FREE ANTIGEN AND ANTIBODY CIRCULATING TOGETHER IN LARGE AMOUNTS (HEMAGGLUTININ AND AGGLUTINOGEN IN THE BLOOD OF TRANSFUSED RABBITS).*

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Plates 12 and 13.

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A singular phenomenon, hitherto undescribed, may frequently be observed in the shed blood of rabbits rendered plethoric by repeated small transfusions from compatible donors.[1] In fresh slide preparations the red corpuscles begin almost at once to clump into masses, and within a few moments the separation of plasma and cells is complete. The blood film, homogeneous at first to the naked eye, is transformed into a mixture of clear fluid and large red flakes. In defibrinated blood allowed to stand at room temperature, the cells fall out rapidly as a red granular sediment, which, in the course of a few hours, may become a solid mass that cannot be broken up without hemolysis. The clumping can occur *intra vasam*, as may be shown by inducing stasis in the rabbit's ear with a tourniquet applied at the base. When the marginal ear vein is opened after ½ hour of such stasis, the blood flowing forth is seen to consist of numerous rather large, dark red flakes in a clear fluid. Under ordinary conditions, the clumping is plainly an extravascular phenomenon, being first

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[1] The method of transfusion has already been described (Robertson, O. H., and Rous, P., *J. Exp. Med.*, 1917, xxv, 665). The rabbits received intravenously, 6 days in 7, 10 cc. of whole citrated rabbit blood obtained by the cardiac aspiration of compatible donors. For each recipient a series of donors were employed in rotation. Their compatability had been determined by the method of Rous and Turner, *J. Am. Med. Assn.*, 1915, lxiv, 1980.

visible in slide preparations some 12 to 40 seconds after the blood is drawn.

Protection of the Organism.

The clumping of the red cells would result in pulmonary emboli and be quickly fatal, were it not in some way prevented *in vivo*. Our first tests had to do with this phenomenon. The problem presented proved unexpectedly easy of solution. The clumping is absolutely conditioned by temperature. If no precautions are taken to keep warm the rabbit's ear when bound with a tourniquet, clumping occurs in the vessels, as already described. But if the bound ear is kept in water at the body temperature, the blood taken from it after half an hour shows no clumping, and this appears only secondarily as cooling occurs. Again, if a little fresh blood is taken into each of two thick-walled glass tubes of capillary bore, one of which has been chilled in the ice box, the other heated to body temperature, a gross clumping will be seen almost at once in the cold tube, whereas in the warm one the blood remains homogeneous during the 3 or 4 minutes before clotting takes place. Blood allowed to drop directly into a few cubic centimeters of cold salt solution shows clumping almost before it can be distributed in the medium; whereas if the solution has the body temperature, the corpuscles remain separate for 24 hours even—the longest time over which we have observed them. If, after 24 hours of warmth, the mixture is cooled in running water, clumping occurs in case the initial dilution of the blood has not been too great.

The Clumping Is a True Agglutination.

The character of the temperature control suggests that the clumping is caused by hemagglutinins. Landsteiner has shown that the isoagglutinins and the weak normal autoagglutinins of several animal species, among them the rabbit, are similarly governed by temperature and have most effect in the cold.[2] The clumping, viewed microscopically, has certainly the appearance of a true agglutination, while the circumstances of its occurrence are such as would favor the development of agglutinins. It is most pronounced when the rabbit

[2] Landsteiner, K., *Münch. med. Woch.*, 1903, l, 1812.

has received from ten to fifteen transfusions. After the first five or six, a change begins to be noticeable in the shed blood. Rouleau formation is more marked than normally. Then, as the injections are continued, the rouleaux of the shed blood, which are strikingly long and perfect, tend to draw together into masses in which they undergo little disorganization (Figs. 1 and 2). Finally, the tendency to clumping becomes so strong that the rouleaux present when the blood is first shed collapse after a few moments into irregular agglomerates of corpuscles lying against one another without definite arrangement. In slide preparations these masses are at first connected by large trunks of cells, but shrinkage soon takes place—doubtless from closer apposition of the cells—and the trunks pull out into thin strands, often only one or two cells thick (Figs. 3 and 4). Within the large plasma spaces thus opened up, there are almost no corpuscles, red or white. When pressure is put on the cover-glass the cells are often stretched into long ropes, but they hold together tenaciously as if made of a sticky, elastic material. When the pressure is released they resume their normal shape.

