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[Reprinted from JOURNAL OF EXPERIMENTAL MEDICINE, Vol. XIII., No. 4, 1911.]

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STUDIES IN ISOAGGLUTINATION.

I. TRANSFUSION AND THE QUESTION OF INTRAVASCULAR AGGLUTINATION.*

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INTRODUCTION.

This paper offers a solution of a special problem in isoagglutination; namely, the question whether transfusion is permissible between persons whose sera agglutinate the red blood cells of each other. It also shows why such transfusions are not regularly followed by serious symptoms.

The isoagglutination of human red blood cells was discovered independently by Landsteiner and by Shattock in 1900. Many workers at first regarded the phenomenon as one of pathological significance. Halban, Ascoli, and others, however, showed that isoagglutination occurred with a large proportion of normal bloods, and Landsteiner discovered the remarkable fact that all human bloods can be divided into three sharply defined groups, according to the way in which they interagglutinate. To these groups was subsequently added a fourth, independently discovered by several observers. The groups are as follows (see table 1):

The serum of the first group, designated in this paper as group I, possesses the power of agglutinating the red cells of members of all the other groups, but the red cells of members of group I are not agglutinated by any human serum. This group includes about 50 per cent. of all persons examined.

The serum of members of the second group (group II) can agglutinate the cells of persons belonging to the third and fourth groups, but not of other members of the second group itself nor, of course, of group I. The cells of mem-

*Received for publication, February 5, 1911. This study is one of a projected series on *isoagglutination*, which in turn constitutes a section of a comprehensive plan of research on the composition of protoplasm as well as the structural and dynamic relationships of cell constituents and products. These investigations are now in progress in the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, and under the auspices of the George Crocker Special Research Fund.

bers of the second group can be agglutinated by sera of individuals of group I and of group III only.

The third group is the reciprocal of the second group; its serum agglutinates cells of persons belonging to members of the second and fourth groups. Its cells are agglutinated by sera of the second and fourth groups, and, of course, of the first group. Members of this group sometimes show slight individual irregularities, the cells now and then failing to be agglutinated by the sera of some members of the second group, although being agglutinated by others, and the sera occasionally agglutinating the cells of some, but not all, other members of the third group itself.

The fourth group, whose members are relatively rare, is characterized by possessing no agglutinin for human red cells and by its cells being agglutinable by the sera of all other groups.

TABLE I.

Interagglutination Reactions of Ten Individuals.

Groups.			Sera. I				II	III				IV
			1	2	3	4	8	6	7	9	10	5
Cells	I	1	−	−	−	−	−	−	−	−	−	−
		2	−	−	−	−	−	−	−	−	−	−
		3	−	−	−	−	−	−	−	−	−	−
		4	−	−	−	−	−	−	−	−	−	−
	II	8	+	+	+	+	−	+	+	+	+	−
	III	6	+	+	+	+	−	−	−	−	−	−
		7	+	+	+	+	+	−	−	−	−	−
		9	+	+	+	+	+	−	−	−	−	−
		10	+	+	+	+	+	−	−	−	−	−
	IV	5	+	+	+	+	+	+	+	−	?	−

Landsteiner himself suggested that the groupings could be explained by assuming the existence of two agglutinins, of which the second group possessed one, the third group the other, the first group both, and the fourth group neither. In each case the cells are susceptible only to that agglutinin which does not exist in the individual's own serum. (Moss has, in a recent paper, made the quite unnecessary assumption that there are three agglutinins.)

The group characteristics are permanent for each individual throughout his life. When concentrated, the agglutinins act almost instantaneously; when diluted they act more slowly. They act in the cold as well as at high temperatures and they are relatively thermostable. Hektoen, who first worked out many of their characteristics, has shown that the agglutinin is absorbed by the cells which it agglutinates.

These agglutinins, like other serum agglutinins, are probably associated with the euglobulin of the blood. I have recently found that when agglutinative serum in collodion sacs is dialyzed against running water, the precipitate which forms in the serum can be redissolved in saline solution and contains the agglutinin in only slightly weakened concentration.

