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THE RHEUMATOID FACTOR IN SERUM AND SYNOVIAL FLUID¶

INTRODUCTION

The agglutination of sensitized sheep cells by a factor in the serum of individuals with rheumatoid arthritis has been the subject of continued investigation since its recognition by Waaler.[11] This reaction, with its modifications, furnishes the useful diagnostic tests for the illness.[1, 2, 3, 4, 9, 13]

The hemagglutination reaction has also been used in the study of the pathogenesis of rheumatoid arthritis. In this connection, efforts were made to absorb the factor from serum[6, 12] or to identify it with a serum protein fraction by virtue of differential solubility[5, 10, 13] or rate of migration.[5, 6, 7] These procedures have not been of significant help in the chemical identification of the rheumatoid factor.

Rheumatoid arthritis is characterized clinically by the prominence of changes in the synovial tissue and sometimes by the accumulation of joint fluid. Therefore, it was decided to investigate synovial fluid with the thought in mind that the joint itself might be the site of formation of the rheumatoid factor or at least a source of this material which is ultimately found circulating in the blood stream. Since the presence of mucin represents a significant difference between synovial fluid and serum, attention was also focused upon it. The viscosity, mucin clot, and mucin nitrogen to glucosamine ratio determinations reported by Ropes and Bauer[8] indicate that the hyaluronic acid of synovial fluid is depolymerized, or incompletely polymerized, in rheumatoid arthritis.

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Such alterations of hyaluronic acid in synovial fluid have not previously been related to the agglutination of sensitized sheep cells by the rheumatoid factor. This study reports the effects of *in vitro* enzymatic treatment of serum and synovial fluid in an attempt to learn if a relationship between the hemagglutination test and the mucin polysaccharide does exist.

MATERIALS

Specimens. Synovial fluid samples were obtained from the knee joints of patients at the Arthritis Clinic of the Grace-New Haven Community Hospital or from patients under the care of private physicians. Aspirations were performed, with aseptic precautions, when clinical evidence of joint effusion existed. The fluids were kept at 4° C. for 12 hours, then centrifuged. The fibrin clot, cells, and debris, if any, were discarded and the supernatant fluid stored at —10° C.

Whenever possible, blood was collected from the individual at the time of the knee tap. For the purpose of this study, however, serum and synovial fluid samples were considered contemporary if collected within one week of each other.

Enzymes. Hyaluronidase and β-glucuronidase were purchased from a commercial source.* The bovine testicular hyaluronidase had a reported assay value of 150-250 Turbidity Reducing Units per milligram. The activity of the bovine liver β-glucuronidase was given as approximately 50,000-70,000 units per gram.

Buffers. A pH 6.0 acetate buffer, 0.15 M with respect to sodium chloride, was prepared by diluting 6 ml. of 0.5 M acetic acid, 19.4 ml. of 0.5 sodium acetate and 8.77 gm. of sodium chloride to 1 liter with distilled water.

A pH 4.5 buffer was prepared from 11 ml. of 0.2 M acetic acid and 9 ml. of 0.2 M sodium acetate.

METHODS

Hemagglutination Test

A recent modification of the hemagglutination test was used.[1] It employs the settled pattern of sensitized sheep cells to indicate the endpoint of the reaction. Results are expressed in units which are the reciprocal of the test material dilution at which the last positive pattern occurred. Less than eight units is a negative test. Except for the inclusion of synovial fluids and the use of enzymes for treatment, the hemagglutination method is that previously described. To insure comparable results the contemporary samples of serum and synovial fluid were always included in the same unit of hemagglutination tests.

Enzyme Experiments

Bovine testicular hyaluronidase was dissolved in pH 6.0 buffer to give final concentration of 0.25, 0.50, 1.0, 2.5, 5.0, and 10.0 mg. per 0.3 ml. of the buffer; 0.3 ml. of the enzyme containing buffer was then added to an equal volume of serum or synovial fluid. The mixture was incubated for one hour at 37° C. Enzyme-treated synovial fluid or serum samples, along with untreated control samples, were assayed by the

* Worthington Biochemical Corporation, Freehold, New Jersey.

hemagglutination test. The final titer and the settled red cell pattern at each dilution were recorded.