If the clumping is caused by a true agglutinin, a separation of this element from the cells should be possible. It has been accomplished by repeatedly washing the cells in warm salt solution in which, as already stated, they are not clumped. Cells thus washed in the centrifuge remain unclumped when sedimented and cooled. But they at once clump when placed in serum obtained from a specimen of the blood defibrinated and centrifuged in the warm.

Attempts were now made to obtain the agglutinin in salt solution by the method Landsteiner employed with the weak, normal autoagglutinin of rabbits.[2] Landsteiner allowed a small quantity of cells in a large quantity of serum to stand over night on ice. Complete agglutination took place. The cell mass was now washed several times in ice cold salt solution and then placed in a little of the fluid at body temperature. After some hours it was centrifuged while still warm. The heating had liberated the agglutinin, which passed into the salt solution, and the latter now possessed the ability to agglutinate cells.

In the case of our transfused rabbits the serum factor responsible for clumping was so strong that there was no necessity for the serum

to preponderate greatly over the cells or for more than a brief chilling and warming.

Experiment 1.—A little of the blood of a transfused rabbit was taken into a test-tube surrounded by a water jacket at 38°C. and was defibrinated with glass beads. From this 0.6 cc. was pipetted off and cooled in ice. The corpuscles rapidly clumped and fell to the bottom of the tube. After 45 minutes centrifugation was done in an ice jacket. The serum was immediately pipetted off and kept. It will be termed Serum A. The cells, which had formed a solid mass, were now twice washed with 3.5 cc. of ice cold salt solution. The cell mass showed no tendency to break up when thus handled. All fluid was now pipetted away, 0.3 cc. of fresh salt solution put on, and the tube transferred to a water bath at 40°C. Within 5 minutes the mass had broken up into a homogeneous cell suspension. After 10 minutes more, an attempt was made to throw down the cells while still warm, but though a warm water jacket was used, the temperature fell sufficiently during the process for some clumping to occur. The tube was therefore warmed again for 15 minutes and again centrifuged rapidly, but now in a jacket of warm paraffin oil. This time the heat was retained, no clumping occurred, and the fluid—Fluid B, as it will be called—was immediately taken off for test. The cell sediment was then twice washed in an excess of warm salt solution and made up to the original blood bulk. The cells remained unclumped.

The following mixtures were now set up:

(*a*) 1 part cell suspension + 3 parts Fluid B.
(*b*) 1 part cell suspension + 3 parts Fluid B + 9 parts salt solution.
(*c*) 1 part cell suspension + 3 parts Serum A + 9 parts salt solution.
(*d*) 1 part cell suspension + 12 parts salt solution.

In (*a*) marked clumping of the cells took place almost at once at room temperature. The other three mixtures were cooled in ice. Strong clumping was observed after a few minutes in (*b*), slight clumping in (*c*), and none at all in (*d*). From the presence of a slight clumping in the mixture (*c*) it is evident that the factor responsible for agglutination was not completely removed from the serum when the defibrinated blood was cooled to 0° C.

Variation with Temperature.

In experiments such as the foregoing, success was obtained only after the necessity for careful maintenance of the essential temperatures had been recognized. During the separation of the agglutinin a moment's accidental cooling or warming was sufficient to fix or liberate it in large part from the cells, thus leading to confusion. When blood was defibrinated in the warm and then gradually cooled in tubes that permitted of microscopic inspection, slight agglutination was

found to appear as the temperature fell from 36° to 35°C. At 33°C. large clumps formed; and at room temperature (22°) the agglutination was massive. When the tube was warmed again, all clumping disappeared at between 35° and 36°C. In the experiment for the separation of agglutinin given in detail above, all agglutinin was not absorbed from the serum at 0°C. This may have been due to insufficient contact of serum and cells owing to the rapid clumping and sedimentation of the latter when suddenly chilled. We have repeatedly noted that a potent serum, if allowed to separate from the clot at room temperature, may contain no agglutinin whatever.

