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LINKED ANTI-A AND ANTI-B ANTIBODIES FROM GROUP O SERA

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IT is accepted that antigen molecules can have more than one combining site, but the existence of a number of combining sites on an antibody molecule is unproven. Heidelberger and Kendall (1935), Kabat and Heidelberger (1937) and Marrack (1938) considered the precipitate formed in the precipitin reaction to be a network of antigen and antibody molecules, each antigen bound to several antibodies and each antibody molecule to two or more antigen molecules, thus postulating antibodies with several combining sites. Meyer and Pic (1937) offer evidence for the presence of specifically different combining groups in a single antibody. They studied sera from rabbits immunised against aqueous extracts of tubercle bacilli ; these sera reacted with both the lipids and the polysaccharide of the tubercle bacilli. By preparation of antibody solutions from lipid or polysaccharide absorbed on kaolin, they obtained from either preparation a solution which reacted with both antigens. They concluded that this was due to the presence in the rabbit sera of bivalent antibodies with a combining site for each of the two antigens.

More recent work tends to favour the idea of univalent antibodies. Landsteiner and van der Scheer (1938) prepared immune sera to azoproteins with two structurally different active groups in the same molecule, viz., succinic and phenylarsenic acids, and to glycine and leucine. When the immune sera were tested by absorption with one or other partial antigens there was no reduction in reactivity with the antigen containing the other determinant group. Thus these authors conclude that there is no evidence for the formation in these sera of antibodies having two different combining sites. Haurowitz and Schwerin (1942, 1943) also conclude from results obtained in precipitation experiments that immunisation with a complex antigen containing two kinds of determinant groups does not produce antibodies with both kinds of specificity.

The present work was the outcome of the observation that when A or B red cells absorbed and subsequently gave up agglutinins originating from human group O sera (containing both anti-A and anti-B agglutinins) the antibody solu-

tion obtained from red cells of either group agglutinated both A and B red cells. In other words, an agglutinin solution was prepared from group A cells which, as well as agglutinating group A cells, agglutinated cells of group B, and *vice versa*.

The simplest explanation of this finding is that at least some of the globulin molecules in group O serum possess both anti-A and anti-B specific combining sites, and so can be removed by either A or B cells.

The fact that antibodies absorbed from group O sera by A or B cells and subsequently recovered, agglutinated both A and B red cells, has been observed by Landsteiner and Witt (1926), Thomsen and Worsaae (1929), and Fischer and Hahn (1935). The agglutination of heterologous cells was attributed to a non-specific reaction, although Landsteiner and Witt suggested that group O sera as well as containing α and β also contain some antibody units which might be written $\alpha + \beta$. Evidence that other blood group antibody molecules may possess combining sites for two different antigens is provided by the work of Race, Sanger and Lawler (1948) with the antibodies anti-C and anti-C^w of the Rh system.

The following experiments were designed to establish the existence in human group O serum of antibody molecules having two specifically different combining sites, anti-A and anti-B.

MATERIALS AND METHODS.

Red cells.—The group A and group B red cells used for titration of antibodies and preparation of antibody solutions were almost always taken from the same two donors throughout. Blood was obtained by venepuncture and placed in a sodium citrate glucose mixture. It could then be used for experiments on two consecutive days, but was not kept longer. Before use the red cells were washed three times in 0·85 per cent saline. Packed cells were used for the preparation of antibody solutions and an approximately 2 per cent suspension in saline for all titrations except haemolysin titres, for which an approximately 5 per cent suspension was used.

Anti-human-globulin serum for Coombs' tests.—A very potent anti-human globulin rabbit serum, kindly provided by Professor Marrack, was used throughout the investigation. This was used at a dilution in saline of 1 : 100.

Purified A and B group-specific substances.—A and B group-specific substances prepared from pseudomucinous ovarian cysts (Morgan and van Heyningen, 1944) according to the method described by King and Morgan (1944) were supplied by Dr. Morgan, and were used for inhibition tests and for intramuscular injection into group O volunteers.

Complement for haemolysin tests.—Dried guinea-pig complement was used with some success, although usually fresh human complement was found to be more satisfactory.