A similar procedure was used with β-glucuronidase. This enzyme was dissolved, however, in pH 4.5 buffer, and the pH was readjusted with 0.1 M sodium hydroxide to approximately 6.5 after incubation and just before assay.

RESULTS

In Table 1 the hemagglutination test results of 19 contemporary serum and synovial fluid samples from 16 patients are given. It is apparent, in the series studied, that the incidence of a positive hemagglutination test in serum is greater than in synovial fluid. In the three instances in which both the serum and synovial fluid gave positive hemagglutination tests, the titer of the synovial fluid was consistently higher by one or two dilutions. From general use in this laboratory, the maximum limit of error for the test is placed at one dilution.

After preliminary studies on β-glucuronidase-treated serum and synovial fluid samples and similar samples treated with hyaluronidase, it was evident that the only treatment which significantly altered the hemagglutination test was the hyaluronidase treatment of certain synovial fluids. Therefore, the principal effort was concentrated on the effect of hyaluronidase on all available synovial fluids.

Preliminary experiments indicated that 1 mg. of hyaluronidase (150-250 T.R.U.) per 0.3 ml. of synovial fluid was a satisfactory enzyme concentration. Higher concentrations failed to elevate the titer further while lower concentrations occasionally failed to give optimal results. Control experiments showed that in the absence of the enzyme neither the pH 6.0 buffer nor the period of incubation altered the hemagglutination test. Furthermore, the buffered enzyme alone did not agglutinate sensitized sheep cells.

Table 1 also gives the synovial fluid hemagglutination test results, before and after hyaluronidase treatment, of the 16 patients with contemporary serums. In those instances (F.G., C.G.) where a positive titer was found in the synovial fluid before enzyme treatment, the action of hyaluronidase resulted either in no change or in a reduction in titer. In cases in which the serum hemagglutination test was positive but the synovial fluid test was negative, enzyme treatment of the synovial fluid resulted in a positive hemagglutination test (A.K., A.Cr., C.Fr., L.A.). The titer of the enzyme-treated synovial fluid in such cases was higher, with one exception (A.Cr.), than the titer of the corresponding serum. In addition, five hyaluronidase-treated synovial fluids had significantly positive hemagglutination tests, while the contemporary serums were negative (M.S., I.T., A.C., A.P.,

TABLE 1. COMPARISON OF THE HEMAGGLUTINATION TEST RESULTS OF CONTEMPORARY SERUMS, SYNOVIAL FLUIDS, AND HYALURONIDASE-TREATED SYNOVIAL FLUIDS

Diagnosis	*Patient*	*Hemagglutination titer**		
		Serum	*Synovial fluid*	*Synovial fluid after hyaluronidase treatment*
Group I				
Rheumatoid arthritis	F.G. 11-21-55	256	512	512
	1-16-56	512	1024	512
	11- 5-56	128	<8	512
	M.S.	<8	<8	8
	A.K.	128	<8	256
	C.G.	512	2048	256
	I.T.	<8	<8	64
	A.Cr.	256	<8	64
	C.Fr.	128	<8	256
	A.C.	<8	<8	8
	V.W. 8-30-56	<8	<8	<8
	9-15-56	<8	<8	<8
Juvenile rheumatoid arthritis	C.F.	<8	<8	<8
Group II				
Possible rheumatoid arthritis	A.P.	<8	<8	64
	H.L.	<8	<8	8
Rheumatoid arthritis or lupus erythematosus	L.A.	64	<8	512
Gonococcal arthritis history—possible rheumatoid arthritis	R.V.	<8	<8	<8
Gouty arthritis—possible rheumatoid arthritis	J.T.	<8	<8	<8
Group III				
Traumatic arthritis	S.S.	<8	<8	<8

* Expressed in units as reciprocals of serum or synovial fluid dilutions. Less than 8 units is a negative test.

H.L.). Three of these were from cases of rheumatoid arthritis and two from cases diagnosed as possible rheumatoid arthritis.

The means by which hyaluronidase produces a positive hemagglutination test was partially elucidated by examination of the actual settled sheep cell patterns. Many synovial fluids had sheep cell patterns which were atypical and could not be assigned a titer. These patterns were neither negative nor typically positive, but represented a third type of pattern which was distinct and easily recognizable. Upon addition of hyaluronidase, such fluids presented typically positive patterns and could be assigned a titer. This titer corresponded to the last dilution in which an atypical pattern appeared in the synovial fluid alone.

