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A STUDY OF IMMUNE AGGLUTININS PRODUCED IN CHICKENS AGAINST
HUMAN ERYTHROCYTES OF INDIVIDUALS BELONGING TO
BLOOD GROUPS O, A, AND B.

by

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B.A., Montana State University, 1934

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quirement for the degree of Master
of Arts.

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1939

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The nature of this problem has made it necessary to depend upon the generosity of individual students for the blood cells used. The cheerful spirit with which this request was met has not only made the work possible, but has been deeply appreciated by the author.

1. Acknowledgment.
2. Historical.
3. Experimental.
4. Discussion.
5. Summary and Conclusions.
6. Procedure Sheet.
7. Graphs.
8. Bibliography.

HISTORICAL.

The discovery of the blood groups has in no small measure added to the progress of modern medicine. The results of transfusion before the work of Landsteiner were not very encouraging. There are records which indicate that this surgical procedure was resorted to on several occasions.

Landsteiner's (22) discovery has, however, established transfusion on a scientific basis, and the untoward results of the past need not be repeated in the future. That the first honors must go to this pioneer investigator is no longer open to question.

That agglutinins which will agglutinate human blood cells occur in the normal blood sera of some animals has been noted by several writers (32,55,42,54,12,10,41). That these same properties or substances may be imparted to the normal blood by artificial means has long been known. This phenomenon, the production of agglutinins, is one of the important observations of the science of immunology. Most of the common laboratory animals can be used to demonstrate their occurrence.

Gruber and Durham (14) made use of the specific quality of agglutination in the differentiation of bacteria. By their method an organism may be identified when placed in a known specific anti-serum. By a process of elimination it is possible to determine the nature of the antigen in question. This is not only true of bacteria but other antigenic substances as well. Probably the

widest application this test has is in the so-called Widal reaction.

Gruber and Durham (14) found, that though an animal be immunized against a certain specific antigen, there is a concomitant production of agglutinins against related antigens. This is generally termed a group reaction and usually depends upon certain biological relationships. In the production of antisera in which these group agglutinins occur, it has been found that the specific or major agglutinins reach a higher titer than the related or minor agglutinins. In titrating such sera it is necessary to absorb out the group specific, or minor agglutinins first and then, determine the agglutinating titer against the specific antigen.

Many investigators, Bordet (3) among them noted that hemolytic serum heated to 56°C. to destroy the hemolysin and complement, would agglutinate specific erythrocytes. Heating the serum removed the hemolysin and complement which would otherwise obscure the action of the agglutinins. Furthermore, this phenomenon of specific hemagglutination is not only demonstrable by the use of immune agglutinins, but it has been observed that certain animals normally possess in their sera specific hemagglutinins against the cells of other species. For example, it has been demonstrated (9) that the normal serum of goats contains specific agglutinins for the red blood cells of rabbits.

Since the work to follow is concerned primarily with hemagglutinins involving human red blood cells and serum, any subsequent references to agglutinins or isoagglutinins will mean hemagglutin-

ins, or isohemagglutinins, unless otherwise indicated.

At this point it would probably be advisable to differentiate between immune hemagglutinins and isohemagglutinins. In a general way it may be said that immune hemagglutinins are built up artificially and isohemagglutinins occur normally. Specifically immune agglutinins are substances or physical properties of a serum which has been secured from an animal injected with a blood foreign antigen. This serum when added to the specific or a closely related particulate antigen results in its clumping and precipitation. On the other hand, isoagglutinins are found normally in the blood sera of individuals within a species and are specific for the tissues or cells of another member of the same species.

Ehrlich and Morganroth (9) the first workers on the problem of isoantibodies, demonstrated that by injecting the erythrocytes of one goat into the blood stream of another, it was possible to produce hemolysins against the red blood cells of some goats but not against all goats and never against the corpuscles of the goat in which the hemolysin had been built up. These are the so-called immune isohemolysins, and this observation foreshadowed the important discoveries made later in the field of isoantibodies.

In 1892, Maragliano (34) first recorded the occurrence of isoantibodies in the human blood. He concluded that the hemagglutinins he observed were the result of disease, and this fallacy was not disproved until the work of Landsteiner was published in 1901. Landsteiner, himself, thought the phenomenon was due to disease, and it was not until sometime after the discovery that he realized the

significance of his findings.

