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Differential Agglutination of Normal and Sensitized Sheep Erythrocytes by Sera of Patients with Rheumatoid Arthritis.

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While performing complement fixation tests for rickettsialpox, the convalescent serum of a patient was observed to agglutinate the unhemolysed sheep erythrocytes. The patient, in addition to having suffered recently from the rickettsial infection, was also afflicted with rheumatoid arthritis in an active state. Further investigation revealed that the serum of this individual would agglutinate normal sheep erythrocytes to a dilution of only 1-16, whereas the same erythrocytes after being "sensitized" by two units of rabbit anti-sheep cell amboceptor were agglutinated to a serum dilution of 1-2048, a 128-fold increase in titer. A study was then made to determine under what circumstances a similar phenomenon was produced with the sera of other persons. The results of this study indicate that a 16-fold difference, or more, between the agglutination titers of normal and sensitized sheep erythrocytes, occurs almost exclusively with the sera of patients with rheumatoid arthritis, and that the magnitude of the difference in titer usually reflects the clinical activity of the disease. The present report outlines the method of performing the differential sheep cell agglutination test and describes the findings in a group of patients with rheumatoid arthritis and a variety of other diseases.

Methods and Materials. The suspensions of normal sheep erythrocytes were prepared

from fresh, defibrinated blood by washing the cells 3 times in saline (0.85% solution of sodium chloride), packing by centrifugation in a graduated tube, and resuspending in saline to make a 1.0% suspension by volume.

The suspensions of sensitized sheep erythrocytes were prepared in a manner somewhat similar to that for the Kolmer complement fixation test. Dilutions of rabbit anti-sheep cell serum ranging from 1-1000 to 1-10,000 were made in saline. Five-tenths ml of each dilution was then mixed with 0.5 ml of a 2.0% suspension of washed sheep cells and to each tube 0.1 ml of a 1-10 dilution of fresh guinea pig complement was added. The tubes were inspected after incubation in a water bath at 37°C for one hour and the highest dilution of the serum producing complete hemolysis was considered to contain one unit of hemolysin. For the agglutination test, a 2.0% suspension of the cells was mixed with an equal volume of rabbit anti-sheep serum diluted so as to contain 2 units of hemolysin. For example, if the unit of hemolysin were contained in a 1-4000 dilution of the serum, then a 1-2000 dilution would be employed for the final preparation of the sensitized erythrocytes.

The sera of the patients were obtained from freshly collected venous blood and the complement was inactivated by heating at 56°C for 30 minutes. Serial doubling dilutions of the serum were made in duplicate in 2 series of 12 tubes containing 0.5 ml of saline. To each tube of one series was added 0.5 ml of the 1.0% suspension of the normal sheep erythrocytes, and to each tube of the other series a similar amount of the 1.0% suspension of sensitized erythrocytes. The final serum dilutions were therefore 1-4, 1-8, 1-16, . . . 1-8192. (For most tests it is unnecessary to carry out serum dilutions with the normal sheep erythrocytes beyond a dilution of 1-128).

The racks containing the tubes were placed first in a water bath at 37°C for one hour and then in a refrigerator at 4°C overnight. Readings were made immediately after removing the racks from the refrigerator by flipping the tubes with the finger and estimating the degree of agglutination with the naked eye. Four-plus agglutination was indicated by a tightly-packed disc of cells and one-plus agglutination by finely granular clumping. The end-point of each titration was read as the serum dilution in the last tube showing one-plus agglutination, and the result was expressed as the reciprocal of that dilution. The agglutination titers of the individual sera for normal and sensitized sheep cells were then compared and the outcome of the test was recorded as the algebraic difference between the titers. For example, if the titer was 16 with normal erythrocytes and 1024 with sensitized erythrocytes, the result was recorded as 64.

Results. Differential sheep cell agglutination tests were performed with the sera of 110 patients. Table I shows the detailed results of the tests in representative cases, which illustrate the character of the actual findings and indicate the basis for calculating the differential agglutination titers.

In Table II are shown the results of the differential sheep cell agglutination test in the entire group of 110 patients, of whom 51 had some form of rheumatoid arthritis, 15 had rheumatic fever, 15 had arthritis other than rheumatoid or rheumatic fever, and 29 had no evidence of arthritic disease whatever.

