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15206

A Simple Method for the Concentration of Rh Agglutinins.

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There are two sources of human anti-Rh serum. Following multiple transfusions of Rh-positive blood certain Rh-negative individuals develop Rh antibodies.[1] The more common source of human Rh antibodies, however, is found in women who have given birth to erythroblastotic children.[2] Rh agglutinins if present are frequently of low titer. The procedure for fractionated precipitation of sera containing antibodies has been developed especially in Dr. Cohn's laboratory at Harvard, and is successfully applied in the case of anti-Rh serum (Diamond). However, this method requires special equipment and training and is not available to everybody. It seemed to be desirable to find means and ways to concentrate Rh agglutinins by a simple method.

Anti-Rh sera were subjected to dialysis in the following manner: The native serum is placed in a cellophane tubing bag under tension, air having been eliminated as much as possible. The bag is then suspended in a battery jar containing a large volume of freshly distilled water. The serum is dialyzed 24 to 30 hours in the icebox at 4°C, the distilled water being changed several times during the procedure. During dialysis at this temperature a precipitate forms. The serum with its precipitate is removed from the cellophane bag and the precipitate is separated from the supernatant fluid by centrifugation. In the experiments to be described, the sediment (precipitate) is dissolved in saline solution and referred to as the globulin fraction. The globulin fraction obtained from 10 cc of each serum is dissolved in 1 cc of saline solution (0.9% NaCl) by vigorous stirring, then kept for 2-3 hours in the icebox. Insoluble particles are removed by centrifugation. The dissolved globulin is preserved in the frozen state at −20°C or

[1] Wiener, A. S., and Peters, H. R., *Ann. Int. Med.*, 1940, **13**, 2306.

[2] Levine, Philip, *Am. J. Obs. and Gyn.*, 1945, **49**, 810.

TABLE I.
Agglutination of Rh Positive Group O Cells by Serum (Dem).

Serum (Dem)	a Native	b Supernatant	c Globulin
1. Undiluted	++++	+++	++++
2. 1:2	+++	++	++++
3. 1:4	+++	+	++++
4. 1:8	+++	+	++++
5. 1:16	++	±	++++
6. 1:32	++	—	++++
7. 1:64	+	—	+++
8. 1:128	±	—	+++
9. 1:256	—	—	++
10. 1:512	—	—	++
11. 1:1024	—	—	±
12. Saline	—	—	—

— = No agglutination.
± = Faint agglutination.
\+ = Slight agglutination.
++ = Marked agglutination.
+++ = Strong agglutination.
++++ = Very strong agglutination.

lyophilized. The supernatant fluid to which a sufficient amount of 10% saline solution is added to bring the final salt concentration to 0.9% is referred to in this paper as the "supernatant." In former years the described procedure served as a rough method to separate the globulin and the albumin fractions of serum. It is now generally agreed that the albumin fraction obtained by dialysis is by no means pure but still contains some globulin.

The analysis of several anti-Rh sera is reported in this paper. One serum (Kru) was obtained from an Rh-negative patient who had received multiple transfusions and who had died after a long chronic illness. A large amount of blood containing an Rh agglutinin of relatively weak potency was collected after the patient's death. The second serum (Dem) and the third serum (Heng) were obtained from Rh-negative women who had given birth to erythroblastotic children; so was a fourth serum which contained "blocking antibodies" only.[5]

The first experiment was carried out in the following way: Decreasing amounts of serum (Dem) and its fractions respectively (volume 0.1 cc) were mixed with 0.1 cc of a 2% suspension of Rh-positive Group O cells. After standing at room temperature for one hour the tubes were centrifuged at medium speed for 1 minute and read for agglutination.

The experiment shows that the globulin fraction, dissolved in one-tenth of the original volume of the serum, contains a potent Rh agglutinin. Characteristic of the agglutination of Rh-positive cells by the globulin fraction is not only an increase in end titer but also an increase in the relative strength or quality of the agglutination showing a 4+ agglutination up to a dilution of 1:32. In contrast, the supernatant fluid is poor in Rh agglutinins when compared with the native untreated serum. From this experiment the conclusion can be drawn that the Rh agglutins are found in the "globulin" fraction as obtained by simple dialysis and only a minor portion remains in the supernatant fluid.

The discovery of the "incomplete antibody" (Race[3,4]) or the "blocking antibody" (Wiener[5]) in the serum of patients expected to contain Rh antibodies has proved to be of great importance. This antibody uniting with Rh-positive cells, thus sensitizing them, will prevent agglutination of Rh-positive cells by the Rh agglutinin. Diamond's and Abelson's[6] investigations have shown that "blocking antibodies" when mixed with whole blood will lead to agglutination of Rh-positive cells on the slide as well as in the test tube. As demonstrated by Diamond and his co-workers[7,8] and Wiener,[9] agglutination of Rh-positive cells by "blocking antibodies" can be easily obtained if undiluted serum or 20% albumin replaces saline solution as a diluent.

The following experiment as recorded in Table II deals with serum (Kru) and its fractions derived by dialysis. The experiment itself was carried out in two parts. In the first part, all dilutions were made in saline solution, and for the second part all dilutions

[3] Race, R. R., *Nature*, 1944, **153**, 771.