Reversibility of the Reaction.

The rapid variation in the clumping with changes in temperature has led us to investigate the reversibility of the agglutination. A sample of blood was defibrinated in the warm, as usual, filtered through gauze, and placed, first on ice, and, when clumping was complete, in water at body temperature. This was repeated as fast as massive clumping or its reverse, complete dissociation, had occurred. After nine coolings and warmings, the cells still clumped and separated as rapidly and completely as at first. There was an entire absence of the gummy change seen when cells are repeatedly clumped by a heteroagglutinin.[3]

Strength of the Agglutinin.

The great variation in the clumping at different temperatures and the rapidity with which the agglutinating principle is fixed or freed has rendered difficult a precise determination of its strength. We have employed a crude method, allowing the blood to fall from the rabbit's ear, drop by drop, into known quantities of warm salt solution, and noting the agglutination when the mixtures have been cooled for some minutes at room temperature. Under these circumstances the dilution of both antigen and antibody vary, but they vary alike, maintaining practically a constant relation to each other. There is not the same likelihood of error in the ingredients as when mixtures are made of cells and serum separated from each other in the warm.

[3] Landsteiner, K., and Reich, M., *Centr. Bakteriol., 1te Abt., Orig.*, 1905, xxxix, 83.

But the temperature of the salt solution must be above 37°C., since even slight cooling results in some clumping of the cells before they can be properly distributed.

The strongest agglutinin found in the transfused rabbits caused well defined clumping in a mixture of one drop of blood with 100 cc. of salt solution; that is, in a plasma dilution of approximately 1: 2,800.[4] No clumping occurred in the 1: 5,600 mixture. The plasma of a second rabbit agglutinated the cells when diluted 500 times. These were exceptional instances. In the majority of cases the plasma failed to clump the cells when it was diluted with more than 20 parts of salt solution.

The clumping phenomenon did not regularly appear in transfused rabbits. Indeed, in ten out of twenty transfused with a special view to its development it was never observed despite the fact that the transfusions were continued far beyond the usual period. Furthermore, in rabbits transfused persistently any agglutinating factor that had developed tended to disappear. A similar disappearance of precipitin following unduly prolonged immunization has been recorded by Tchistovitch[5] and Nuttall.[6] Sudden reductions in the plethora of the recipients, accomplished by bleeding, failed to induce or increase the clumping phenomenon, as did also a use of donors with cells agglutinable by the recipient's plasma.

Agglutination and Anemia.

The peculiar temperature control of the agglutination in the transfused animals has an obvious, if superficial, likeness to that occurring in paroxysmal hemoglobinuria. And the fact that the animals with the strongest agglutinin developed a sudden anemia, in the midst, so

[4] The percentage volume of the blood plasma was reckoned from a comparison of the rabbit's hemoglobin with that of normal rabbits of which the cell plasma ratio had been established with Epstein's hematocrit. In these normal animals the cells averaged 42 per cent and the plasma 58 per cent of the blood volume. Twenty drops of blood were assumed to make 1 cc. This was the case in actual tests.

[5] Tchistovitch, T., *Ann. Inst. Pasteur*, 1899, xiii, 406.

[6] Nuttall, G. H. F., Blood immunity and blood relationship, Cambridge, 1904, 127.

to speak, of their plethora, has in this respect a special interest. The hemoglobin of the rabbit with an agglutinin active in a 1:2,800 serum dilution fell after the fifteenth transfusion from 128 (Sahli) to 75 per cent in the course of 4 days, despite the injection on each of these days of the usual 10 cc. of blood. The transfusions were now stopped, and the hemoglobin fell to 37 per cent in 2 days more, after which a gradual recovery ensued. The animal at no time manifested symptoms of distress. Some of the blood changes in this rabbit and others of like sort have already been described by Robertson in another connection.[7] In these instances the hemagglutinin was at its greatest strength when the anemia developed, while in animals with a weak agglutinin or none, an anemia was never observed, but, on the contrary, plethora was maintained for weeks after the transfusions had been stopped.