In 1901 Landsteiner (22) published a brief account of his work on isoagglutination of the human red blood cells. He pointed out that individuals fall into one of three different groups. Later work by students (7) of Landsteiner disclosed a fourth group which has proved to be very rare in comparison with the other three.

In his original work Landsteiner (22) demonstrated that the twenty two individuals examined could be grouped on the basis of isoagglutination. These groups were indicated by the letters A, B, and C. He found that the sera from group A people agglutinated the corpuscles of group B, but not of group C; that the corpuscles of group A were agglutinated by the sera of both B and C; that the sera from group B individuals agglutinated the corpuscles of A, but not those of C; on the other hand, the corpuscles of group B were agglutinated by both A and C. The sera of group C agglutinated the cells of both A and B and its corpuscles were not effected by the sera of either A or B. The addition of the fourth group by Decastello and Sturli (7) in 1902 added to this original classification. The corpuscles of this latest member were agglutinated by all the other groups, but its serum agglutinated none of the cells of the other groups.

Since 1900 many different investigators have probed into the various aspects of blood grouping, and a great fund of valuable information has been made available.

Looking back into the past it is apparent that the observations of Gruber and Durham, Ehrlich and Morganroth culminated in the

remarkable discovery of isohemagglutination in the human by Landsteiner in 1900.

Several men have put forth their claims or have been championed by loyal followers as the first discoverers of isohemagglutination. Furuhashi (11) has described a blood grouping technique in use among the Japanese seven hundred years ago. Actually his account would lead one to believe that it is just another of those mystical proceedings so common among unenlightened people.

Jan Jansky in 1907 (17) carried out similar work in this field. Not aware of the work of Landsteiner, he introduced a system of classification which has added nothing to the subject. His work has been acclaimed by a few, J. A. Kennedy (55) for one. Jansky himself, made no claims to priority.

Moss 1908 (35) is another contender for the honor, but his experimental work, like Jansky's was done at a later date than 1900. Ignorant of previous publications, Moss (35) devised a third form of classification and has as a result added to the confusion. At the present time, Moss's system is used in most of the hospitals of the United States, while Jansky's is widely used throughout Europe. The classification as formulated by Landsteiner is used sporadically in many parts of the world.

Probably the only individual who observed agglutination in human blood and antedated the work of Landsteiner, was S. C. Schattcock (44). Schattcock, while working with the blood sera of patients suffering from various acute and chronic diseases mixed with the blood of normal individuals, literally stumbled over this

phenomenon. He was attempting to demonstrate the differences occurring in various conditions and was concerned primarily with what is termed "rouleaux formation". Schattock undoubtedly observed the clumping of agglutination, but he failed to interpret his findings correctly.

To clear up the confusion wrought by the several methods used in designating the four groups, Von Dungern and Hirschfeld (47) suggested a classification similar in most respects to that of Landsteiner, and which is commonly known as Landsteiner's classification. By this method the groups are named according to the antigenic structure of the red cells. Thus O, A, B, and AB are probably more acceptable at the present time. The agglutinins where present are referred to as alpha or beta. For a better understanding of the three methods of classification in use the following table is drawn up.

Table No. 1.

Table showing relationship of the several systems of human blood group classification according to the methods of Landsteiner, Jansky and Moss.

| Landsteiner | | | $\alpha\beta$ | β | α | o |
|-------------|-----|---|---------------|---------|----------|-----|
| Jansky | | | 1 | 11 | 111 | 1V |
| Moss | | | 4 | 2 | 3 | 1 |
| O | 1 | 4 | - | - | - | - |
| A | 11 | 2 | + | - | + | - |
| B | 111 | 3 | + | + | - | - |
| AB | 1V | 1 | + | + | + | - |

The numbers directly below the names of Moss and Jansky, indicate the method used by each in designating the agglutinogens present in the human erythrocytes. Under Landsteiner's name will be seen the alphabetical characters used by that worker for the same purpose. The agglutinins when present are designated by the Greek characters alpha and beta.

