# CHEMICAL, CLINICAL, AND IMMUNOLOGICAL STUDIES ON THE PRODUCTS OF HUMAN PLASMA FRACTIONATION.

## XIII. THE SEPARATION AND CONCENTRATION OF ISOHEMAGGLUTININS FROM GROUP-SPECIFIC HUMAN PLASMA [1,2]

By L. PILLEMER,[3] J. L. ONCLEY, M. MELIN, J. ELLIOTT,[4] AND M. C. HUTCHINSON[5]

*(From the Department of Physical Chemistry, Harvard Medical School, Boston, and the Army Medical School, Washington)*

(Received for publication February 17, 1944)

At the beginning of the twentieth century, Landsteiner (1, 2) reported the presence of normal isohemagglutinins in human serums and their corresponding agglutinogens in the red cells. On the basis of these observations, normal human blood is classified into 4 groups, *i.e.*, Groups O, A, B, and AB. Continued research has served to confirm Landsteiner's observations and postulates. Landsteiner found further that an isohemagglutinin and its homologous agglutinogen could not exist together in the same blood, and that their relationships are reciprocal; *i.e.*, the presence of one indicates the absence of the other.

With the advent of whole blood transfusions as an important therapeutic procedure, it became necessary to determine the blood groups of both donors and recipients prior to transfusion. A number of methods have been described for assigning an unknown blood to one of the 4 blood groups. In practice, they involve the collection and preservation of 2 grouping serums, containing, respectively, avid Anti-A and Anti-B agglutinins, preferably in high titer. The isohemagglutinin serums are mixed with the cells of the unknown blood under appropiate conditions which will give prompt true agglutination if A and/or B cells are present. The cells of a Group O blood are not agglutinated by either Anti-A or Anti-B serum; the cells of a group A blood are agglutinated by the Anti-A but not by the Anti-B serum; the cells of a Group B blood are agglutinated by an Anti-B serum but not by an Anti-A serum; and the cells of an AB blood are agglutinated by both Anti-A and Anti-B serums. It is not within the scope of this paper to present blood grouping methods in detail and the reader is referred to the many excellent blood grouping manuals for further information (3, 4).

It is, however, apparent that specific avid high-titered grouping serums are necessary for accurate blood grouping. The collection of high-titered serums from special donors has been the major means of procuring such serums in the past. Recently, Wiener (5) has been able to increase both the titer and the avidity of the plasma isohemagglutinins by intravenous injection of dried human plasma into special donors. Witebsky (6) has also been able to accomplish the same effect by the injection of small doses of A and B specific substances into Group A and B individuals. Immunization of rabbits and absorption with group-specific human red blood cells has also led to the production of satisfactory grouping serums.

Recently, the necessity arose at the Army Medical School to obtain large quantities of potent grouping serum. The difficulties in obtaining donors with sufficiently high-titered isohemagglutinins in their serum made it necessary to investigate the possibility that chemical or physical concentrations of plasma from random donors of proper group might offer a means of procuring a sufficient quantity of uniform high-titered grouping material.

[1] This work has been carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

[2] This paper is Number 20 in the series "Studies on Plasma Proteins" from Harvard Medical School, Boston, Massachusetts, on products developed by the Department of Physical Chemistry from blood collected by the American Red Cross.

[3] First Lieutenant, Sanitary Corps, Army of the United States.

[4] Captain, Sanitary Corps, Army of the United States.

[5] Second Lieutenant, Sanitary Corps, Army of the United States.

Thalhimer and Myron (7) have shown that the isohemagglutinins could be separated and concentrated from serum or plasma by ammonium sulfate precipitation. Pillemer (8) later showed that the concentration of the isohemagglutinins from serum could be very easily accomplished by the use of methanol as a precipitant. The globulins separated by this method exhibited marked avidity for incompatible red cells and showed a 4- to 8-fold isohemagglutinin concentration over serum.

As reported earlier in this series of papers, among the variety of plasma components with diverse physiological and immunological functions, that are available as by-products of the human serum albumin program, are included the isohemagglutinins (9). Boyd, working in the Department of Physical Chemistry, Harvard Medical School, has noted that Fraction II + III, obtained as a by-product in the fractionation of human plasma for production of serum albumin, has isohemagglutinin activity (10). Since large amounts of Fraction II + III would be available as a by-product, it was thought advisable to extend the studies which had been started at the Army Medical School with the possibility of obtaining high-titered grouping material from Fraction II + III. The following report is a summary of the results obtained with products prepared at the Harvard Fractionation Laboratory. It will be seen that a potent grouping reagent, which can be distributed as a white powder, has been prepared routinely as a by-product of the human serum albumin program.[6]

## COLLECTIONS AND ASSAY OF GROUPING SERIES

The initial step in the preparation of an isohemagglutinin-containing fraction from human plasma consists in the collection of blood from donors of a single group, either A or B, for a given pool. While each pool has thus been derived from donors of the same blood group, no attempt has been made to select high-titered plasmas. Of course, initial titration of each individual plasma, in order to exclude low-titered plasmas, will lead to a much more potent grouping material than is currently obtained. The titers of the pooled plasmas processed have varied from 1:32 to 1:128 and from this material, final products having titers from 1:512 to 1:2048 have been obtained.