No adequate search has yet been made for an hemolysin in the plasma of the animals becoming anemic, but we have chilled, without result, two plethoric rabbits possessing a weak agglutinin (active in a 1:5 dilution of the whole blood) in the hope of initiating a drop in the hemoglobin. The chilling was accomplished by means of ice cold water, in which the well shaved ear of the rabbit was submerged for ½ to 1 hour. Throughout this period the circulation in the cold ear was exceptionally good. The rectal temperature fell to 37°C., considerably below the normal for the rabbit, but not low enough to produce the *in vitro* agglutination of blood corpuscles.

Persistence of the Agglutinin.

The agglutinating principle, once it has appeared in a blood, persists for a long period and is relatively uninfluenced by the disappearance of plethora, or by sudden intercurrent anemia of the sort just described, or by moderate bleedings. In a rabbit, for example, in which the hemoglobin fell from 125 to 27 per cent in the course of a few days, with a gradual return to the normal of 90 per cent, the agglutinin persisted throughout. 110 days after the normal hemoglobin had been finally reached, the blood still showed clumping when di-

[7] Robertson, O. H., *J. Exp. Med.*, 1917, xxvi, 221.

luted with two volumes of salt solution. This was 133 days after the last transfusion.

SUMMARY.

In rabbits transfused almost daily with the whole citrated blood of other rabbits, an extraordinary condition often develops, which manifests itself in an almost immediate clumping together of all the red cells in specimens of the shed blood. This clumping is due to one or more true agglutinins, of which the strength may be such as to cause clumping in a 1: 2,800 plasma dilution.

The agglutinating principle circulates with the corpuscles against which it is effective; but under ordinary circumstances intravascular clumping fails to occur because the union of antigen and antibody can take place only at a temperature several degrees below that of the body. If the temperature is sufficiently lowered, as when a tourniquet is applied to the rabbit's ear, intravascular clumping ensues. In defibrinated blood, gradually cooled, clumping is first noted as the temperature of 35°C. is approached; and at room temperature (22°) the corpuscles will often come together in a short time into a single, solid mass. At 0°C. the agglutination is still more marked. The reaction seems to be completely reversible, for when the blood is warmed again, the clumps break up and disappear at between 35° and 36°C. Cooling and warming with the resultant clumping and dissociation can be carried out many times on the same blood specimen without apparent change in the corpuscles or in the rapidity of the reaction. The response to temperature changes is extremely prompt.

Once it has been elicited, the agglutinating principle may persist for a long time after the transfusions are stopped. In one instance it was still strong 133 days after the last transfusion. During this period the plethora was succeeded by a severe anemia, which in turn was recovered from. In many rabbits no agglutinin develops, and a continuance of the transfusions will not elicit it. Indeed, when present it tends to disappear if the transfusions are persisted in.

In several of the animals in which the agglutinin was strongest, the plethora was suddenly succeeded by severe anemia, despite continued transfusions. The character of the temperature control of

the agglutination, which somewhat resembles that of the hemolysin in paroxysmal hemoglobinuria, has led us to consider whether the blood destruction might not be due to accidental chilling of the animal. Efforts to induce a fall in the hemoglobin by placing the rabbit's ear in ice water have as yet been unsuccessful. Thus far no adequate search for an hemolysin has been made.

The object of the present paper has been to describe a condition in which large amounts of free antigen and antibody circulate together in the organism, and to demonstrate the factor which prevents their union, the results of which could easily be fatal. The causes of the condition will be dealt with in a subsequent communication.

EXPLANATION OF PLATES.