The peculiar groupings are not only permanent with the individual but they

are hereditary. In 1908, in a paper[1] by Dr. A. A. Epstein and the author, we stated that these agglutinins were inherited and followed the Mendelian law. At that time I had examined only five families and was unable to prove completely the assertion. Recently Von Dungern and Hirschfeld have examined seventy-two families and have conclusively proved that the agglutinins are hereditary and follow Mendel's law. From this explanation of the general nature of isoagglutination, I return to the special subject of the present paper.

Landsteiner, Hektoen, and, in fact, almost all who have written on the subject, as well as Crile in his work on transfusion, have expressed the opinion that isoagglutination might be a danger in transfusion. Nevertheless, the surgeons have performed a large number of transfusions without making any serum tests, and in most of the cases there have been no accidents. There have been accidents and untoward symptoms, however, both in published and unpublished cases.[2]

The only direct observations on the present question are those in a recent communication by W. Schultz.[3] Schultz made intravenous injections in ten cases of small amounts (5 to 250 cubic centimeters) of defibrinated blood. In his first case the serum of the patient was agglutinative as well as hemolytic toward the donor's red cells. After the injection of 50 cubic centimeters of blood there was a collapse, followed by vomiting, diarrhea, fever, and edema of the face and hands. In case 6, however, in which there was neither agglutination nor hemolysis between the two bloods, there was also a collapse after the injection of only 5 cubic centimeters of blood. In case 4 there was mutual agglutination but no hemolysis. Schultz, made cautious by the first experience, injected only 5 cubic centimeters of defibrinated blood. No ill effects followed. Shultz concluded that agglutinative blood is an absolute contra-indication to transfusion.

The numerous experiments by Flexner, Pearce, Coca, and others, demonstrating that various lesions and symptoms, including death, could be produced by the agglutinative sera or agglutinable cells of foreign species, do not bear directly on the present subject.

[1] *Tr. of the New York Path. Soc.*, 1908, viii, 117.

[2] The results of a study of transfusions with regard to agglutination and hemolysis tests made at Mt. Sinai Hospital during the past two years, will be published separately by Dr. Kaliski and the author.

[3] *Berl. klin. Wchnschr.*, 1910, xlvii, 1407.

EXPERIMENTAL PART.

I have found that the explanation of the inconsistency between the very sharp test tube agglutination, and the frequent absence of symptoms on transfusion of agglutinative blood, depends on the quantitative relations between agglutinin and agglutinable cells, and non-agglutinable cells.

While performing some experiments for the purpose of determining the bulk of agglutinable cells that is necessary for complete absorption of all the agglutinin from a given serum, I noticed that when the amount of red cells exceeded a certain ratio to the amount of serum, all the agglutinin was absorbed without, however, any agglutination taking place, so far as the naked eye could determine.

This was confirmed by several experiments, of which one only is referred to in table II. In this experiment blood cells belonging to group II were obtained in normal salt solution containing 1 per cent. of sodium citrate. The cells were washed once in salt solution, then again sedimented by centrifuging for about ten minutes at high speed. All the supernatant fluid was decanted and the concentrated cells were used in the experiment. An actual count (with Thoma-Zeiss hemocytometer) showed 10,000,000 red blood cells per cubic millimeter. It has been calculated (P. Weber) that there is only space for 13,000,000 human red cells in one cubic millimeter, so the suspension used might be designated as a 75 per cent. suspension.

The serum used was of class I. Its exact agglutinative titer was not determined, but the serum was known to be active in a dilution of at least 1 to 12. The mixtures were made in the proportions stated, kept at room temperature for thirty minutes, again thoroughly mixed, and kept in an ice box over night. On the following day the serum was pipetted off and its agglutinative power tested against a 5 per cent. suspension of the same supply of red cells.