Two methods of assay have been employed in this laboratory in the determination of the isohemagglutinin activity of the plasma pools and the final isohemagglutinin globulins. These will be briefly mentioned here.

### SPEED AND INTENSITY OF AGGLUTINATION (AVIDITY)

*Group A Serum (Anti-B).* One drop (0.05 ml.) of serum or isohemagglutinin containing globulin solution is mixed on a slide with a drop (0.05 ml.) of a fresh group B cell suspension (5 per cent). The slide is constantly rocked, and the time necessary for beginning visible macroscopic agglutination as well as the time required for complete agglutination is recorded.

*Group B Serum (Anti-A).* In general, this is the same as for the Anti-B serums except that the avidity is tested against $A_1$, $A_2$, and $A_2B$ cells.

### ANTIBODY CONTENT (TEST TUBE TITER)

The plasma or globulin preparation is geometrically diluted in a series of test tubes so that each tube contains 0.2 ml. of solution in dilutions 1:1, 1:2, 1:4, 1:8, 1:16, and on in the same progression up to 1:4096. Two-tenths of a ml. of a 2 per cent suspension of incompatible red cell suspension (Group A in the case of testing agent Anti-A and Group B in the case of an Anti-B serum) is added to each tube. The contents of the tubes are well agitated and the tubes then centrifuged at 1000 R.P.M. for 1½ minutes. The packed cells in each tube are gently shaken up from the bottom of the test tube and the degree of agglutination recorded: 4 plus = one large clump; 3 plus = 2 to 4 medium size clumps;

[6] As will be shown in a subsequent paper of this series, eight preparations of isohemagglutinin globulin prepared at the Harvard Pilot Plant have been evaluated at the request of the Subcommittee on Blood Substitutes of the National Research Council by Dr. Elmer L. DeGowin and a panel of investigators chosen by Dr. DeGowin. The reports from this panel were invaluable in that the preparative group were mainly guided by their appraisals, and are summarized in the following paper in this series (11).

2 plus = several medium size and small clumps; one plus = several very small clumps.

## PROPERTIES OF THE ISOHEMAGGLUTINATING GLOBULINS

A purified euglobulin fraction of plasma, in which the isohemagglutinin activity is mainly concentrated and which may be dispensed in a dried stable state, has been prepared routinely at the Harvard Pilot Plant. The dried powder dissolves readily upon the addition of proper amounts of distilled water and is highly active for blood grouping purposes.

The separated isohemagglutinin containing globulins represents about 5 per cent of the proteins present in plasma. Electrophoretically, this protein fraction is composed mainly of gamma and beta globulins. Work is in progress on the further purification of this fraction in order to establish the chemical identity of the isohemagglutinins. It should be pointed out here that as yet no chemical or physical differences have been encountered between the Anti-A and Anti-B isohemagglutinins, respectively.

TABLE I

*The concentration of isohemagglutinin globulins over plasma*

| Run | Plasma | | | Fraction III-1 | | |
|---|---|---|---|---|---|---|
| | Avidity | | Test tube titer | Avidity | | Test tube titer |
| | B[a] | C[b] | | B[a] | C[b] | |
| | *seconds* | | | *seconds* | | |
| 89A | 30 | 150 | 1 : 32 | 5 | 30 | 1 : 512 |
| 90B | 15 | 90 | 1 : 64 | 5<br>15 | 30-$A_1$[c]<br>60-$A_2$ | 1 : 1024<br>1 : 256 |
| 91A | 30 | 150 | 1 : 64 | 5 | 30 | 1 : 1024 |
| 94B | 15 | 90 | 1 : 64 | 5<br>15 | 30-$A_1$<br>60-$A_2$ | 1 : 1024<br>1 : 256 |
| 92A | 30 | 150 | 1 : 32 | 5 | 30 | 1 : 512 |
| 9094B | 15 | 90 | 1 : 64 | 5<br>15 | 30-$A_1$<br>60-$A_2$ | 1 : 1024<br>1 : 128 |
| 9193A | 30 | 150 | 1 : 32 | 5 | 30 | 1 : 512 |
| 104B | 30 | 90 | 1 : 64 | 5<br>15 | 30-$A_1$<br>60-$A_2$ | 1 : 1024<br>1 : 256 |

[a] Beginning agglutination.
[b] Complete agglutination.
[c] Indicates type of A cell employed.

