A Sensitive Gelatin Agglutination Test for Detecting Antisperm Agglutinins.* (19740)

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As a preliminary step in the study of antibodies against spermatozoa in fertile and sterile women it was necessary to develop a highly sensitive technic capable of demonstrating the presence of small amounts of antibodies. Among other serological tests the detection of anti-human sperm agglutinins offered a promising field, although previous investigators with mammalian spermatozoa had obtained only relatively low agglutination titers. Therefore, various methods of increasing the delicacy of the agglutination test by modifying the standard technics were tried.

These studies led to the development of a new type of test, the gelatin agglutination technic, which is capable of detecting sperm agglutinins in titers about 100-fold higher than with the ordinary agglutination tests. The technic is described since this test may have possible application in the detection of agglutinins against other organisms than spermatozoa. The rationale of the test is based on the use of live motile spermatozoa as antigens and the employment of a viscous medium in the form of 2.5% gelatin. This medium prevents the actively motile spermatozoa, once agglutinated, from disentangling themselves from the weakly bound floccules in high antiserum dilutions. It also retards sedimentation, thus rendering unnecessary redispersal of the delicately flocculated sediment.

Technic of test. Antigens. Donors with numerous motile spermatozoa and readily liquefying semen are used for the preparation of the antigen. Specimens of semen with large amounts of cellular debris are rejected to minimize nonspecific agglutination. A fresh ejaculum is allowed to stand until liquefaction is complete, usually in from 30 to 60 minutes. A sperm count is done and the specimen is diluted to approximately 60 million spermatozoa per ml with physiological sodium chloride solution or Baker's buffered glucose solution(1). The diluted semen is allowed to stand undisturbed for about an hour at room temperature to permit settling of clumped and immobile spermatozoa and cellular debris. The supernate, containing the actively motile spermatozoa, is carefully removed, a repeat sperm count is made, and the suspension is adjusted to 40 million spermatozoa per ml. An equal volume of gauze-filtered 10% gelatin in physiological sodium chloride solution is then added, giving a sperm concentration of 20 million per ml and a gelatin content of 5%. The gelatin solution and gelatin-sperm mixture are maintained at about 36-37°C to facilitate pipetting.

Test. Serial dilutions of the sera to be tested are made with physiological sodium chloride solution, the range depending upon the expected titers. A total of 0.3 ml of each dilution is first placed in a 65 x 5 mm precipitation tube and an equal volume of the spermatozoa-gelatin antigen is then added giving a final concentration of 10 million spermatozoa per ml in 2.5% gelatin. The use of these small precipitation tubes not only conserves reagents, but also provides a 30 mm column of fluid, which raises the sensitivity of the test by increasing the frequency of contact among the slowly sedimenting floccules. Comparison with the controls is also simplified, since the viscous gelatin medium tends to prevent settling of the unagglutinated spermatozoa. The contents of the tubes are well mixed and incubated at 36-37°C for 2 hours. This period is the most satisfactory from the standpoint of maximal agglutination and minimal sedimentation in the controls. The gelatin-prepared antigen plus diluent as well as known positive and negative sera comprise the controls. During incubation the antigen control tubes may be checked for the appearance of a granular precipitate, indicative of pseudoagglutination. Such nonspecific agglutination

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occurs infrequently, but, if noted, incubation is terminated and the results of the test are immediately recorded.

Reading. At the end of the incubational period the tests are read. The tubes may then be placed, for subsequent observation, in the refrigerator where rapid solidification preserves the agglutinates. The criteria for agglutination are the clarity of the dispersing phase and the size and character of the floccules. In true agglutination the dispersing phase is much clearer than in the controls. Finely dispersed granulations are occasionally encountered in negative serum controls but rarely in titers above 1:4, and are readily distinguishable from true agglutination. Final results are recorded as +, ±, and —, although positive reactions may be expressed in terms of 1- to 4-plus if desired.

Discussion. Antigen. The agglutination of human spermatozoa by antibody, while involving the same principles as the agglutination of bacteria, is modified by the number, the motility, and the anatomical characteristics of the spermatozoa. If too few spermatozoa are present in the antigen, the reduced frequency of collisons per unit volume results in the formation of small floccules. If too many are used, the amount of antibody available for each spermatozoon is reduced and an excessive turbidity is produced that may obscure small agglutinates in the higher serum dilutions. The optimal concentration is 20 million spermatozoa per ml for the antigen, which gives a final concentration of 10 million per ml in the test. There is no advantage in the use of washed spermatozoa over diluted whole semen for antigens, since the seminal plasma does not interfere with the reading of the test, when the final dilution of semen is 1:10 or higher. Actively motile spermatozoa are essential for the test. The higher the motility the greater is the number of collisons per unit time, thus facilitating agglutination. Furthermore, active spermatozoa give controls of homogeneous turbidity, whereas inactive spermatozoa show a greater tendency toward sedimentation. In the absence of the gelatin menstruum the large size of the spermatozoa causes agglutinates to sediment rapidly although the weakly bound floccules of intertwining heads and tails are easily disrupted by slight shaking and jarring, especially in the presence of small amounts of antibody where the sperm motility alone may be sufficient to overcome the agglutinative forces.