From table II it may be seen that one volume of the cell suspension absorbed all the agglutinin from at least sixteen volumes of serum, and that as one volume of cells was still completely agglutinated by thirteen volumes of serum, the cells were able to absorb more agglutinin than was actually necessary to agglutinate them. Controls with non-agglutinable cells showed no absorption of agglutinin. On the other hand, above this concentration of one part of

cells to thirteen parts of serum, the agglutination diminished in intensity, all the agglutinin being still absorbed; and when the concentration of four volumes of cells to six volumes of serum was reached, agglutination could no longer be observed, either macroscopically or on microscopic examinations of a drop of the mixture. It was at first thought that in these concentrated cell mixtures the agglutinin was distributed among the cells in such a way that too little of the active substance went to each cell to produce the visible physical change known as agglutination. Later, however, when the work was repeated and the microscopic examinations were made in a different way, namely, by diluting the mixture with an excess of salt solution or of Hayem's fluid, it was seen that some agglutination also occurred in the more concentrated mixtures, but that the clumps were extremely small (four to five cells) and had been masked previously by the excess of densely concentrated non-agglutinated cells.

TABLE II.

The Absorption of Agglutinin by Agglutinable Cells.

Volumes.		Agglutination.	Absorption of agglutinin.
Serum.	Cells.		
4	6	−	All absorbed.
5	5	−	" "
6	4	−	" "
7	3	+ (microscopic).	" "
8	2	+ (naked eye).	" "
10	1	almost complete.	" "
13	1	complete.	" "
14	1	"	" "
15	1	"	" "
16	1	"	" "
17	1	"	Partly absorbed.
18	1	"	" "
19	1	"	Not absorbed.
20	1	"	" "
21	1	"	" "

In transfusion, however, two other substances may accompany agglutinable cells and agglutinative serum (plasma). These are non-agglutinative serum (or plasma) and non-agglutinable cells. The question therefore arises whether non-agglutinative serum exerts any hindering effect on agglutination. Hektoen states that the addition of increasing quantities of serum from one group to

sera of the other groups does not hinder agglutination of the proper corpuscles. The author has reëxamined the question by directly comparing the results of dilution with saline solution and dilution with neutral (non-agglutinative) serum (table III).

Two specimens of blood (labeled E and K) obtained by defibrination were used, the one of class II and the other of class III.[4] The cells of one (K) were washed and made up to a 5 per cent. suspension in 0.9 per cent. saline solution. Two parallel series of dilutions of the agglutinative serum E were then made; in one series saline solution was the diluent; in the other, neutral (non-agglutinative) serum K.

TABLE III.

Agglutination in Diluted and in Mixed Sera.

Proportions of			Actual dilution of active serum.	Agglutination.	
Active serum.	Diluent.	Cell suspension.		With *saline* as diluent.	With *serum* as diluent.
2	7	3	1 : 6	Complete: 2 or 3 clumps.	Complete: numerous small clumps.
1	5	2	1 : 8	Complete: 2 or 3 clumps.	Complete: numerous small clumps.
2	13	5	1 : 10	Complete: 2 or 3 clumps.	Complete : numerous small clumps.
1	8	3	1 : 12	Complete: 2 or 3 clumps.	Complete: numerous small clumps.
2	19	7	1 : 14	Complete: 2 or 3 clumps.	Complete : numerous small clumps.
1	11	4	1 : 16	Complete: 2 or 3 clumps.	Complete : numerous small clumps.
2	25	9	1 : 18	Complete: 2 or 3 clumps.	Complete : numerous small clumps.
1	14	5	1 : 20	Numerous small clumps.	Very fine clumps.
1	17	6	1 : 24	Fine clumps.	Clumps seen only through microscope.
1	20	7	1 : 28	Very fine clumps.	Clumps seen only through microscope.
1	23	8	1 : 32	Clumps visible only through microscope.	No agglutination.
1	26	9	1 : 36	No agglutination.	No agglutination.

To each test tube was added exactly the same proportion of cell suspension; namely, one-fourth of the total volume. The actual dilution of the agglutinative serum is shown in the fourth column of table III. It is seen that when saline solution was the diluent, agglutination was still observable at a dilution of 1 to 32; when

[4] It was not determined which specimen was of class III; and the determination would be of no significance in the present instance.

serum was the diluent, it occurred at a dilution of 1 to 28. There is then only an extremely slight interference, if any, on the part of neutral serum.