It will be noted that Moss and Jansky cannot agree on the numbering method consequently, the first and fourth groups are reversed in the classifications used. Since they picked arbitrary figures to represent their groups, the systems of Moss and Jansky are prone to lead to confusion. On the contrary, the method used by Landsteiner shows in a clear, concise way the relation existing between the agglutinins and agglutinogens.

Since the original work in 1900 many new facts have been uncovered. Von Dungern and Hirschfeld (47) have demonstrated the inheritance of the isoagglutinogens as Mendelian dominant characters. Bernstein (1) and many others (16,23,27,37,46,52) have delved into the field of inheritance and have uncovered some rather confusing results. In 1924, Bernstein (1) postulated the existence of three allelomorphic genes A, B and R. According to this plan there is only one locus for the genes determining the blood groups in a single pair of chromosomes, at which locus only one of the three genes may be situated. This type of heredity, is not uncommon and is known as multiple allelomorphism. It is obvious that each germ cell will contain only one of the three genes, A, B, or R. Six different genotypes will result from the combination of

sperm carrying A, B, or R with the three possible kinds of ova. According to this theory, genes A and B are both dominant over R, so that the possible Genotypes corresponding to each of the four blood groups are as shown in the table below.

TABLE No. 2.

| Phenotype | Genotype | |
|-----------|------------|---------------|
| | Homozygous | Heterozygous. |
| AB | | AB |
| A | AA | AR |
| B | BB | BR |
| O | RR | |

The agglutinogens A and B cannot appear in the blood of a child unless present in the blood of one or both parents, on the other hand, if either parent belongs to group AB, his or her genotype is AB, so that half of the germ cells will contain gene A and half gene B. The child therefore, cannot belong to group O since the child's genotype will include an A or B. If either parent belongs to group O, all of the children must possess at least one R gene, so that children of group AB are impossible. In a like manner, group AB parents with a group O child, and group O parents with group AB child, are not possible.

Blood grouping has found a wide application other than its importance and use in transfusion (5,31,38). In forensic medicine, it has been used to establish non-paternity (50,51,52). This test has been found useful, especially in parts of Europe. Blood group is used by the anthropologist as a useful tool along with other physical criteria, to link races and to establish origins (2,13,36, 27).

More recent work has indicated that there are additional substances in the human erythrocytes which can be demonstrated by specific immune sera (21,26,52,43,28). The hereditary factors M, N, and P have been added by Landsteiner and others. Schiff (43) has described two other antigenic factors in human red cells and it is possible that the list will continue to grow.

Isoagglutinins are not confined to the human race but have been demonstrated among many of the lower animals (32,33). When present they may be very irregular and in large numbers.

It has been found that rabbits injected with group A human erythrocytes will consistently produce a high titer specific for group A cells. On the other hand, rabbits injected with B type human red blood cells elaborate agglutinins specific for B type cells, but never will a single rabbit produce a high titer for both A and B cells if either one type or the other is injected alone. Of course due consideration must be taken of other antigenic principles such as the M and N factors. This observation has been confirmed by numerous investigators (15,20,32).

Two Japanese experimentors G. Tsuchida and M. Nagase obtained some very unusual results by injecting human red blood cells of the various groups into chickens. They found that 20% of chickens showed type differences according to the agglutinating qualities of human blood cells. They were able to obtain an immune serum having a titer of 1-16,284 against human B type cells, also by absorbing anti-A serum with A cells it was possible to secure anti-B immune serum, in other words, both anti-A and anti-B sera could

be obtained from the same chicken. Further that if more than four injections were made the titer would go down, These interesting observations of Tsuchida and Negase have prompted the work that is to follow.

If the conclusions reached by these investigators prove true, it will no longer be necessary to use two animals for the production of A and B typing sera. It may indicate some antigenic relationship between A and B type erythrocytes demonstrable in the immune serum of chickens. It will also indicate the point at which the highest titer can be obtained. There may be some solution for the fall in titer after the fourth injection, a solution applicable to the entire field of immunology. It may indicate new applications for isohemagglutination.