[4] Race, R. R., and Taylor, G. L., *Brit. Med. J.*, 1944, **2**, 756.

[5] Wiener, A. S., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 173.

[6] Diamond, L. K., and Abelson, N. M., *J. Lab. and Clin. Med.*, 1945, **30**, 204.

[7] Diamond, L. K., and Abelson, N. M., *J. Lab. and Clin. Med.*, 1945, **30**, 668.

[8] Diamond, L. K., and Denton, R. L., *J. Lab. and Clin. Med.*, 1945, **30**, 821.

[9] Wiener, A. S., *J. Lab. and Clin. Med.*, 1945, **30**, 662.

TABLE II.
Agglutination of Rh_1 Group O Cells by Serum (KRU).

	I. All dilutions in saline			II. All dilutions in serum		
Serum (KRU)	a Native	b Supernatant	c Globulin	a Native	b Supernatant	c Globulin
1. Undiluted	++++	—	++++	++++	++	++++
2. 1:2	+++	—	++++	+++	++	++++
3. 1:4	+++	—	++++	+++	++	++++
4. 1:8	++	—	++++	++	+	++++
5. 1:16	+	—	++++	+	±	++++
6. 1:32	±	—	++++	±	±	+++
7. 1:64	—	—	+++	—	—	+++
8. 1:128	—	—	++	—	—	++
9. 1:256	—	—	+	—	—	+
10. 1:512	—	—	—	—	—	±
11. 1:1024	—	—	—	—	—	±
12. Saline	—	—	—	—	—	—

(cells, serum and its fractions) were made in undiluted serum.

Decreasing amounts of serum (Kru) and its fractions (volume 0.1 cc) were mixed with 0.1 cc of Rh_1 cells belonging to Group O. After standing for one hour at room temperature the tubes were centrifuged and read.

Experiment II again demonstrates the fact that Rh agglutinins appear in the globulin fraction. In this case the "supernatant" appears to be completely free of agglutinins. However, when diluted in serum the "supernatant" reveals the presence of antibodies of the type referred to as "blocking" or "incomplete antibodies." It should be pointed out that the untreated serum when diluted in serum does not reveal a higher antibody titer than when diluted in saline solution. One might therefore come to the conclusion that serum (Kru) is free of "blocking antibodies," but actually the presence of such antibodies can be demonstrated by splitting the serum into fractions by dialysis and using serum as a diluent instead of saline. The relative effectiveness of the globulin fraction in respect to the agglutination of Rh-positive cells in a saline medium can therefore be explained not only by concentration of the Rh agglutinins, but also by the fact that the accompanying "blocking antibodies" have not been concentrated to the same extent. The same results as those reported in Table II were observed when Rh_2 and Rh_0 cells were used instead of Rh_1 cells.

The presence of "blocking antibodies" in serum (Kru) manifests itself easily if a different order of experiment is used as shown in Experiment III.

Decreasing amounts of (a) serum obtained from an Rh-negative mother who had an erythroblastotic child and whose serum contained "blocking antibodies" only, (b) a normal serum (as a control), (c) the supernatant fraction of serum (Kru) (volume 0.15 cc) were mixed with 0.05 cc of a 6% suspension of Rh_1 cells belonging to Group O. After incubation for 30 minutes in the water bath at 37°C, 0.05 cc of undiluted anti-Rh serum (Diamond B-2X) were added. The tubes after being shaken well were kept for an additional hour at 37°C, and centrifuged.

The blocking effect of the supernatant (Kru) which equals in strength the blocking property of an untreated serum of an erythroblastotic woman known to contain "blocking antibodies" is evident from this experiment. The question arose whether the "incomplete" or "blocking antibody" was to be found also in the globulin fraction, or whether this antibody would remain in the supernatant following dialysis. For that reason serum (Heng) containing "blocking antibodies" exclusively was selected and subjected to dialysis, using the technic as described above. The untreated serum as well as the two serum fractions obtained by dialysis were then examined in the following way:

Decreasing amounts of whole serum (Heng) and its serum fractions respectively (volume 0.1 cc) were mixed with 0.1 cc of a 2%

TABLE III.
Inhibitory Effect of "Blocking Antibodies" on Agglutination of Rh_1 Cells by Rh Agglutinins.

Serum containing blocking antibodies	a Blocking serum	b Normal serum control	c Serum (KRU) supernatant
1. Undiluted	—	+++	—
2. 1:3	—	+++	—
3. 1:9	+	+++	±
4. 1:27	++	+++	+
5. 1:81	+++	+++	++
6. 0	+++	+++	+++

suspension of Rh_1 cells belonging to Group O. The experiment was carried out in two parts: Serum dilutions and blood cell suspensions were made (I) with saline solution and (II) with undiluted normal human serum. Tubes were kept for one hour in a water bath at 37 C, and then centrifuged for one minute at a medium speed. The resulting agglutination is shown in Table IV.