The qualitative differences between the two series, however, are more significant than the quantitative. Where saline solution was the diluent there were, in all the dilutions below 1 to 20, only two or three large compact clumps. Where serum was the diluent, on the other hand, there were very many small clumps.

The second question, whether the presence of non-agglutinable cells in the mixture interferes with agglutination, was investigated by making mixtures of whole defibrinated bloods in varying proportions, instead of serum and washed cells. These mixtures imitated then, as closely as can be done in a test tube, the actual conditions in transfusion (table IV).

TABLE IV.

Mixtures of an Agglutinative and an Agglutinable Whole Blood. Defibrinated Blood of Class I (Agglutinative Serum, Non-Agglutinable Cells) and of Class II (Agglutinable Cells, Non-Agglutinative Serum).

Proportions.		Agglutination.*
Blood (class II).	Blood (class I).	
1	9	+
2	8	+
3	7	+
4	6	+
5	5	+ (greatest number of clumps).
6	4	+ (smaller numer of clumps).
7	3	+ (only a few clumps).
8	2	−
9	1	−

*The clumps were all microscopic in size.

The mixed bloods were thoroughly shaken at once and again in about thirty minutes, at room temperature. They were then put on ice and observations were made at the end of twenty-four hours.

None of the mixtures exhibited macroscopic agglutination; all of them formed, when shaken, thick emulsions. Through the microscope it was seen, however, that microscopic agglutination had occurred in seven of the nine mixtures. The presence of non-agglutinable cells in the mixtures, then, did not prevent agglutina-

tion, but did change entirely the nature of the agglutination;—instead of a few large clumps, innumerable microscopic clumps were produced. This was particularly true of the first five mixtures in the series; in the other mixtures the absorption of agglutinin by the excess of agglutinable cells present also played a part; and in the last two, they apparently prevented agglutination, as in the experiments referred to in table II.

This, of course, is extremely significant for transfusion; a few large clumps might cause serious or fatal embolism. A considerable number of microscopic clumps, becoming plugged in widely scattered capillaries might cause few or no symptoms. The microscopic appearances were controlled by making a similar series of mixtures of each of the specimens of blood used with another blood of its own class. In none of these were any clumps observed.

From this experiment it is seen, then, that when the proportion of agglutinative to agglutinable blood is small (1 to 4 or less), no agglutination occurs, all the agglutinin being absorbed by the excess of substratum. When the proportion is reversed, agglutination occurs but is microscopic. When the bloods are mutually agglutinative (one of class II, the other of class III), one finds, likewise, that the blood shows no agglutination to the naked eye but shows microscopic clumps in all proportions (table V). In this instance both of the inhibitory factors are in play; either the one or the other variety of red cells being always present in too great a proportion to be agglutinated completely. The excess of cells functionates as non-agglutinable, and interferes (probably mechanically) with the agglutination of the other species of red cells, which, in many of the mixtures, are present in small amounts compared to their agglutinative serum, and would otherwise be agglutinated completely.

There is no doubt, then, that concentrated non-agglutinable cells interfere with the agglutination of actually agglutinable cells present in the same mixture. This interference is probably mechanical; it does not occur when both varieties of cells are present in small amounts.

Additional experiments were performed to determine whether similar conditions prevail when one or the other blood is very

TABLE V.

Mixtures of Mutually Agglutinative Bloods.

Proportions.		None of the mixtures showed any agglutination to the naked eye, but all did microscopically.*
Blood 8 (class III)	Blood 3 (class II).	
9	1	Numerous small compact clumps.
8	2	Small clumps of 10 to 30 cells.
7	3	A few small clumps.
6	4	"
5	5	"
4	6	"
3	7	"
2	8	"
1	9	More numerous compact clumps, 20 to 24 cells.