Rheumatoid Arthritis. In 27 cases of rheumatoid arthritis with clear-cut subjective and objective evidence of active joint disease the difference between the titers of the normal and the sensitized sheep cells was never less than 16, and in 23 cases the difference in titer ranged from 32 to 512. On the other hand, in 16 cases of rheumatoid arthritis all of whom apparently were in remission, 11 gave differential titers of 16 or less, and only 5 had titers of 32 or higher.

The sera of 3 children with juvenile rheumatoid arthritis (Still's disease) were examined. One of these children, in remission, had a differential titer of 4, while the other 2, whose disease was active, gave values of 128.

The findings in 5 cases of Marie-Strumpell arthritis were of considerable interest, especially because of the questionable relation-

TABLE I.
Representative Results of Differential Sheep Cell Agglutination Test.

Clinical diagnosis	Cell suspension*	Serum dilution 4	8	16	32	64	128	256	512	1024	2048	4096	8192	Differential agglutination titer
Rheumatoid Arthritis—	NS	3+	2+	0	0	0	0	0	0	0	0	0	0	256
Active	SS	4+	4+	4+	4+	4+	4+	4+	3+	2+	1+	0	0	
Rheumatoid Arthritis—	NS	4+	4+	3+	2+	0	0	0	0	0	0	0	0	128
Active	SS	4+	4+	4+	4+	4+	4+	4+	4+	3+	3+	2+	0	
Rheumatoid Arthritis—	NS	4+	4+	3+	1+	0	0	0	0	0	0	0	0	4
Inactive	SS	4+	4+	4+	3+	2+	1+	0	0	0	0	0	0	
Rheumatoid Arthritis—	NS	2+	0	0	0	0	0	0	0	0	0	0	0	8
Inactive	SS	4+	3+	2+	1+	0	0	0	0	0	0	0	0	
Rheumatic Fever	NS	3+	2+	1+	0	0	0	0	0	0	0	0	0	8
	SS	4+	4+	3+	2+	2+	1+	0	0	0	0	0	0	
Undifferentiated Joint	NS	4+	4+	3+	1+	0	0	0	0	0	0	0	0	2
Disease	SS	4+	4+	3+	2+	1+	0	0	0	0	0	0	0	
Infectious Mononucleosis	NS	4+	4+	4+	4+	3+	2+	1+	1+	0	0	0	0	0
	SS	4+	4+	4+	4+	3+	2+	1+	1+	0	0	0	0	

* NS = normal sheep erythrocytes.
SS = sensitized sheep erythrocytes.

TABLE II.
Differential Agglutination of Normal and Sensitized Sheep Erythrocytes by Sera of 110 Patients.

Clinical diagnosis	No. of cases	Differential agglutination titer 0	2	4	8	16	32	64	128	256	512
Rheumatoid Arthritis—Active	27					4	4	7	8	3	1
" " Inactive	16	2	1	4	1	3	2	1		2	
Juvenile Rheumatoid Arthritis (Still's Disease)	3			1					2		
Marie-Strumpell Arthritis	4		1		3						
" " " with Peripheral Joint Involvement	1								1		
Rheumatic Fever	15	1	6	1	7						
Arthritis Other than Rheumatoid or Rheumatic Fever	15	3	6	5	1						
Infectious Mononucleosis	3	2	1								
Other Disease Without Evidence of Arthritis	26	6	8	8	2	2					

ship of this disease to typical rheumatoid arthritis. Four of these patients had what we consider negative tests, with titers of 8 or less. The remaining patient had a titer of 128, and this individual was the only one with evidence of peripheral joint involvement.

Rheumatic Fever. In 15 cases of rheumatic fever, most of them active with elevated sedimentation rates and antistreptolysin titers, the sheep cell agglutination test never gave differential titers exceeding 8. Several of these patients were suffering from the adult type of rheumatic fever which is often difficult to distinguish clinically from rheumatoid arthritis.

Arthritis Other Than Rheumatoid Arthritis and Rheumatic Fever. Fifteen patients were studied in this group, with the following diagnoses: Undifferentiated arthritis of mild character, 5 cases; degenerative joint disease (osteoarthritis), 2 cases; dermatomyositis, 2 cases; lupus erythematosus disseminatus, 2 cases; and one case each of gonococcal arthritis, gout, arthritis associated with lymphogranuloma venereum, and Sudek's atrophy. None of these patients had titers exceeding 8.