EXPERIMENTAL

Since the work of G. Tsuchida and M. Negase is of considerable importance because of its bearing on the antigenic structure of the human erythrocytes A and B, it has seemed advisable to carry out an experiment along similar lines and to extend the work of these investigators, if possible. It has been the hope that the present paper would answer some of the questions they left unanswered.

The conclusions reached are set forth in the pages to follow.

The problem has many different angles, and an effort will be made to enumerate them as briefly as possible.

1. To induce the formation of immune agglutinins in the serum of chickens by injecting human erythrocytes;
2. To demonstrate both species specific and type specific agglutinins in the chicken serum, the type specific agglutinins demonstrable only after absorption with the proper type of cells;
3. To determine the highest point of the titer both with regard to the entire course of injection and to the intervals between injections;
4. To find the optimum number of injections and the effect of subsequent injections on the titer;
5. To procure both A and B typing sera from the blood of a single immune chicken.
6. To explain the results from the data obtained.

Since the materials used are simple and readily available they can be dismissed without further consideration, with the single

exception of the experimental animals.

At the beginning of the experiment the chickens were tested to demonstrate, if possible, the presence of any normal agglutinins for human red blood cells. About 2 c.c. of blood was secured from each chicken and placed in small tubes in the ice box for several hours. When the clot had contracted the serum was removed, and this was used for the tests. A drop of 2.5% suspension human red blood cells of types A, B, and O was placed upon a clean slide, and a drop of chicken serum was added. The slides were examined at short intervals and any changes noted. In none of the chickens could normal agglutinins be definitely shown. The fact that, after standing, the red blood cells seemed to adhere to the slide would indicate some reaction had taken place. Whether this change was simple adhesion or some antibody reaction could not be determined. Certainly it was not agglutination, since microscopic examination showed the cells widely scattered but adherent to the glass. The O type cells seemed to be especially affected, but, as with the other types, it was not the characteristic clumping of agglutination. Blood from four chickens of the same variety was examined and the adhesion to the slide again sought. It was found that the same action occurred if the slides were permitted to lie unmolested for fifteen or twenty minutes. If the cells were loosened from the slide, with a needle, they again formed a homogeneous suspension.

Three groups of three chickens each were injected with washed human erythrocytes. The first three chickens received cells from

persons belonging to group O. The second group of three chickens were injected with cells from group A persons, and the last series of three were immunized with cells from individuals belonging to group B. Ten chickens were used during the course of the experiment, and the tenth served as a control. This animal was injected with whole milk and was titrated against red blood cells in the same manner as the other nine. This was to rule out any chance of non-specific antigenic action.

During the course of the experiment it was necessary to keep the chickens under suitable conditions. They were confined in pens and supplied with a good mixed diet. Special attention was given to this latter item, since, as will be seen, it was highly desirable to provide a good supply of available calcium to decrease the blood clotting time.

One of the great difficulties encountered during the prolonged course of bleedings was the annoying hemorrhage which resulted from the needle puncture. A number 26 needle was used and pressure was applied as soon as the required amount of blood was withdrawn. Despite these precautions, considerable blood was lost in hemorrhage. Various methods were tried to overcome this difficulty. Small pledgets of cotton were applied with pressure, for as long as fifteen minutes, only to have the hemorrhage continue. Ice was applied with indifferent success. The only means of controlling the condition, without prolonged pressure, was through the free administration of calcium in the form of milk or dried eggshells. Blood which would stand in the hemolytic tubes for ten or fifteen

minutes without clotting would, 24 hours after the fowl had obtained calcium through its diet, congeal readily.

The chickens used were of the Barred Rock strain and not more than five months old. They appeared to be in good health at the start of the experiment, but some of them were quite small. It was hoped that by selecting large chickens the mortality would not be as high and that a greater amount of blood could be secured from them. It was also reasoned that the blood loss would be made up more rapidly in the larger birds.

For the injections the suspensions were made up of washed human blood cells. The cells were secured from individuals of the required types, by bleeding from any one of the numerous veins which course along the inner surface of the arm at the bend of the elbow. The blood obtained in this way was defibrinated and centrifugalized to remove the plasma. After the plasma had been drawn off, the cells were washed and centrifugalized in four different changes of normal saline solution. At the final washing the packed cells were made up to a 2.5% suspension for the injections.