*The microscopic examinations were made by diluting with Hayem's fluid, in which further agglutination can not occur.

anemic, as is practically always the case in patients requiring transfusion. From the defibrinated whole bloods of normal people, artificial anemic bloods were made by pipetting off one volume of the sedimented red cells and suspending it in nine volumes of the corresponding serum. These anemic bloods then contained less than one million red cells per cubic millimeter. Mixtures were made (table VI) in such a way that in one series (A) the anemic blood was agglutinative, in the other (B) the anemic blood was agglutinable. From the results in series B it appears that, if the anemic patient's cells are agglutinable by the donor's serum, a considerable amount (at least one-fourth as much as the total blood volume of the patient) can be transfused before the danger of intravascular agglutination occurs. If, as in A, the contrary is true, and the anemic patient's blood is agglutinative toward the full-blooded donor's cells, the likelihood of intravascular agglutination is much greater and disappears only when a large amount (more than the volume of the patient's blood) is transfused. The same should be true for mutually agglutinative bloods.

This would depend, of course, to a considerable extent on the rapidity of mixing of transfused bood. The assumption need not be made that mixing occurs very rapidly, because when the hindering factors are present—dilution of agglutinative serum, excess of agglutinable cells, interference by non-agglutinable cells—any agglutination that does take place occurs very slowly.

TABLE VI.

Series A.

Mixtures of Anemic Blood No. 10 (Agglutinative, Class I) and Full Blood No. 1 (Agglutinable, Class II).

Proportions.		Agglutination.
Anemic No. 10.	Full No. 1.	
9	1	Fine clumps seen with naked eye.
8	2	"
5	5	Microscopic clumps only.
2	8	Doubtful, a few small clumps.
1	9	No clumps.

Series B.

Mixtures of Full Blood No. 10 (Agglutinative, Class I) and Anemic Blood No. 1 (Agglutinable, Class II).

Proportions.		(Microscopic clumping only.)
Full No. 10.	Anemic No. 1.	
1	9	No clumps.
2	8	No clumps.
5	5	Many sharply defined clumps.
8	2	Smaller number of clumps.
9	1	Very few clumps, but all sharply defined.

Above and beyond all the considerations presented here, there remains a possibility that the isoagglutinins are substances which, like fibrin ferment, are formed only after the blood leaves the vessels. This, though possible, seems highly improbable. The agglutinins are present no matter how the cells and serum are obtained,—whether by defibrination, ordinary clotting, citration, and the use of oxalate or hirudin. Furthermore, I have recently found them to occur in most transudates and exudates.

CLINICAL PART.

I have recently seen several transfusions which confirm the view that in the presence of an excess of agglutinable cells all the agglutinin can be absorbed without doing harm.

In the first case the patient was a young woman with a bleeding gastric ulcer. Her blood belonged to the second or third agglutinative class, while that of the donor, who was chosen in the emergency, belonged to the first; that is, the donor's serum agglutinated the patient's cells, but his cells were not agglutinated by the patient's

serum (the same instance as that in table VI, series B). Sufficient blood was transfused to carry the patient's hemoglobin from 18 at the beginning to 40 per cent. at the end of the transfusion. By calculation, this must have been about forty per cent. as much as the patient's own blood volume.[5] No untoward symptoms occurred and the patient subsequently recovered. On examining the patient's serum, obtained thirty-six hours after the transfusion, it was found to contain no agglutinin of class I; it did not agglutinate her cells obtained either before or after the transfusion. All the agglutinin of class I which had been transfused into her had disappeared.

A confirmation of the view expressed here is also furnished by a case recently described by J. G. Hopkins.[6] The transfusion was instituted for the relief of an extreme anemia resembling the primary pernicious type. The patient's serum agglutinated the donor's cells but the donor's serum did not agglutinate the patient's cells (the more dangerous instance, as shown in table VI, series A). Immediately following transfusion, smears of the peripheral blood showed large numbers of polymorphonuclear leucocytes containing red blood cells.

After the transfusion, the patient was incontinent of feces, and irrational. Six hours later he developed hemiplegia and coma. Nine hours after the transfusion he died.

"The post-mortem serum did not agglutinate the donor's corpuscles". "The agglutinins present when the serum was first examined were evidently bound". Hopkins is "unable to determine the meaning of the blood-picture in this case" and believes that "what was observed was probably phagocytosis of the transfused red cells by the phagocytes of the recipient".