Other Disease Without Evidence of Arthritis. There were 29 patients in this group, all of whom were patients on the general medical wards, with a variety of diseases none of which was associated with any arthritic involvement. Two of these patients were found to have a differential agglutination titer of 16, while the remaining 27 all had a titer of 8 or less. Three cases of infectious mononucleosis were included, which are assigned a separate place in Table II.

Correlation of Differential Sheep Cell Agglutination with Erythrocyte Sedimentation Rate and Streptococcal Agglutination. Erythrocyte sedimentation rates were performed by the Westergren method in nearly all patients at about the same time that blood was drawn for the differential sheep cell agglutination test. As might be expected, most of the patients with active rheumatoid arthritis who had high differential agglutination titers also had high sedimentation rates. However, many of the other patients exhibited equally high sedimentation rates but had differential agglutination titers of less than 16. Consequently, it would appear that there is no correlation between the height of the sedimentation rate and the results of the differential sheep cell agglutination test.

It is well established that many patients with rheumatoid arthritis, especially those with active, progressive disease, possess an antibody in their serum which will agglutinate Group A hemolytic streptococci.[1,2] Indeed, the streptococcal agglutination test is employed as an adjunct in the diagnosis of rheumatoid arthritis. Streptococcal agglutination tests were performed in all of the cases of rheumatoid arthritis and the results were compared with those of the differential sheep cell agglutination test. In general, the patients with active rheumatoid arthritis with differential agglutination titers of 16 or higher also gave positive streptococcal agglutination tests. However, there was no correlation between the positivity of the two reactions and, in addition, several cases of active rheumatoid arthritis with high differential sheep cell titers had either negative or doubtful streptococcal agglutination tests. It therefore seems probable that the substance in the serum responsible for the differential agglutination of normal and sensitized sheep erythrocytes is different in nature from that responsible for the agglutination of Group A hemolytic streptococci.

Recent reports have suggested that the agglutination of streptococci by the sera of patients with rheumatoid arthritis is a nonspecific or panagglutinative effect, since the same sera may also agglutinate collodion particles.[3] In our experience we have found no direct relationship between these reactions.[4] Furthermore, with 10 sera selected at random from the present group of patients, the differential sheep cell agglutination titer bore no relation to the agglutination of collodion

[1] Nicholls, E. E., and Stainsby, W. J., *J. Clin. Invest.*, 1931, **10**, 323.

[2] Dawson, M. H., Olmstead, M., and Boots, R. H., *J. Immunol.*, 1932, **23**, 187.

[3] Wallis, A. D., *Am. J. Med. Sci.*, 1946, **212**, 713.

[4] Lipman, M. O., Coss, J. A., Jr., and Ragan, C., in preparation.

particles, either plain, coated with nutrient broth, or coated with the broth filtrate of cultures of hemolytic streptococci.

The Serum Fraction Containing Agglutinin for Sheep Cells. Differential sheep cell agglutination tests were carried out with the electrophoretically separated beta-gamma globulin fractions of the serum from cases of active and inactive rheumatoid arthritis, and the results are shown in Table III. It will be observed that the differential agglutination of normal and sensitized erythrocytes by the purified globulin fractions resembles that seen with the whole serum of similar cases.

Discussion. When fresh, heat-inactivated human sera are tested for their ability to agglutinate both normal sheep erythrocytes and sheep erythrocytes "sensitized" by their exposure to small amounts of rabbit anti-sheep cell serum, the sensitized erythrocytes are almost always agglutinated to a higher titer than are the normal erythrocytes. With the sera of patients suffering from rheumatic fever, various arthritic disorders other than rheumatoid arthritis, and diseases unassociated with joint involvement, the difference between the agglutination titers of normal and sensitized sheep erythrocytes is usually of a low order and thus far has not been observed to exceed an algebraic difference of 16. These findings are in marked contrast to those with the sera of patients suffering from rheumatoid arthritis, especially when the disease is in an active state. In such patients the differential titer is never lower than 16—the upper limit of the "normal" values—and is usually considerably higher. On the other hand, in patients with clinically inactive rheumatoid arthritis, the differential agglutination titer falls within normal limits in the majority of cases. The conclusion therefore seems valid that one of the unique features of active rheumatoid arthritis is the presence in the serum of a substance which will agglutinate sensitized sheep erythrocytes to a markedly higher titer than it will agglutinate normal sheep erythrocytes. This substance occurs in the globulin fraction of the serum and does not seem to be associated with factors which are responsible for alterations in the sedi-

TABLE III.

