.

Mechanical trapping of cells in a fibrin clot not responsible for non-specific agglutination. It seemed possible that the agglutination of normal leukocytes by autologous serum was caused by simple mechanical trapping of the leukocytes in tiny pieces of fibrin clot formed from the small amounts of free fibrinogen inevitably present in the cell suspension when cells had been obtained from whole blood. To test this possibility, leukocyte suspensions were prepared from defibrinated blood, and to the cells during a usual incubation was added 0.02 ml of autologous plasma. The fibrinogen in the plasma was promptly clotted by the excess of calcium present. Microscopically, one saw occasional red and white cells trapped loosely in the clot. However, at no time did any leukocyte clumping occur, either inside or outside the clot. In addition, white cells were taken from whole blood and washed 2 times in saline (with added EDTA) to remove all but traces of free fibrinogen. Such cells were still clumped promptly on addition of autologous untreated serum though there was so little free fibrinogen present that no visible fibrin clot ever formed in such a preparation. Thus, mechanical trapping of leukocytes in a clot does not apparently cause the phenomenon of non-specific agglutination of white blood cells.

Discussion. The results of these experiments can be most easily interpreted by assuming that fibrinogen is adsorbed on the surface of some leukocytes, and that when polymerization of the fibrinogen occurs, as in the clotting process, cell surfaces are joined by fibrinogen-fibrinogen bonds, and "agglutination" of the leukocytes takes place. Such non-specific agglutination can be prevented by inhibiting the clotting process (*e.g.* by decalcifying test serum as well as cell preparation), or by using white blood cells which do not contain adsorbed fibrinogen, that is, by using white cells separated from defibrinated blood (see below).

More direct evidence that leukocytes do adsorb fibrinogen was provided by Seligmann, *et al.*(1), who found, using sensitive immu-

nologic technics, fibrinogen to be present in the extracts of washed leukocytes prepared from whole blood, but not in the extracts of leukocytes prepared from defibrinated blood. That normal leukocytes adsorb plasma protein whereas normal erythrocytes apparently adsorb almost none is not surprising in light of the old observation(2) that the white blood cell has a hydrophilic polar surface, in contrast to the red blood cell which has a hydrophobic non-polar surface that will not adsorb more than traces of protein in its normal uninjured state. The non-polar character of the erythrocyte surface is one important factor permitting the anti-globulin (Coombs) test to be so useful, since normal red cells give a negative test, and only an abnormal red cell surface results in a positive test. Because the normal leukocyte surface does contain adsorbed protein one would anticipate that current efforts to apply the anti-globulin test to leukocytes will not be successful.

The observation(3) that normal globulin is present in or avidly adheres to normal human leukocytes together with the observations already reported and discussed here suggest that normal leukocytes may have several kinds of adsorbed plasma proteins on their surface. Obviously, the possibility exists that a "leuko-agglutinin" may in fact be acting against adsorbed plasma protein (on a white blood cell behaving like a collodion particle) rather than against the leukocyte itself. This possibility must at present be considered always in interpretation of leuko-agglutinins.

Because of the inability to demonstrate fibrinogen attached to the surface of white cells prepared from defibrinated blood, some authors have concluded that the fibrinogen must be deposited on leukocytes only *in vitro*. However, when one defibrinates blood, a large fraction (usually about one-half) of the leukocytes originally present in the blood are inevitably removed with the clot, and the leukocytes removed might have contained adsorbed fibrinogen *in vivo* and so have been separated with the clot. Furthermore, Allison, Smith and Wood(4), on the basis of their observations of the leukocytic sticking reaction in the early stages of thermal inflammation, have suggested that partially polymerized fibrinogen may be present on leukocyte surfaces at this time. The explanation for either why all leukocytes do not adsorb fibrinogen *in vivo* or for the alternate possibility that leukocytes only adsorb fibrinogen *in vitro* is not clear.