Titration of anti-A and anti-B antibodies.—The method used for titrating antibodies was essentially as described by Taylor and Ikin (1939). Doubled dilutions of test serum were made in saline with a Pasteur pipette graduated to deliver a constant volume (about 0·03 ml.). To each of the dilutions an equal volume of a 2 per cent suspension of appropriate red cells was added and the tubes shaken. The titrations were made in duplicate and allowed to stand at room temperature for 2–3 hours, after which the contents of the tubes were examined for agglutination both macroscopically and microscopically. The method of scoring the

degree of agglutination is shown in the key to Table I. The presence of evenly distributed clumps of three or more cells was accepted as evidence of agglutination. The titre of a serum was expressed as the reciprocal of the highest dilution of serum causing agglutination.

Titration by anti-globulin technique.—The anti-human globulin test of Coombs, Mourant and Race (1945) was developed for the detection of a type of Rh antibody which sensitized red cells suspended in saline without causing them to agglutinate. Red cells sensitized by these Rh antibodies and washed free from serum were agglutinated on exposure to the serum of a rabbit which had been previously immunised against human globulin, or against human whole serum.

This test is applicable in certain circumstances to the antibodies of the ABO system (Boorman and Dodd, 1946), and has proved invaluable in the present work, where antibody is apparently concerned which sensitizes the red cell more readily than it causes agglutination.

The titrations were carried out as described and kept at room temperature for at least 1 hour. The red cells in each tube were then freed from serum by two washings in saline, after which the cell sediment from each tube was mixed on a slide with one drop of suitably diluted anti-human-globulin serum freshly made up for each batch of tests. The slides were left on the bench for an interval of from 5–10 min. with occasional rocking and then examined for agglutination. The degree of agglutination was scored in the usual manner. When Coombs' titrations were performed on antibody eluted from red cells the washing stage was omitted.

Titration of anti-A and anti-B haemolysins.—Sera containing haemolysins were titrated using the Pasteur pipette technique and guinea-pig complement or fresh human serum as diluent instead of 0·85 per cent saline. It was found that the best results were obtained when the sera were titrated within 3 hours of venepuncture without the addition of complement. This finding suggested that the complement in the test serum itself was more efficient in the production of haemolysis than added complement of either human or animal origin. After the addition of one unit volume of 5 per cent red cells to each tube, the tests were incubated for approximately two hours at 37°. They were then removed from the incubator, shaken and allowed to stand at room temperature until the red cells had sedimented or the tubes were centrifuged. The degree of haemolysis in each tube was then read.

Elution of antibody from the red cell surface.—The method was one adapted from Landsteiner and Miller (1925). In the first experiments equal parts of a group O serum and packed washed red cells of group A and B respectively were mixed and left at room temperature for at least one hour. Later it was found that the potency of the antibody finally recovered was improved if the red cell volume was reduced and the absorption stage carried out at 4°. A mixture of 10 drops of packed washed cells and 2 ml. of serum was therefore placed in the ice chest to absorb for 1 hour.

The cells, which were usually strongly agglutinated, were then carefully washed at 4°. About 8 ml. of saline was used for each washing and the number of washings given related to some extent to the potency of the original group O serum, the minimum number being four and the maximum six. After each washing the agglutinated cells, which were often compacted into a solid mass by the action of the centrifuge, were broken down as completely as possible by vigorous shaking.

In this way an attempt was made to ensure that no antibody remained caught up mechanically in the agglutinated cell mass. In each experiment the last washing of the red cells was kept and tested for antibody together with the eluted antibody. After the cells were thoroughly washed 2 ml. saline was added, and after thorough mixing the suspension was placed in a water-bath at 56° for 10 minutes. During this time the suspension was continually agitated. The tubes were then transferred quickly to centrifuge cups previously heated to 56° and lagged with cotton-wool soaked in water at 56°. They were then centrifuged strongly for a short time and the supernatant fluid (the eluate) which contained the liberated antibody removed as quickly as possible from the cells. Antibody solutions thus prepared were always tested for antibody as far as possible on the day on which they were prepared, because it was found that they underwent a certain degree of deterioration, even if stored at − 40°.

EXPERIMENTAL.