We have here a case, then, in which apparently all the agglutinin in the body was absorbed by the transfused red cells. It seems at least probable that the death was due to intravascular agglutination, and that the phagocyted red cells were such as had already been

[5] Patient's hemoglobin, at beginning = 18 per cent.; at end = 40 per cent. Donor's hemoglobin, at beginning = 90 per cent. Let the relation of volume transfused to the patient's original blood volume = $x : 1$; then $18 + 90x = 40(x + 1)$ and $x = 22/50 = 0.44$.

[6] Hopkins, *Arch. Int. Med.*, 1910, vi, 270.

affected by the agglutinative serum and were in effect foreign bodies, although on account of the hindering factors mentioned in the present article, they had not yet become actually agglutinated.

Since the publication of Hopkins' paper I have had the opportunity of studying the blood of three transfusions with regard to phagocytosis. In the first two cases a number of candidates were examined, and a donor was chosen who belonged to the same agglutinative group as the patient. In these cases no phagocytosis of red cells was observed.[7] In the third case the surgeon elected to disregard the blood tests. The patient's serum was agglutinative toward the donor's red cells, but the donor's serum was not agglutinative to the patient's cells. The patient's serum was also very slightly hemolytic.

Blood smears made at the end of transfusion showed that many of the polynuclear leucocytes were phagocyting red cells in the peripheral circulation of the patient. The leucocytes contained from one to three red cells each. No agglutinated red cells could be seen in the smears, nor were any seen in a fresh drop of blood diluted with sodium citrate in normal salt solution.

The examination of cells and serum after the transfusion showed a remarkable condition of affairs (table VII).

TABLE VII.

P_1 = Patient's blood taken before transfusion.
P_2 = Patient's blood taken at end of transfusion.
D = Donor's blood.

		Sera.		
		P_1	P_2	D
Cells.	P_1	−	−	−
	P_2	+	−	−
	D	+	−	−

The serum after transfusion no longer agglutinated the donor's cells; as in the two cases above, the agglutinin had all been absorbed.

[7] For one of these cases I am indebted to Dr. Kaliski, with whom I shall at a later date publish more detailed accounts of all the cases referred to.

On the other hand, the patient's cells after transfusion were agglutinated markedly by his own serum obtained before transfusion, but not, of course, by his own serum after transfusion. This proves definitely that the donor's cells in the patient's circulation had not lost their agglutinability, but that most of them were not agglutinated simply because there was not enough agglutinin.

The absence of phagocytosis of red cells in two transfusions of non-agglutinable blood, and its occurrence in two transfusions of agglutinable blood, indicate clearly that there is a close connection between agglutination and phagocytosis. It further makes it probable that the transfusion of agglutinable blood, even if no accident occurs, is useless, as the agglutinable cells are foreign and do not remain in the patient's circulation.[8]

CONCLUSIONS.

1. Intravascular agglutination can occur, and is the probable cause of occasional untoward symptoms, or even death, following transfusion of agglutinative blood. In the majority of cases, however, it does not occur, or if it does, it causes no symptoms. This is dependent on the influence of three factors: (1) concentration of the agglutinin; (2) absorption of the agglutinin by an excess of agglutinable cells; (3) interference with agglutination by an excess of non-agglutinable cells, so that when clumps occur they are microscopic in size.

2. If, for a given transfusion, a non-agglutinative donor, i. e., a donor whose blood is of the same agglutinative class as the patient's, can not be obtained, then it is safer to use a person whose serum is agglutinative toward the patient's cells than one whose cells are agglutinated by the patient's serum.

3. Tests for agglutination, as well as for hemolysis, ought to be made before transfusion. When time does not permit this, one has

[8] Since writing the above, I have had an opportunity to confirm further these views in two cases. One was a transfusion between persons of the same agglutination class. No phagocytosis was seen. The other was a transfusion in which the bloods were mutually agglutinative. Extensive intravascular phagocytosis of erythrocytes occurred. Other observations on these cases will be published later.

to weigh the possible dangers of agglutination or hemolysis against the dangers of letting the patient go without transfusion.

4. Agglutinable cells when transfused are taken up by the phagocytes in the patient's blood; and, for this reason, the transfusion of agglutinable blood, even when no accident happens, can be expected to do little permanent good.