That EDTA is highly effective in inhibiting fibrinogen polymerization and therefore non-specific clumping of leukocytes is not surprising in view of the work of Rosenfeld and Janszky(5). They found that EDTA, in addition to preventing thrombin generation, specifically inhibited fibrinogen-fibrin transformation, an effect they attributed to the potent decalcifying action of EDTA. We have supposed that the greater effectiveness in preventing non-specific leukocyte agglutination of EDTA over other decalcifying anticoagulants results because EDTA binds calcium more tightly than do the other agents. Thus, EDTA may inhibit blood clotting more completely than, for example, the usual amounts of sodium citrate. The inevitable eventual clumping of leukocytes taken from whole blood may result from the fact that fibrinogen polymerization occurs constantly, though very slowly, in any whole blood or plasma removed from the body. Also one would expect that the leukocytes themselves, which are known to contain thromboplastic substances(6) and proteolytic enzymes, might accelerate fibrinogen polymerization as they break up and release these clot-promoting substances.

The non-specific agglutinates of leukocytes may look precisely like the leukocyte clumps produced by pathologic sera. Consequently, just as in all agglutination tests, one should not infer the presence of an antibody simply because one observes clumped cells. Although there are probably other mechanisms of non-specific agglutination of white blood cells(7), the mechanism here described is a common and important one, and inhibition of this one kind of non-specific agglutination (by adding EDTA to the test sera) has enabled a satisfactory leuko-agglutinin test(8) to be constructed.

Because platelets regularly adsorb fi-

brinogen(1), it is probable that our findings are relevant to the platelet as well as to the leukocyte.

Summary and conclusions. Fibrinogen and other plasma proteins are apparently adsorbed on the hydrophilic surface of normal leukocytes, thereby complicating the interpretation of leuko-agglutinin tests. Normal leukocytes separated from whole blood (but not leukocytes separated from defibrinated blood) were rapidly and non-specifically agglutinated when suspended in normal untreated serum. Polymerization of adsorbed fibrinogen appeared to be the mechanism of such non-specific agglutination, which could be prevented by adequate decalcification of the suspending serum. Addition of EDTA to all test sera has permitted construction of a satisfactory leuko-agglutinin test using white cells separated from whole blood.

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Effect of D-Sorbitol on Absorption of Vitamin B_{12} by Pernicious Anemia Patients.* (24314)

LEON ELLENBOGEN, VICTOR HERBERT† AND WILLIAM L. WILLIAMS

Nutrition and Physiology Dept., Research Division, Amer. Cyanamid Co., Pearl River, N. Y., and Dept. of Hematology, Montefiore Hospital, N. Y. City

It has been previously demonstrated that sorbitol in solution with Vit. B_{12} will increase oral absorption of Vit. B_{12} in non-pernicious anemia subjects as measured by an increase in serum levels. Increased absorption of Vit. B_{12} by means of sorbitol has been reported to occur in experiments with young, healthy adults(1), and intact rats(2), while limited experiments with pernicious anemia patients were inconclusive(3). Since pernicious anemia is the primary state in which increased Vit. B_{12} absorption is desired, and since it was speculated that sorbitol may have an intrinsic factor-like action, experiments were undertaken on the effect of sorbitol on absorption of orally administered Vit. B_{12} in pernicious anemia patients in remission.

Method. Each pernicious anemia patient was fed an oral dose of 2 μg of Vit. $B_{12}Co^{58}$ containing 0.25 μc of radioactivity, first alone, then together with a potent intrinsic factor concentrate, then together with crystalline D-sorbitol and finally together with both intrinsic factor concentrate and D-sorbitol. In each of these 4 tests the medication was mixed and diluted to 100 ml, and following ingestion the container was rinsed with tap water and the rinsings fed to the subject. Immediately and 24 hours after each oral dose, 1000 μg of unlabeled Vit. B_{12} was given intramuscularly. The 4 excretion studies on each patient were repeated with a second series of studies using 30 μg oral doses of $B_{12}Co^{58}$ (containing 0.5 μc of radioactivity). In all instances except where noted the amount of sorbitol used was 10 g. Radioactivity of each 24-hour urine specimen was determined with a special beaker of one liter capacity adapted to fit over and around a NaI well type scintillation crystal(4) or by counting 200 ml in

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† Senior Research Fellow, N. Y. Heart Assn. Present address: Mt. Sinai Hospital, N. Y. C.