It will be seen from Table I that the isohemagglutinins in plasma have been concentrated approximately 16 times over plasma. Since approximately 50 to 60 ml. of this material are obtained per liter of plasma, this means that almost quantitative yields have been achieved.

### *Speed of reaction (avidity) between red cells and the isohemagglutinins*

On a glass slide the products will produce macroscopic agglutination in 5 to 15 seconds and complete agglutination of incompatible cells in 30 to 60 seconds.

### *Specificity of reaction*

No rouleaux or false agglutination with compatible cells have been observed in any of the preparations thus far carried out.

### *Stability*

The material is stable under ordinary conditions. Tests have run for 10 days at 50° C., 53 days at 37° C., and 4 months at room temperature (+25° C.). The outcome of further tests will be needed to establish definitely the criteria for stability.

### *Reproducibility*

A uniform product has been obtained from all pooled type-specific plasma processed at the Harvard Pilot Plant. There is reason to believe that this will continue to be true on large scale production since the material can be concentrated or adjusted to the desired specified activity. Furthermore, preliminary selection of only high-titered plasma will doubtless guarantee an even more satisfactory product.[7]

### *Subgroups specificity*

Due to the large number of individual plasmas in each pool, satisfactory agglutination of the

[7] Upon the request of the National Research Council, two preparations of Anti-A and Anti-B isohemagglutinin globulin, respectively, were prepared by the Harvard Pilot Plant to serve as reference standards in evaluating subsequent preparations of isohemagglutinins. The Anti-A has a titer of 1 : 1024 and the Anti-B of 1 : 512. These standards meet the requirements and specifications of the Armed Forces and were approved by DeGowin and his group.

weak subgroup A cells should be achieved. If deemed necessary, preliminary selection of B plasmas will assure this.

*Ease of manipulation of grouping tests*

Since the material can be dispensed in a dried state which can readily be reconstituted into a stable solution, definite concentrations of it can be added to the red cells, thereby achieving a high degree of uniformity in grouping tests.

Since some service laboratories are not equipped or not readily prepared to do either test-tube or microscopic blood grouping tests, the ready availability of a stable, specific, rapid reagent for macroscopic use on slides offers decided advantage.

*Physical properties*

The grouping reagent prepared by these methods also lends itself well for blood grouping purposes because of its suitable viscosity and clarity.

## SUMMARY

A specific, stable, and highly active isohemagglutinating material has been prepared as a by-product of the human serum albumin program. A concentration of isohemagglutinating activity over plasma of 16 times has been achieved. The properties of this material are described.

## BIBLIOGRAPHY

1. Landsteiner, Karl, Zur Kenntnis der antifermentativen, lytischen und agglutinierenden Wirkungen des Blutserums und der Lymphe. Centralbl. f. Bakt., 1900, **28**, 357.
2. Landsteiner, Karl, Über Agglutinationserscheinungen normalen menschlichen Blutes. Wien. klin. Wchnschr., 1901, **14**, 1132.
3. Schiff, F., and Boyd, W. C., Blood Grouping Technique. Interscience Publishers, New York, 1942.
4. Wiener, A. S., Blood Groups and Transfusion. Charles C. Thomas Publishing Company, Springfield, Illinois, 3rd Ed., 1943.
5. Wiener, A. S., Personal communication.
6. Witebsky, M., Personal communication.
7. Thalhimer, W., and Myron, S. A., Globulin fractions of A and B agglutinating serums for blood typing; rapid card technic furnishing permanent record. J. A. M. A., 1942, **118**, 370.
8. Pillemer, L., The separation and concentration of the isohemagglutinins from human serums. Science, 1943, **97**, 75.
9. Cohn, E. J., Oncley, J. L., Strong, L. E., Hughes, W. L., Jr., and Armstrong, S. H., Jr., Chemical, clinical, and immunological studies on the products of human plasma fractionation. I. The characterization of the protein fractions of human plasma. J. Clin. Invest., 1944, **23**, 417.
10. Enders, J. F., Chemical, clinical, and immunological studies on the products of human plasma fractionation. X. The concentration of certain antibodies in globulin fractions derived from human blood plasma. J. Clin. Invest., 1944, **23**, 510.
11. DeGowin, E. L., Chemical, clinical, and immunological studies on the products of human plasma fractionation. XIV. Appraisal of isohemagglutinin activity. J. Clin. Invest., 1944, **23**, 554.