A few synovial fluid samples (M.S., A.C., H.L.) gave a clear-cut negative pattern in the hemagglutination test, but after treatment with hyaluronidase the hemagglutination test became typically positive at a titer of eight units.

In addition to the sixteen patients from whom both synovial fluid and serum were available, synovial fluid from fourteen other patients was studied. The hemagglutination titers of 43 synovial fluids, before and after hyaluronidase treatment, are given in Table 2.

To evaluate the specificity of the effect of hyaluronidase it was necessary to study its effect on synovial fluids of nonrheumatoid origin. Group 3 of Table 2 contains nine such patients. The post-mortem synovial fluid served as a normal control. The occurrence of a positive hemagglutination test in one case of traumatic arthritis indicates either that the effect of hyaluronidase is not entirely specific for rheumatoid arthritis or that in this case the diagnosis may change with time to that of rheumatoid arthritis.

DISCUSSION

The low incidence of a positive hemagglutination test in untreated synovial fluid and the difficulty and trauma associated with obtaining synovial fluid would seem to make it an undesirable material for the application of the routine diagnostic hemagglutination test. However, when synovial fluid is treated with hyaluronidase, prior to assay, the incidence of a positive hemagglutination test is increased, and under these circumstances synovial fluid provides at least as much information as the usual serum hemagglutination test. It may provide additional diagnostic information, but this cannot be established until more data on the specificity of the effect of hyaluronidase on the hemagglutination test are available.

Although it is unlikely that hyaluronidase acts *in vitro* to produce the rheumatoid factor, it either makes it more available for reaction with sensi-

TABLE 2. COMPARISON OF THE HEMAGGLUTINATION TEST RESULTS OF SYNOVIAL FLUIDS AND HYALURONIDASE TREATED SYNOVIAL FLUIDS

Diagnosis	*Patient*		*Hemagglutination titer*	
			Synovial fluid	*Synovial fluid after hyaluronidase treatment*
Group I				
Rheumatoid arthritis	F.G.	10-31-55	1024	512
		11-21-55	512	512
		12- 5-55	1024	1024
		1-16-56	1024	512
		3- 5-56	1024	512
		5-21-56	1024	512
		11- 5-56	<8	512
	M.S.	2-27-56	<8	8
		2-29-56	<8	<8
		3- 7-56	<8	<8
		2-26-56	<8	<8
	A.K.		<8	256
	C.G.		2048	256
	I.T.		<8	64
	A.Cr.		<8	64
	C.Fr.	4-16-56	<8	256
		5-28-56	<8	512
	A.C.	5-29-56	<8	8
		6- 6-56	<8	<8
	A.J.		<8	128
	H.H.		<8	<8
	V.W.	8-30-56	<8	<8
		9-15-56	<8	<8
Juvenile rheumatoid arthritis	F.H.		<8	16
	J.W.		<8	<8
	C.F.	9- 7-56	<8	<8
		9-12-56	<8	8
Group II				
Probable rheumatoid arthritis	D.G.		<8	<8
Possible rheumatoid arthritis	A.P.		<8	64
	N.E.		<8	16
	H.L.		<8	8
Rheumatoid arthritis or lupus erythematosus	L.A.		<8	512

Diagnosis	Patient	Hemagglutination titer: Synovial fluid	Hemagglutination titer: Synovial fluid after hyaluronidase treatment
Gonococcal arthritis history—possible rheumatoid arthritis	R.V.	<8	<8
Gouty arthritis possible rheumatoid arthritis	J.T.	<8	<8
Group III			
Gouty arthritis	L.C.	<8	<8
Degenative joint disease	R.P.	<8	<8
	E.M.	<8	<8
Scleroderma	G.P.	<8	<8
Traumatic arthritis	S.S.	<8	<8
	C.K.	<8	<8
	G.R.	<8	<8
	N.O.	<8	16
Post-mortem*	J.C.	<8	<8

* No history or clinical signs of joint disease.

tized sheep cells or it may depolymerize an inhibitory mucopolysaccharide. It is unclear exactly how this is accomplished, but it would appear that the effect must be ascribed to some chemical change induced by hyaluronidase. Hyaluronidase reduces the viscosity of synovial fluid by depolymerizing the polysaccharide, hyaluronic acid. The physical change in viscosity itself does not seem to be the responsible alteration, since preliminary experiments indicate that hyaluronidase can convert atypical hemagglutination patterns to typically positive ones even when a high viscosity is artificially maintained with glycerol or methocel.