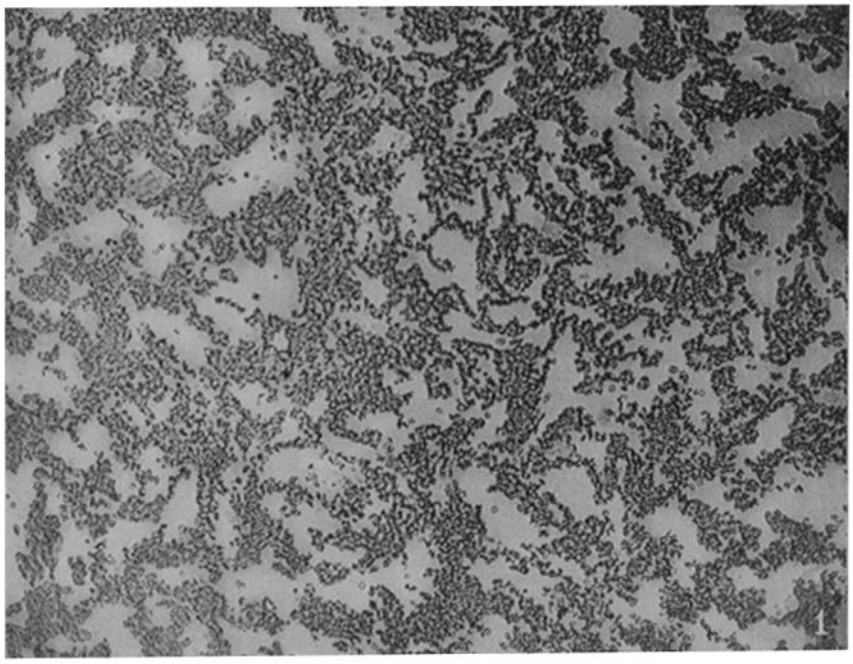
PLATE 12.

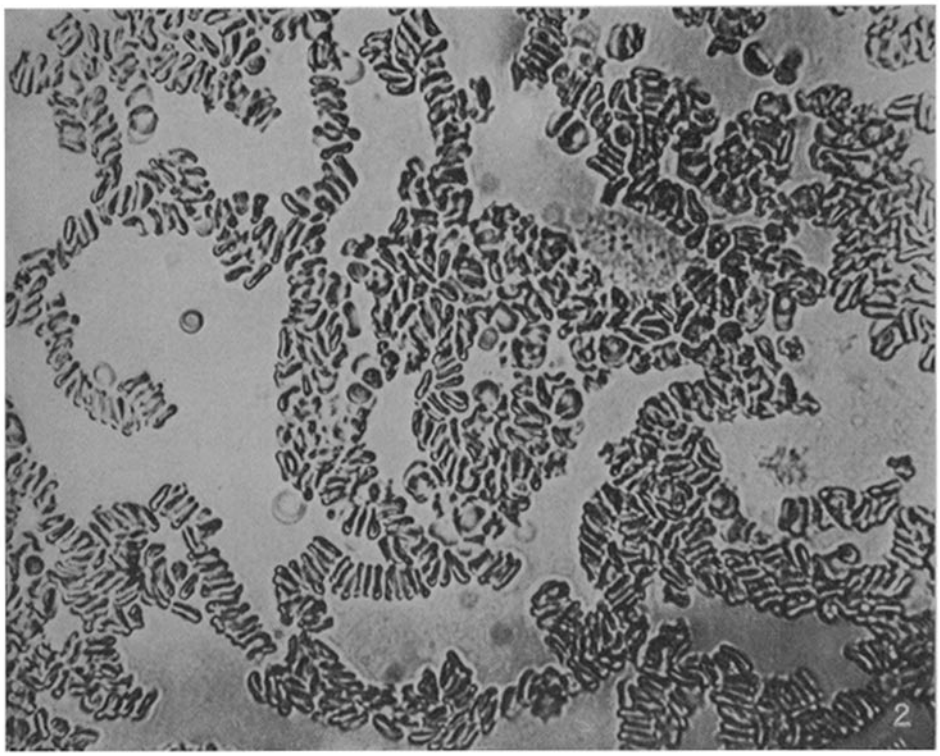
FIG. 1. A weak clumping phenomenon. The rouleaux are largely intact.