These cell suspensions were injected in 2 cc. amounts and were repeated every nine days until ten injections had been made.

The injections were made into the chickens by way of the large vein crossing the bone at the second joint on the inner surface of the wing. The administration of the cells by this route was accompanied with little difficulty, after the technique had been mastered. Great care was taken to be sure the needle was in the vein. Every effort to aspirate blood into the syringe resulted in

failure. This was especially true after the titer had reached a high value. It was found that agglutination occurred which blocked the small bore needle used, thus necessitating withdrawal. Several unsuccessful attempts to inject or bleed at any point where the vein was not securely fastened to the skin were abandoned. Hemorrhage readily occurred in the loose subcutaneous tissue, and the formation of hematomata would invariably take place. It is true that absorption in this animal is very rapid and the clot is soon resorbed, still the discoloration produced by the extravasated blood obscured the vein and led to difficulty in subsequent work.

Despite the fact that the inoculations were made at such long intervals no ill effects were observed, with a single exception, one chicken shortly after it had been injected and returned to the cage, died. This was the only instance of untoward effects as a result of the injections.

The titrations were made every day, with the exception of the ninth, when the cell suspension was administered. The blood for the titrations was secured from the same location on the inner surface of the wing. It was found that 2 cc. amounts were enough to yield sufficient serum for the titrations. The blood was drawn and placed in small hemolytic tubes to clot. When the blood had coagulated, it was rimmed and placed in the ice box over-night. At the end of that time the serum was removed and inactivated at 56°C for thirty minutes. This was done to destroy the hemolysin which otherwise would obscure the action of the agglutinins.

The dilutions of the serum with saline ranged from 1 to 2 on up to 1 part in 2048. Two tenths of each dilution was placed in each of three different tubes, and a drop of a 2.5% red blood cell suspension was added to each. In this way the groups A, B, and O were titrated against each serum. The following protocol may help to make this clear.

TABLE NO. 3.

Protocol showing serum dilutions used in titrating the red blood cells. The first vertical column indicates the type of cells used, while the succeeding columns to the right show the serum saline dilutions.

| One drop of 2.5% suspension of Human cells | 1 part of Chicken serum in 2 parts saline | | | |
|---|--|--------|---------|-------|
| | 1 to 4. | 1 to 8 | 1 to 16 | |
| A. | .2cc. | .2cc. | .2cc. | .2cc. |
| B. | " | " | " | " |
| O. | " | " | " | " |

After the addition of the cells the tubes were shaken and incubated at room temperature for 15 minutes. At the end of that time they were examined and then placed in the incubator at 37°C for fifteen minutes. After the incubation the tubes were read macro- and microscopically. The microscopic reading was recorded on the data sheet.

In the absorption experiment the red blood cells used were washed as for injections, and, after the final packing, the saline was pipetted off. The serum of the chicken was added to the appropriate blood cells and agitated to facilitate thorough mixing.

The mixture was then placed in the ice box and left for at least thirty minutes. At the end of that time the mixture was centrifuged and the serum tested for agglutinins. If non-specific agglutinins were still present, the procedure was repeated. This work required considerable care to prevent over-absorption, which did not seem to remove all the agglutinins but did cut down the titer. After absorption the serum was titrated in the same manner as the unabsorbed serum.

In the absorption experiments the cells were added to either the homologous or heterologous serum, depending upon the result desired. That is, if the serum of a chicken injected with B type cells was absorbed with B type cells the homologous agglutinins would be removed; on the other hand, if A type cells were used to absorb the same serum the heterologous agglutinins would be removed.

In the work here outlined it has again been demonstrated that it is possible to produce both A and B typing sera from a single chicken immunized with either A, or B cells. In the course of the experiment it was shown that chickens immunized with B type cells and absorbed with B cells became anti-A and vice versa. The titer after absorption was much lower than in the original serum.

DISCUSSION

As a result of the work followed out in this experiment, it has been shown that chickens of the Barred Rock variety can be used successfully in this type of work.

