

## Agglutination of Ax Blood Cells with So-called Anti-C agglutinin in Human O sera

Nobuyoshi MATSUDA

*Department of Clinical Pathology,  
(Director: Susumu Shibata)  
Yamaguchi Medical school*

(Received February 5, 1966)

Hooker-Anderson, Dölter and Okabe<sup>1-3)</sup> demonstrated by serological experiments using rabbit sera immunized with human blood cells that group A and B blood cells had a common antigen in addition to A or B antigen. Hibino, making additional serological studies, confirmed their findings and first denominated this common antigenic substance "C-antigen"\* and supposed that the antigenic structures of group A, B, AB and O blood cells could be expressed by AC, BC, ABC and O,<sup>4)</sup> respectively.; Namely, only group O blood cells would lack C antigen.

On the other hand, Wiener evaluated their experimental results<sup>1-4)</sup> in view of Landsteiner's law,<sup>5)\*\*</sup> and advocated the so-called C-theory<sup>6)7)</sup> as against the ABO blood group system so far universally accepted, on the assumption that group O human sera must contain anti-C agglutinin in addition to anti-A and anti-B agglutinins.

Previously, we reported on the first pedigree with sub-group Ax blood from Japanese family and its blood cells showed a specific nature of being agglutinated only by group O human sera.<sup>8)</sup>

When this fact was considered according to Landsteiner's law, the following surprising result could be expected; namely that their blood cells might have C antigen though neither A\*\*\* nor B antigen were present and this C antigen might have reacted with anti-C agglutinin in group O human sera to cause agglutination. As was expected, serological experiments using Ax blood cells from our pedigree revealed that the agglutination of Ax blood cells with group O sera occurred by reaction between C antigen and anti-C agglutinin, to give proof of the validity of Wiener's C-theory. In the following, we shall report the results obtained.

### Material and method

Blood cells and sera: group A, B, AB and O blood cells and sera were obtained

\* Wiener distinguished antigens from blood factors and called "C antigen" blood factor C.<sup>9)</sup>

\*\* Between serum agglutinin and blood cell antigen, there is a reciprocal relation. Namely, some human sera have agglutinin incompatible with the blood cell antigen. For instance, group A human serum has anti-B agglutinin incompatible with A antigen contained in its blood cells.

\*\*\* Actually, Ax blood cells absorbed anti-A agglutinin (group B sera) and eluted it by heating to 56°C.<sup>9)</sup> It is, therefore, assumed that Ax cells might have very weak A antigen.

from by the staffs of the Department of Clinical Pathology, Yamaguchi Medical School and the students of the Attached Medical Technician School of Yamaguchi Medical School and supplied by the Hiroshima Blood Bank. Ax blood cells and sera were obtained from the proband and her younger brother already reported.<sup>8)</sup>

Agglutination was performed according to test tube centrifugation technique,<sup>10)</sup> at room temperature (15°–20°C) unless otherwise specified.

#### Experiment 1.

Avidity for Ax blood cells of the serum of rabbits immunized with human group A blood cells: To determine if any antibody other than anti-A agglutinin produced by immunization with group A blood cell causes agglutination of Ax blood cells.

#### Method

##### 1) Preparation of rabbit anti-human group A blood cell sera:<sup>11)</sup>

Through the ear vein of rabbits with  $\alpha\beta$  serum type,<sup>12)13)</sup> 5 ml of 10% human group A blood cells in saline solution was injected 5 times at intervals of 2 days. One week after the last injection, the animals were bled. The immune rabbit serum thus obtained was inactivated for 10 minutes at 56°C and then agglutinins in this serum that clump group O human blood cells were completely removed by repeated absorption with mixed group O blood cells of 25 persons.\* Then the serum was put into test tubes 1°, 2° and 3°.

2) Anti-A, anti-B and anti-Ax agglutinin\*\* of the serum test tube 1° were titrated by serial dilution.

3) Reaction of eluates from group B and O blood cells with various kinds of blood cells:

To the serum (each: one volume) in test tubes 2° and 3°, group B and O human blood cells (packed cells, each: 1/3 volume) were added, respectively. These were kept at room temperature for 2 hours, (absorption of agglutinins) and centrifuged for 3 minutes at 3000 rpm. The supernatant was discarded and the sediment was washed in saline solution more than 4 times until albutix became no longer positive for the supernatant protein.

To the washed aggregates (I volume) thus obtained, saline equal in amount to that of the original serum (3 volume) was added, and the mixture, being shaken occasionally, was incubated for 5 to 10 minutes at 56°C (eluate of agglutinin), and was immediately centrifuged for 3 minutes at 3000 rpm. Thus, the supernatant (eluate of agglutinins) 2° and 3° were obtained. Anti-A, anti-B and anti-Ax agglutinin of the supernatant 2° and 3° were titrated by serial dilution.