Differential Sheep Cell Agglutination Tests with Electrophoretically Separated Beta-Gamma Globulin Fractions of Sera from Patients with Active and Inactive Rheumatoid Arthritis.

Clinical diagnosis	Cell suspension	Dilution of globulin fraction*							Differential agglutination titer
		10	20	40	80	160	320	640	1280	
Inactive Rheumatoid Arthritis	NS	++++	++++	+	0	0	0	0	0	4
	SS	++++	++++	+++	++	+	0	0	0	
Active "	NS	0	0	0	0	0	0	0	0	128
	SS	++++	++++	++++	+++	+++	++	+	0	

* Dilutions expressed as final dilutions of original serum.

mentation rate of erythrocytes or the agglutination of Group A hemolytic streptococci. It is probably unrelated to the conglutinin reaction of Bordet and Gay[5] since complement is not required for its effect. The precise nature of the substance and its significance with regard to the rheumatoid state remain to be determined. In any event, the present observations indicate that the sheep cell differential agglutination test may be of value in determining the activity status of patients suffering from rheumatoid arthritis. It may also prove to be helpful as an aid in the differential diagnosis between rheumatoid arthritis and other diseases with arthritic manifestations, such as rheumatic fever.

Summary. A differential sheep cell agglutination test is described wherein the agglutination titers of human serum for normal and sensitized sheep erythrocytes are compared. High differential titers have been found to occur almost solely with the sera of patients suffering from active rheumatoid arthritis. Some of the features of this serological phenomenon are pointed out, together with its possible practical applications.

[5] Bordet, J., and Gay, F. P., *Ann. Inst. Pasteur*, 1906, **20**, 467.

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Phospholipid Metabolism in Diabetes: Turnover Rate of Plasma Phospholipids in Completely Depancreatized Dogs.

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The increased amounts of fatty acids that appear in both liver and plasma of dogs deprived of all islet tissue indicate that diabetes in this animal is characterized by a severe disturbance in fat metabolism.[1,2] It is shown here, however, that the turnover of plasma phospholipids is not altered in the diabetic dog. Apparently, plasma phospholipids do not participate in the redistribution of fat that occurs in the diabetic dog.

Experimental. Depancreatized Dogs. After complete excision of the pancreas the dogs were injected with insulin and fed twice daily a mixture containing lean meat, raw pancreas, sucrose, and vitamin supplements.[3]

Synthesis of Labeled Plasma Phospholipids. To provide labeled plasma phospholipids, 2-3 millicuries of radioactive phosphate were administered by stomach tube to fasted normal dogs. Twenty-four hours later the dogs were bled, the heparinized blood centrifuged, and the plasma containing the radioactive phospholipid separated.

Methods of Analyses. Phospholipid P^{31} and phospholipid P^{32} were determined in plasma in a manner described elsewhere.[4] The method used for determination of plasma total-fatty acids has also been recorded elsewhere.[5] At the end of the period of observation, the whole liver was excised, thoroughly ground, and analysed for its total-fatty acid content.[6]

Experiment 1. 50 cc of plasma containing labeled phospholipids were injected intravenously into a normal and a depancreatized dog, the latter of which had been deprived of insulin but not food for 3 days. Samples of blood were removed from both dogs at several intervals and the plasma analysed for

* Fellow of the American Cancer Society (recommended by the Growth Committee).

[1] Gibbs, G. E., and Chaikoff, I. L., *Endocrinology*, 1941, **29**, 885.

[2] Bloor, W. R., Gillette, E. M., and James, M. S., *J. Biol. Chem.*, 1927, **75**, 6.

[3] Montgomery, M. L., Entenman, C., and Chaikoff, I. L., *Am. J. Physiol.*, 1944, **141**, 216.

[4] Zilversmit, D. B., Entenman, C., Fishler, M. C., and Chaikoff, I. L., *J. Gen. Physiol.*, 1943, **26**, 333.

[5] Chaikoff, I. L., and Kaplan, A., *J. Biol. Chem.*, 1934, **106**, 267.

[6] Kaplan, A., and Chaikoff, I. L., *J. Biol. Chem.*, 1935, **108**, 201.