Series 1 *: Antibody eluted from A and B cells after these had been allowed to absorb antibody from immune group O sera.*

Twenty-two cases were investigated ; of these 12 sera contained immune anti-A antibody and 10 immune anti-B antibody. Eight of the sera containing immune A antibodies were from group O mothers who had been carrying a foetus of group A, while 3 were from group O individuals injected with purified A group specific substance, and 1 was from a group O woman who had received an incompatible transfusion of 500 ml. of group A blood. Of the 10 sera containing immune anti-B antibodies 6 were from group O mothers carrying a group B foetus, and 4 were from individuals who had received an intramuscular injection of group specific substance B. The individuals immunised by either A or B

TABLE I.—*Typical Protocol showing Details of Titrations.*

	Original serum.				Antibody solutions.							
					From A cells.				From B cells.			
Serial dilutions.	Anti-A.		Anti-B.		Against A.		Against B.		Against A.		Against B.	
	S	*Co*	*S*	*Co*	*S*	*Co*	*S*	*Co*	*S*	*Co*	*S*	*Co*
1 : 1	c	c	c	c	v	v	v	v	v	v	v	v
1 : 2	c	c	c	c	+ +	v	v	v	+ +	v	+	v
1 : 4	v	v	v	v	+ +	v	+	v	+	v	(+)	v
1 : 8	v	v	v	v	(+)	(+)	gw	v	(+)	v	w	v
1 : 16	+ +	v	+ +	v	w	w	w	+	w	v	−	v
1 : 32	+ +	v	+ +	v	−	w	−	w	−	(+)	..	+ +
1 : 64	+	v	+	v	..	−	..	−	..	w	..	(+)
1 : 128	+	v	+	v	..	..	..	..	..	−	..	(+)
1 : 256	(+)	v	w	+ +	..	..	..	..	..	..	..	−
1 : 512	gw	+ +	−	w								
1 : 1000	w	w	..	−								
1 : 2000	−	−										
1 : 4000												

S = agglutinin titre. *Co* = Coombs' titre.

Read macroscopically : *c* = complete agglutination ; surrounding fluid clear. *V* = visual agglutination ; surrounding fluid pink.

Read microscopically : + + = very large clumps—few free cells. + = large clumps—more free cells. (+) = smaller clumps with many free cells. gw = slightly smaller clumps with many free cells. w = evenly distributed clumps of 2–5 cells. − = no agglutination.

TABLE II.—*Anti-A and Anti-B Titres of 22 Immune Group O Sera, together with Titres of Antibody Solutions Prepared from these Sera.*

Cases.	Immune antibody.	Titre of original serum.				Titre of antibody solution from A cells.				Titre of antibody solution from B cells.			
		Against A cells.		Against B cells.		Against A cells.		Against B cells.		Against A cells.		Against B cells.	
		S	Co	S	Co	S	Co	S	Co	S	Co	S	Co
1. Jasper	Anti-A	1,000	16,000	64	128	32	4,000	–	2	16	1,000	16	8
2. Grayson	Anti-B	512	4,000	1,000	8,000	32	64	32	128	2	4	2	32
3. Dark	Anti-A	512	1,000	64	256	–	64	–	32	4	128	1	8
4. Sparks	,,	32,000	128,000	1,000	2,000	128	8,000	4	8	64	16,000	4	8
5. Taylor	,,	4,000	64,000	32	128	16	2,000	–	4	32	512	1	512
6. Cooper	Anti-B	256	1,000	1,000	8,000	32	32	64	128	32	32	32	32
7. Golding	Anti-A	2,000	8,000	256	512	32	32	–	4	16	32	4	16
8. Prince	Anti-B	64	128	512	1,000	4	8	1	16	1	8	2	8
9. McRae	Anti-A	4,000	16,000	1,000	1,000	2,000	2,000	64	128	64	128	16	1,000
10. Scott	,,	4,000	32,000	128	1,000	64	1,000	1	8	2	8	16	32
11. Pearson	,,	2,000	4,000	64	128	32	64	2	128	16	64	8	16
12. Young	Anti-B	128	512	1,000	4,000	32	64	64	256	4	8	1,000	2,000
13. Howe	Anti-A	2,000	8,000	16	32	32	128	–	–	8	64	8	128
14. Tysson	Anti-B	256	2,000	1,000	4,000	32	256	8	32	4	16	64	1,000
15. Zeitlin	,,	1,000	1,000	4,000	8,000	4	16	8	64	2	8	64	256
16. Sharp	Anti-A	1,000	1,000	256	512	8	16	16	32	16	32	8	128
17. Butcher	Anti-B	128	32,000	16,000	32,000	64	128	64	128	32	64	64	256
18. Adams	Anti-A	512	16,000	64	256	128	512	4	128	128	256	16	256
19. McTrustery	,,	4,000	512,000	256	512	128	8,000	8	16	128	256	16	32
20. Gore	Anti-B	1,000	2,000	2,000	8,000	8	64	32	256	1	1	64	1,000
21. Sterile	,,	512	8,000	2,000	32,000	128	256	128	256	2	8	64	1,000
22. Fitzgerald	,,	512	2,000	8,000	16,000	64	512	8	64	2	32	32	512