#### Experiment 2.

##### Agglutination inhibition test using salivas:

\* The agglutinin concerned was considered to have been removed completely when all group O blood cells taken from 5 person selected at random were no longer agglutinated.

\*\* Means agglutinin titer for group Ax blood cells. Hereinafter, the same.

To determine whether or not agglutinins in group O human sera that clump Ax blood cells are neutralized by group specific substance in group A, B, AB or O human saliva.

#### Method

##### 1) Preliminary treatment of salivas.<sup>15)</sup>

Group A, B, AB and O human salivas (Secretors from group A, B, AB and O) were taken, placed in boiling water bath for 10 minutes and then centrifuged to obtain transparent supernatant.

2) Neutralization of Anti-Ax agglutinin in group O sera by addition of salivas: Then, 15 rows of serial dilutions in saline solution of group O human serum were prepared and divided into 5 group (I-V) of each 3 series. Group A, B, AB and O human salivas were added to the tubes of group II, III, IV and V, respectively, in the same amount as the diluted serum, and the tubes were left standing for 2 hours at room temperature.

3) Measurement of the residual agglutinin titers: Then, 2% A, B and Ax blood cells in saline solution were added to the tubes of the 3 series of each of groups I-V in  $\frac{1}{2}$  volume to one volum of the serum or mixed fluid of serum and saliva, respectively, to measure agglutinin titers.

#### Results

The results of Experiment 1 are shown in Table 1. Rabbit anti-human A blood

Table 1: Titration of anti-A, B and Ax agglutinins of the rabbit anti-human A blood cell serum absorbed with group O human blood cells and of the eluates derived from group B (and O) blood cells treated with this immune serum.

Blood cells \ Serum	Agglutinin titers			
	Befor immunization	After immunization 1°	2°	3°
Group A	32	8192	16	(—)
Group B	4	128	32	(—)
Group Ax	—	128	8	(—)

1° : Immune serum obtained after treating rabbit serum prepared against human group A blood cells with group O human blood cells until it fails to agglutinate 5 group O blood cells selected at random.

2°: Eluate from group B blood cells agglutinated by serum 1.

3°: Eluate from group O blood cells treated with serum 1.

(—): no agglutination.

cell serum, absorbed with group O blood cells agglutinated all of group A, B and Ax blood cells and their agglutinin titers were 8192, 128, and 128 respectively. As was expected, group O blood cells were not agglutinated.

Then, the agglutinin titers for group A, B and Ax blood cells of eluate from group B blood cells treated with the immune rabbit serum were 16, 32 and 8, re-

spectively. On the other hand, the eluate derived from group O blood cells did not agglutinate the blood cells of any these groups at all.

The serum of rabbits immunized with human group A blood cells, diluted in saline solution after complete removal of agglutinins that clump human group O and B blood cells so as to present a titer of 1 : 32 to group A blood cells, did not agglutinate group Ax blood cells.

The results of agglutination inhibition tests using salivas are shown in Table 2.

Table 2: Agglutination inhibition tests using salivas: Agglutination to group A, B and Ax sera, of human group O serum in which the corresponding antibodies were neutralized by group specific substances in salivas.

Group O human serum Group (blood cells)		Dilution series										
		1:1	2	4	8	16	32	64	128	256	512	1024
I (Control)	Group A	+++	+++	+++	+++	+++	+++	++	++	+	?	-
	Group B	+++	+++	+++	+++	+++	+++	+++	++	++	+	-
	Group Ax	+++	+++	++	++	+	-	-				
II (Group A Saliva added)	Group A		-	-	-	-	-	-	-	-	-	-
	Group B		+++	+++	+++	++	++	+	+	+	±	
	Group Ax		-	-	-	-	-					
III (Group B Saliva added)	Group A		+++	+++	+++	+++	++	++	+	+	-	-
	Group B		±	-	-	-	-	-	-	-	-	-
	Group Ax		-	-	-	-	-					
IV (Group AB Saliva added)	Group A		+	-	-	-	-	-	-	-	-	-
	Group B		+	-	-	-	-	-	-	-	-	-
	Group Ax		-	-	-	-	-					
V (Group O Saliva added)	Group A		+++	+++	+++	+++	+++	+++	++	+	-	-
	Group B		+++	+++	+++	+++	+++	+++	++	+	±	-
	Group Ax		+++	++	++	+	-	-				

Agglutination is expressed in six degree using the following marks.

+++, ++, + Distinct clumps, grossly.

± Through less evident, agglutination appears to be present grossly and distinct clumps are observed microscopically.

? Difficult to determine the persence of clumps both grossly and microscopically. Intermediate degree between ± and -.

- No agglutination is noted even microscopically.