Special care must be taken in selecting the chickens used, and only large, young, healthy birds should be procured. If too small, they do not react well to the drain of repeated bleedings and do not yield a large volume of blood on completion of the work. Infections are liable to spring up among them, especially respiratory diseases. This factor alone may give rise to considerable inconvenience.

As a result of the tests carried out on the chickens before the first injection, it was decided that they belonged to the O group, since their sera reacted negatively from an agglutination standpoint.

Repeated injections do not seem to cause any marked symptoms, regardless of how the injections are spaced. Only one chicken seemed to react to the foreign material introduced. It is to be noted that the animal struggled violently after the injection and died almost immediately. It is impossible to assign a definite cause for its death. Autopsy showed a very scanty supply of blood present.

The highest titer obtained was during the late stages of the experiment. The chickens injected with type O cells seemed to reach a higher titer than those which had received either types A or B.

From the attempt that was made to analyze the trend of the titer from one injection to the next, it was found that, in a general way, the titer was highest just before an injection and lowest just after one. However, since it is not safe to draw definite conclusions from generalizations, this might be a disputed point.

It is true that between injections three and four, a high peak was reached, followed by a decline after the fourth injection. At the present time it is impossible to say how much was due to the reaction of the animals to inoculation, and how much was due to disease. The chickens were suffering from a respiratory infection for about four weeks during the course of the experiment. The subsequent rise, at least, would indicate that continued injections were not the only factors involved.

It was found that the injections could be carried on for as long as three months without materially lowering the titer. It is true that the graph shows considerable fluctuation after the fourth injection, but there are later increases which do not coincide with the results obtained by the Japanese workers.

It was also found that the titer, after absorption, is considerably less than before, which does not entirely agree with previous work.

The absorption experiments were carried out in varying combinations. Great difficulty was at first encountered in the complete removal of the undesired or specific agglutinins. The first titers after absorption were almost as high as the unabsorbed, and it was not until the technique was mastered that the readings became

dependable. It was found that if the chickens had received O type cells that their sera could be completely neutralized from the standpoint of agglutination, on the addition of any of the cell types.

In chickens receiving A type cells the sera could be absorbed with cells from individuals of group A, leaving anti-B agglutinins, or the sera could be absorbed with B, leaving anti-A agglutinins. The titer in this case would be much higher than in the preceding.

If O type cells were used for the absorbing agent, both anti-B and anti-A agglutinins could be demonstrated in the serum. This is due to the fact that type O has neither the A nor B agglutino-gen and as a result the alpha and beta agglutinins are not removed.

Every effort should be made to insure that the needle enters the vein at the time of injection. Otherwise, the area around the injection site becomes so dense and tough from scar tissue formation as to embarrass later attempts to penetrate the same location. After the needle has entered, the suspension should be introduced slowly, and after the titer has reached a high value, aspiration of blood back into the syringe should be avoided.

Eggs secured from the hens during the period of inoculation were tested for agglutinins with negative results.

It would be highly desirable to type a large group of individuals with the absorbed sera and with testing fluids from known A and B sources. In this way it would be possible to check the results and arrive at some definite conclusions. The work was not

carried that far, however, and for that reason some important questions still remain unanswered.

Since great care was taken in preparing the cells for injection, washing in four changes of saline, and since the donors were all typed before the cells were used, a possibility of accident from these sources was ruled out.

SUMMARY
AND
CONCLUSIONS

1. The sera of fourteen chickens were tested against A, B and O type human cells with no marked evidence of agglutination. The cells adhered to the glass slides after standing a few minutes but could be re-suspended by mechanical agitation.

2. It is possible to build up an immune serum to a titer of 1 to 2048 by immunizing O type chickens.

3. If the chickens are properly cared for, the injections may be continued for as long as three months without lowering the titer.

4. In general, it may be said that the lowest point in the titer curve between injections is directly after the administration of the injection, and the highest point, usually, just before the succeeding inoculation. This is merely a generalization and does not follow in every case.

5. Anti-A sera, when absorbed with A cells, becomes anti-B sera. Absorption of anti-A sera with B cells results in a high anti-A titer. Absorption of anti-A sera with O cells leaves a high titer against both A and B. Absorption of Anti-A with a combination of A and B results in removing all agglutinins. If a combination of O A cells is added to anti-A sera, anti-B results. B O absorption of anti-A serum leaves a high agglutinin titer against A. The same results can be secured by using anti-B serum in place of anti-A. If immune sera from chickens injected with O cells is absorbed with any type cells, the agglutinins

are all removed.