Gelatin menstruum. The viscous gelatin medium, while allowing the spermatozoa to agglutinate, retards their separation from the weakly bound floccules in the high antiserum dilutions and protects the agglutinates from mechanical shaking and jarring. The viscous medium also retards sedimentation in both the test and controls so that a direct comparison of the antiserum dilutions and controls may be made at the conclusion of incubation without resuspending the sediment. A gelatin concentration of 5% in the antigen (2.5% in the final dilution in the test) gives the most satisfactory results and is nontoxic to spermatozoa. Higher concentrations are impractical, since they are difficult to manipulate, and lower concentrations do not give sufficient viscosity to produce maximal titers. For example, when the same antisperm serum was tested against antigen suspensions which were prepared from one ejaculum but contained varying amounts of gelatin, the agglutinin titers were 1:8,000 for a 0.5% gelatin antigen, 1:32,000 for a 2.5%, and 1:128,000 for a 5%.

Application. The gelatin agglutination test, primarily designed for serological work with spermatozoal antibodies, has proved highly effective in detecting small amounts of antisperm agglutinins, since it gives titers about 100-fold higher than obtained with the usual agglutinative technics. The titers of 7 anti-human-sperm rabbits' sera with this technic ranged from 1:16,000 to 1:256,000 with a mean of about 1:60,000. The test appears to be highly specific since agglutination rarely occurs in dilutions above 1:4 with the normal sera of man, rabbits or guinea pigs, or with rabbits' antisera against various types of unrelated antigens. The gelatin test should prove of value in cases where the concentration of sperm agglutinins is too small to be detected by the ordinary macroscopical or microscopical agglutination tests. It should also be useful in the study of iso-agglutinins, and in the differentiation and determination

of relationship between the spermatozoa of different species.

The basic principle of the test, however, would seem to have a general application to any agglutinative technic involving large motile organisms, where the agglutinates formed are so fragile as to be readily disrupted by shaking or jarring. It may perhaps be of value in the study of trypanosomes and other large flagellated protozoa. Although gelatin has proved suitable for the enhancement of sperm agglutination by increasing the viscosity of the medium, different agents may be better suited for other motile organisms.

Summary. 1. An improved macroscopical agglutination test for the detection of agglutinins against human spermatozoa is described. 2. The test employs an antigen of actively motile spermatozoa, and a final concentration of 2.5% gelatin to increase the viscosity of the medium. 3. The viscous gelatin medium prevents dissolution of the weakly bound sperm agglutinates in the higher antiserum dilutions, and retards sedimentation so as to render resuspension unnecessary. 4. The new method appears to be highly sensitive and specific. Agglutinative titers ranging from 1:16,000 to 1:256,000 with a mean of about 1:60,000 have been obtained with 7 anti-human-sperm rabbits' sera. These titers are over 100-fold higher than those obtained with the usual agglutinative technics. 5. The possible applications of the test are suggested.

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Relation between Adrenal and Pituitary Glands and Urinary Excretion of Neutral Reducing Lipids in the Rat.* (19741)

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Adrenal cortical stimulation in the rat is, at present, evaluated by adrenal ascorbic acid or cholesterol depletion in acute experiments (1) and by adrenal weight and morphological changes in chronic experiments(2). During the course of studies on the role of the adrenal in experimental hypertension, it was thought desirable to evaluate adrenal cortical activity by measuring the urinary excretion of adrenal cortical hormones or their metabolites. Several chemical procedures(3-5) have been developed for the determination of these substances in human urine. The method of Heard and Sobel(3) is based on the ability of neutral lipid extracts of urine to reduce phosphomolybdic acid. This method has been shown to correlate with clinical and biological evidence of altered adrenal cortical activity in human beings. In the present study, a modification of this method has been applied to evaluate adrenal cortical activity in the rat.

Experimental procedure. Urine collection. The method for urine collection has been described(6). Rats of the Slonaker-Addis strain were taken off the stock diet and placed in urine collection cages at 5 P.M. During the overnight collection period the animals received *ad libitum* a 15% glucose solution in 0.4% sodium chloride containing 0.5% of a mixture of B vitamins (Betaplexin®). There may be some theoretical objections to the use of this solution in experiments designed to test adrenal cortical function. Nevertheless, it was necessary in order to assure a copious flow of uncontaminated urine. Normal rats on over-

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