When group A, B, AB or O saliva was added to group O human serum (dilution series), agglutination inhibitions peculiar to each group appeared. Group A human saliva neutralized anti-A and anti-Ax agglutinins, group B human saliva, anti-B and anti-Ax agglutinins, and group AB human saliva, anti-A, anti-B and anti-Ax agglutinins (a significant drop\* in agglutinin titers was noted).

However, none of the agglutinins showed significant drop of agglutinin titer by addition of group O human salivas. The essentially same results were obtained in additional agglutination inhibition tests using each of the salivas taken from 15 persons (A : 6, B : 3, AB : 4 and O : 2). Namely, antibodies that agglutinate group Ax blood cells were neutralized by group A, B and AB human salivas but not by group O human salivas at all.

## DISCUSSION

The eluate derived from Ax blood cells treated with group O human sera agglutinates group A, B and Ax blood cells<sup>8)</sup>. As to the antibody responsible for this agglutination, there has been difference in view; one scholars<sup>6)</sup> consider it to be anti-C agglutinin. The others<sup>16)</sup> maintain it to be anti-A + B agglutinin\*\*.

Serological studies performed on group Ax blood cells gave possible definite proof of validity of the former.

Experiment I revealed that the main agglutinin in the eluate derived from group B blood cells was an antibody that reacted equally with group A, B and Ax blood cells, namely immune anti-C agglutinin<sup>4)11)</sup>.

This is clear from the fact that the agglutinin titers to group A, and Ax blood cells, of the eluate derived from group B blood cells were almost equal\*\*\*.

It is, therefore, suggested that group Ax blood cells might have the same C antigen as group A and B blood cells. However, the question on the molecular level remains to be open.

Agglutinin (naturally occurring antibody) in group O human sera which is responsible for the agglutination of Ax blood cells was neutralized by group A, B and AB human salivas but not by group O human salivas at all. Accordingly, it is very likely, in view of the existence of antigen expressed by AC, BC and ABC in group A, B and AB human saliva, respectively<sup>13)</sup>, that there might be anti-C as

---

\* Drop of 2 in terms of test tubes.

\*\* This is a single hybrid agglutinin which reacts with both A- and B-antigen and exists within group O human serum. This view, first held by Landsteiner-Witt,<sup>17)</sup> is supported by many others.<sup>14) 18-20)</sup>

\*\*\* The agglutinin titers for group A and B blood cells of the eluates prepared from group B blood cells are higher than those for group Ax blood cells, by, in terms of test tubes, one and two tubes, respectively. This is perhaps due to anti-B like agglutinin (factor in rabbit serum before immunization that agglutinates group B human blood cells) and immune anti-A agglutinin secondarily absorbed to specific antigen-antibody complex and eluated from it.

well as anti-A and anti-B agglutinins in human O sera.

Only two of the 450 persons from whom group O human sera were obtained gave sera that did not agglutinate Ax blood cells, and in one of the 2 cases, there is reason to believe that the titer of anti-C agglutinin in the serum was so low that it could no longer agglutinate Ax blood cells<sup>10)</sup>. (Regrettably, no detailed study could be made of the other case.) Therefore, group O human sera has anti-C agglutinin with almost no exception and as a rule, a reciprocal relation exists between C antigen of the blood cells and anti-C agglutinin of the serum.

The non-participation of anti-A + B agglutinin in the agglutination of group Ax blood cells is obvious by the fact that the agglutinability of Ax blood cells by group O human sera remains unchanged<sup>10)</sup> whether or not those blood cells are blocked by anti-A (group B) serum and that eluates from group A blood cells agglutinated by group O human serum agglutinates both group A and B blood cells though it does not agglutinate group Ax blood cells<sup>10)</sup>.

#### SUMMARY

From group B blood cells agglutinated by rabbit anti-human A blood cell sera, eluates of immune anti-C agglutinin were prepared. This fluids agglutinated A<sub>1</sub> and B blood cells to almost the same extent but not agglutinate group O blood cells. Namely, Ax blood cells have C antigen in common with group A and B blood cells.

Agglutinins (naturally occurring antibody) in group O human sera that agglutinate Ax blood cells were neutralized by group A, B and AB human salivas which had C antigen in common but not by group O human salivas which were without the common antigen. In other words, the agglutination of Ax blood cells takes place by interaction between C antigen and anti-C agglutinin. Group O human sera agglutinate Ax blood cells with almost no exception. The soundness of Wiener's C-theory was thus demonstrated.

I should like to thank Prof. S. Shibata and Assist. Prof. H. Takahashi for their generous support, guidance and encouragement during the course of this work.

Deep appreciation is also expressed to Miss Kazue Matsumoto (Hiroshima Blood Bank) for her constant devoted technical assistance in this study.