FIG. 2. A weak clumping phenomenon. Marked rouleau formation.

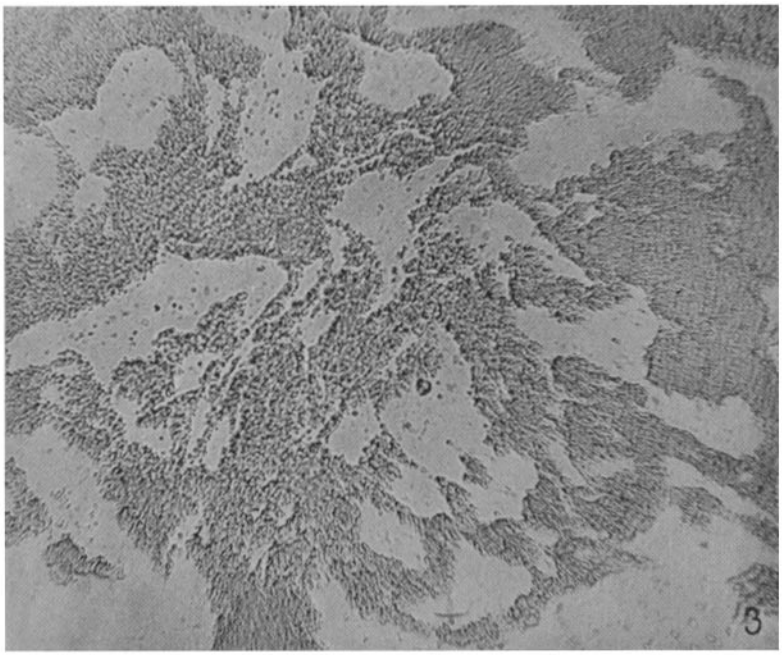
PLATE 13.

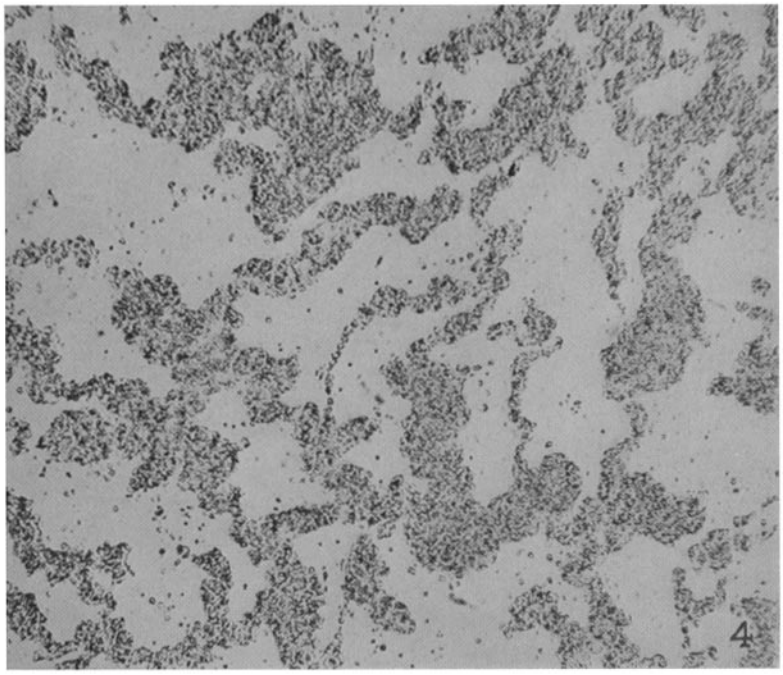
FIGS. 3 and 4. The clumping phenomenon in pronounced form. In the large serum spaces there are almost no free cells.





(Rous and Robertson: Free antigen and antibody.)





(Rous and Robertson: Free antigen and antibody.)