The native serum (Heng) as well as the serum fractions obtained by dialysis do not give any visible agglutination with Rh_1 cells Group O when diluted in saline solution. However, if undiluted normal serum is used as a diluent, agglutination of Rh-positive cells occurs in rather high dilutions. The fraction referred to as "supernatant" shows definite agglutination indicating that the "blocking antibody" is present almost to the same extent as in the untreated serum. The globulin fraction also contains some "blocking antibody." Considering the fact, however, that we are dealing here with an approximately 10 times concentrated fraction it becomes obvious that a large portion of the "blocking antibody" has remained in solution (supernatant) following dialysis.

The serum fractions obtained by dialysis were also examined for the presence of isoagglutinins anti-A and anti-B. Somewhat irregular results were obtained. The impression was that dialysis would divide the isoagglutinins more or less equally between the globulin fraction and the supernatant, though in many instances the supernatant fluid contained more isoagglutinins anti-A and anti-B than the globulin fraction.

Conclusions. The dialysis of serum containing Rh antibodies permits the division of such a serum into two fractions, the precipitate, containing mostly globulins, and the supernatant, containing the albumin fraction and a certain amount of the globulin fraction. The sediment, when dissolved in physiological saline solution using one-tenth of the original volume of the serum prior to treatment, is shown to contain the major portion of the Rh agglutinins. A rather potent Rh testing serum can be obtained by this procedure, where in many instances anti-Rh sera contain agglutinins of such weak potency that the native

TABLE IV.
Titration of Rh "Blocking Antibodies" in the Different Fractions of Serum (Heng) Against Rh_1 Cells.

	a Native		b Supernatant		c Globulin	
	I. Diluted with saline	II. Diluted with serum	I. Diluted with saline	II. Diluted with serum	I. Diluted with saline	II. Diluted with serum
1. Undiluted	—	+	—	+	—	++
2. 1:2	—	++	—	++	—	+
3. 1:4	—	+++	—	++	—	+
4. 1:8	—	++	—	+++	—	+
5. 1:16	—	++	—	+++	—	++
6. 1:32	—	++	—	++	—	++
7. 1:64	—	++	—	++	—	++
8. 1:128	—	+	—	+	—	++
9. 1:256	—	±	—	±	—	+
10. 1:512	—	—	—	±	—	+
11. 1:1024	—	—	—	—	—	—
12. Saline	—	—	—	—	—	—

serum would not be strong enough to serve as a diagnostic test serum. The method is especially recommended for this purpose. The "incomplete" or "blocking antibody" has been found to be associated to a large extent with the supernatant, indicating that at least a partial separation of the Rh agglutinin from the "blocking antibody" has been accomplished by dialysis. The effectiveness of the resulting Rh agglutination by the globulin fraction is therefore not only attributed to simple concentration of the Rh agglutinins, but also to the partial removal of the Rh "blocking antibody." The isoagglutinins anti-A and anti-B are not concentrated to the same extent as the Rh agglutinins which constitutes an additional advantage of the globulin preparation over the untreated serum.

15207

Results of Inoculating Okinawan Horses with the Virus of Japanese B Encephalitis.

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It has been reported previously[1] that a considerable percentage of Okinawan horses, from which samples of serum were obtained during an epidemic of Japanese B encephalitis, possessed antibodies against the virus responsible for the epidemic. This observation suggests that horses may occupy the role of intermediary hosts for the virus. The importance of this role in the epidemiology of the disease in human beings depends to a large extent upon whether the virus is capable of circulating in the blood stream of infected horses, from which source it might be transmitted to man by mosquitoes. In order to obtain information concerning the matter, four Okinawan horses were inoculated with the virus of Japanese B encephalitis on 16 September, 1945. The present report is concerned with the results of the experiment.

Materials and Methods. Through the cooperation of the Commander of Naval Construction Troops, a screened, wooden-frame stable was built on the outskirts of the village of Ishikawa. This building contained four stalls, a separate room for laboratory studies, and a concrete floor with drainage outlets leading to a nearby pit. Throughout the course of the experiment, the interior was thoroughly sprayed each day with DDT.

Four horses were selected from the corral at Ishikawa. Like virtually all other horses on Okinawa, these were malnourished, undersized animals whose general physical condition was obviously poor. Three of the horses were approximately one year old; the fourth was a Mongolian pony approximately 12 years old. Each horse was inoculated intravenously with 2 cc of a 10% suspension of mouse-brain tissue, infected with a strain of Japanese B encephalitis virus isolated during this epidemic from a fatal case of encephalitis.[2] Specimens of blood were obtained from each horse before the injection of virus and at intervals of 16 hours, 3, 6, 9, and 12 days after inoculation. Neutralization tests were performed with the pre-inoculation sera in order to determine the status of immunity in each animal at the time of injection. The specimens of blood taken after inoculation were injected intracerebrally and intraperitoneally into mice, in order to determine the presence or absence of virus. In the instances where a virus was recovered

* The ideas set forth herein are those of the authors and are not necessarily those of the Bureau of Medicine and Surgery of the Navy Department.

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1 Hodes, H. L., Thomas, L., and Peck, J. L., *Science,* in press.

2 Hodes, H. L., Thomas, L., and Peck, J. L., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 220.