#### REFERENCES

1. Hooker, S. B. and Anderson, L. M.: The specific antigenic properties of the four groups of human erythrocytes. *J. Immunol.*, **6**: 419-444, 1921.
2. Dölter, W.: Untersuchungen über die gruppenspezifischen rezeptorendes Menschen blutes und ihre Atikörper. *z. f. Immunfsg.*, **43**: 95-127, 1925.
3. Okabe, K.: Studien über die Antigenfunktionen menschlicher Blutkörperchen verschiedener Gruppen. *z. f. Immunfsg.*, **58**: 22-53, 1928.

4. Hibino, M.: Study on the production of group specific immune antibodies with human blood cells. Chapter 3. Antigenicity common to group A and B blood cells. *Juzen Igakkai Zasshi* (J. of Juzen Medical Society) **40**: 4002-4014, 1935.
5. Landsteiner, K.: Individual difference in human blood. (Nobel lecture) *Science.*, **73**: 403-409, 1931.
6. Wiener, A. S.: The blood factor C of the A-B-O system, with special reference to the rare blood group C. *Ann. Eugen.*, **18**: 1-8, 1953.
7. Wiener, A. S.: Principles of blood group serology and nomenclature: A critical review. *Transf.*, **1**: 308-312, 1961.
8. Matsuda, N., Takahashi, H. and Shibata, S.: A co-called Ax subgroup of blood type in a Japanese family. *Medicine and Biology.*, **70**: 299-304, 1965.
9. Wiener, A. S. and Wexler, I. B.: The mosaic structure of red blood cell agglutinin. *Bact. Rev.*, **16**: 69-87, 1952.
10. Matsuda, N.: Scheduled to be published in *Yamaguchi Igaku*. (J. of Yamaguchi Medical Association)
11. Kobayashi, C.: Study of C group substance. *Tokyo Igaku Zasshi* (Tokyo Journal of Medical Sciences) **56**: 381-456, 1942.
12. Hibino, M.: Study on the production of group specific immune antibodies with human blood cells: Chapter 2 Mutual relation between the presence of normal antibodies and relative difficulty in the production of immune antibodies in various kind of animals. *Juzen Igakkai Zasshi*, **40**: 1308-1337, 1935.
13. Ueyama, O.: Study on C group substance. *Hanzaigaku Zasshi* (Japanese Journal of Legal Medicine Criminology) **14**: 407-425. 1940.
14. Dodd, B. E.: Linked anti-A and anti-B antibodies from group O sera. *Brit. J. Bact. Path.*, **33**: 1-18, 1952.
15. Mollison, P. L.: *Blood transfusion in clinical medicine*. Blackwell. (Oxford) 1961, pp. 383.
16. Dunsford, I.: A critical reviews of the ABO sub-groups. *Congr. Int. Soc. Blood Transf.*, Rome, 1958, pp. 685-691.
17. Landsteiner, K., and Witt, D. H.: Observation on the human blood groups; irregular reactions. Isoagglutinins in sera of group IV. The factor A<sup>2</sup>. *J. Immunol.*, **11**: 221-246, 1926.
18. Bird, G. W. G.: Observations on hemagglutinin "linkage" in relation to isoagglutinins and autoagglutinins. *Brit. J. Exp. Path.*, **34**: 131-137, 1953.
19. Milgrom, F.: Recherches sur la structure des isoanticorps groupax. *Rev. Immunol.*, **16**: 86-109, 1952.
20. Jones, A. R. and Kaneb, L.: Some properties of cross reacting antibody of the ABO blood group system. *Blood*, **15**: 395-403, 1960.
21. Fukao, T.: Study on group specific precipitation: Relation between "serum group" and group specific immune precipitin of rabbits (Report 3). *Juzen Igakkai Zasshi*, **42**: 2737-2760, 1935.
22. Kikkawa, K.: Serological study on so-called 3rd heteroprecipitin and group specific precipitin in anti-blood cell immune serum of chicken (Report 1), group specific precipitin in anti-human blood cell immune sera of chicken and antibody in relation to antigen parts (anti-C precipitin and anti-C agglutinin) common to group A and B blood cells. *Juzen Igakkai Zasshi* (Journal of Juzen Medical Society), **43**: 3404-3446, 1936.
23. Thomsen, O. and Worsae, E.: Über die möglichkeit eines zusammenhanges zwischem den im Serum der O. Gruppe enthaltenen Isoagglutininen: "Anti-A" ( $\alpha$ ) und "Anti-B" ( $\beta$ ). *Z. f. Rassenphysiol.*, **21**: 1929-1930, 1929.
24. Ogata, T. and Matsuhashi, T.: Problems of special and cross reactivity of blood group antibodies. *Proc. 8th. Conger. Int. Soc. Blood Transf.*, Tokyo. pp. 208-210.