6. It is possible to obtain both A and B typing sera from an O type chicken by injecting with A cells. This same result may be obtained by injecting an O type chicken with B cells.

7. Injections may be carried on for as long as three months without causing a consistent fall of titer.

It is difficult to evaluate the results obtained. In the first place it is evident that there was scant possibility for an error to occur with regard to the type of cells injected. All donors were typed before the cells were used and in many cases the same individuals were used for several injections, in this way they were rechecked many times.

There is a possibility that a small amount of serum was carried over with the cells, but this possibility is very remote. The cells were washed in four changes of normal saline and the cells were carefully stirred up after each addition was made. The fact that the sera from O type chickens did not after absorption with A type cells, agglutinate B cells and visa versa, would rule out this accident.

It is conceivable also, that the reaction depends upon some of the immune factors which have been demonstrated by Landsteiner (21) and Schiff. Or that it is a new factor demonstrable only with the immune serum of chickens. In other words; if all the blood groups possess a common antigen their inoculation into the blood of an animal, in this case a chicken; would give rise to agglutinins which would agglutinate all human cells regardless of

group. On the other hand, the absorption of this serum by the cells of any group would remove all the agglutinins for all the groups. In the human erythrocytes however, there probably exists an antigenic complex various fractions of which can be demonstrated in the immune sera of different animals.

That there is an antigenic complex which is common to both A and B cells must also be considered. This theory is not as attractive however, as the preceding and it may be that this is merely a new demonstration of an old principle. Just what part the M and N factors played in this experiment was not determined. It is entirely possible that in this case there is an immune agglutinin demonstrable only in the A and B cells.

It was with considerable reluctance that the problem was relinquished at this point but a definite solution must await further investigation.

LEGEND

Data Sheet

The figures 1 to 10 in the first vertical column, on the extreme left, are used to designate the chickens. The column to the right of this, and in which the letters A, B and O appear in red letters show the type of cells injected. The third column to the right in which the letters A, B and O occur in black type, indicate the type of cells against which the sera were titrated.

The long vertical columns of figures, which are grouped in threes, represent the titers secured with each serum against cells A, B and O.

Each page represents nine days and constitutes eight bleedings and one injection.

Each injection is followed by eight days of titrations, with the exception of the last series, in which two courses of three consecutive injections were made. It will be noted that the main course of injections end on Feb. 14. Six weeks later, on March 28, the chickens are again titrated. Three injections follow this single titration, and the chickens are allowed to rest until the seventh of April when three injections and a week of titrations end the series.

In some instances the sera were absorbed with different types of human red cells before the regular titrations were carried out, this is indicated in the titration columns by the abbreviation abs. followed by the type of cell or cells used.

LEGEND

Graphs

The graphs herein contained, have been prepared with the object of indicating the trend of the titer during the period of immunization.

The number of the chicken from which the serum was obtained, appears in the upper left hand corner.

The figures on the left margin reading up from 0 to 1024, represents the blood serum in various dilutions.

The numbers in the lower margin beginning with 11 and traversing the entire length of the graph, are the days on which the injections and titrations were made. The first injection was made on Nov. 11. The days on which injections were made are indicated in red. The days reserved for titration appear in black.

As regards the graphs proper; the blue line represents the agglutinating strength, or titer, of the chicken serum against human red blood cells of type A. The blue line runs the entire length of the graph and in those instances in which the titer for B and O cells are the same as for A the red or green stars above the line indicate this fact.

The red line shows the titer of the serum against human red blood cells of type B. Where A and B have a common titer, the red star above the blue line is used.

The green line on the graph indicates the titer of the serum against human red blood cells of type O; and in these instances in which, O has a common titer with B or A or both, the green star above the common line is used.

There is a break in the graph beginning with the 15 of Feb. and ending with the 28th of March. The chickens were titrated on the 28th and injected on the three following days. At the end of a week, three additional injections are followed by a week of titration ending the series.

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