SUMMARY

1. A comparison of the hemagglutination test results is made with serum and synovial fluid samples obtained from the same individual.

2. In cases in which the serum hemagglutination test was positive but the synovial fluid did not produce the usual endpoint, hyaluronidase treat-

ment of the synovial fluid resulted in a positive pattern of settled sheep cells. In addition, after hyaluronidase treatment, some synovial fluids gave positive hemagglutination tests of low titer, while the corresponding serum test did not react.

3. The effect of hyaluronidase on this hemagglutination test of synovial fluid suggests a correlation between the rheumatoid factor, or an inhibiting substance, and the mucin polysaccharide.

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REFERENCES

1. Boisvert, P. L., Brockett, J. B., and de Forest, G. K.: The hemagglutination test in rheumatoid arthritis. I. Use of red cell pattern as endpoint in the method. *Yale J. Biol. Med.*, 1956, *28,* 622-628.
2. Heller, G., Jacobson, A. S., and Kolodny, M. H.: A modification of the hemagglutination test for rheumatoid arthritis. *Proc. Soc. exp. Biol., N. Y.,* 1949, *72,* 316-323.
3. Heller, G., Jacobson, A. S., Kolodny, M. H., and Kammerer, W. H.: The hemagglutination test for rheumatoid arthritis. II. The influence of human plasma fraction II (gamma globulin) on the reaction. *J. Immunol.,* 1954, *72,* 66-78.
4. Heller, G., Jacobson, A. S., Kolodny, M. H., and Schuman, R. L.: The hemagglutination test for rheumatoid arthritis. I. An immunological analysis of the factors involved in the reaction. *J. Immunol.,* 1952, *69,* 27-40.
5. Heller, G., Kolodny, M. H., Le Pow, I. H., Jacobson, A. S., Rivera, M. E., and Marks, G. H.: The hemagglutination test for rheumatoid arthritis. IV. Characterization of the rheumatoid agglutinating factors by analysis of serum fractions prepared by ethanol fractionation. *J. Immunol.,* 1955, *74,* 340-350.
6. Lamont-Havers, R. W.: Nature of serum factors causing agglutination of sensitized sheep cells and group A hemolytic streptococci. *Proc. Soc. exp. Biol., N. Y.,* 1955, *88,* 35-38.
7. Robinson, A. K., Stulberg, C. S., and Kuyper, A. C.: Identification of the substance active in sheep cell agglutination test for rheumatoid arthritis. *Proc. Soc. exp. Biol., N. Y.,* 1954, *85,* 4-7.
8. Ropes, M. W. and Bauer, W.: *Synovial fluid changes in joint disease.* Cambridge, Harvard University Press, 1953.
9. Rose, H. M., Ragan, C., Pearce, E., and Lipman, M. O.: Differential agglutination of normal and sensitized sheep erythrocytes by sera of patients with rheumatoid arthritis. *Proc. Soc. exp. Biol., N. Y.,* 1948, *68,* 1-6.
10. Svartz, N. and Schlossmann, K.: A serum cold precipitable hemagglutinating factor in rheumatoid arthritis. *Acta med. scand.,* 1954, *149,* 83-89.
11. Waaler, E.: On the occurrence of a factor in human serum activating the specific agglutination of sheep blood corpuscles. *Acta path. microbiol. scand.,* 1940, *17,* 172-188.
12. Wager, O.: On the factor producing agglutination of sensitized red cells and its relation to the agglutination of hemolytic streptococci in rheumatoid arthritis sera. *Ann. Med. exp. Fenn.,* 1950, *28,* Supp. 8.
13. Ziff, M., Brown, P., Lospalluto, J., Badin, J., and McEwen, C.: Agglutination and inhibition by serum globulin in the sensitized sheep cell agglutination reaction in rheumatoid arthritis. *Amer. J. Med.,* 1956, *20,* 500-509.