TABLE III.—*Results Proving that Anti-A is Present in Antibody Solutions Prepared from Group B Cells, and Anti-B in Antibody Solutions Prepared from Group A Cells.*

Case	Titre of antibody solution from A cells after neutralization with A substance. Against A cells.	Against B cells.	Titre of antibody solution from B cells after neutralisation with B substance. Against A cells.	Against B cells.
17. Butcher	–	8	..	..
9. McRae	..	..	16	–
18. Adams	..	..	2	–
19. McTrustery	..	..	Not tested	Not tested
20. Gore	Not tested	Not tested	..	..

Case.	Tests against random Group A samples. 1	2	3	4	5	6	7	8	9	10	Tests against random Group B samples. 1	2	3	4	5	6	7	8	9	10
17. Butcher	–	–	–	–	–	–	–	–	–	–	w	w	+	+	+	+	+	+	+	+
9. McRae	v	v	v	v	v	v	v	v	v	v	–	–	–	–	–	–	–	–	–	–
18. Adams	+	+	w	+	+	(+)	+	+	+	+	–	–	–	–	–	–	–	–	–	–
19. McTrustery	++	++	++	++	++	++	++	++	++	++	–	–	–	–	–	–	–	–	–	–
20. Gore	–	–	–	–	–	–	–	–	–	–	++	++	++	++	++	++	++	++	++	++

purified substance received one dose of approximately 1·5 mg. of group specific substance by intramuscular injection. After eight to ten days a bleeding was obtained, and the immune serum thus obtained was used in these experiments.

The eluted antibody preparations were titrated against A and B red cells and examined also by the Coombs' technique. The specimens were also tested for their capacity to agglutinate group O cells.

Results.—A typical protocol is shown in Table I. This shows results of titrating antibody solutions (eluates) made from A and B cells which had absorbed antibody from immune group O sera. The results show that the antibody solution prepared from either group A or group B red cells is able to agglutinate *both* types of red cell. This is particularly well demonstrated when the Coombs' titration technique is used. Only a negligible quantity of antibody was present in the saline of the final washing. Results of similar experiments with the 22 immune group O sera are summarised in Table II. In every instance the recovered antibody agglutinates the heterologous cells. This is true whether the antigenic stimulus is afforded by a foetus *in utero,* blood transfusion (1 case only), or injection of group specific substances. None of the antibody solutions caused agglutination of group O cells.

Comment.—These findings are best explained by postulating the presence of both anti-A and anti-B antibodies in the antibody solutions whether these are derived from A or B red cells. As mentioned above, this could occur if there existed in the group O sera certain globulin molecules which possessed both anti-A and anti-B combining groups, thus enabling them to be removed from solution by either A or B red cells and causing the antibody solutions to agglutinate both A and B red cells.

The fact that the antibody solutions did not on any occasion agglutinate group O cells showed that the agglutination of heterologous cells, whether these were A or B, was not due to the presence in the group O sera of antibodies belonging to other blood groups systems.

Series 2 : Experiments to prove that antibody solutions from group A cells prepared as in Series 1, in addition to anti-A contain anti-B, and those from group B cells, in addition to anti-B contain anti-A.

These experiments involved neutralization of antibody solutions prepared as in Series 1 with the appropriate group-specific substance A or B. Thus, if the antibody had been recovered from group A cells, the anti-A activity of the solution was inhibited by the addition of group-specific substance A. The neutralised antibody solution was then tested against bloods of groups A and B. A similar experiment was made by neutralising the anti-B in an antibody solution prepared from B cells and testing this also against A and B bloods.

Results.—The results are shown in Table III, where it will be seen that an antibody which only agglutinated cells of group B was present in the solutions neutralized with group-specific substance A. This antibody can only be anti-B and therefore even though other antibodies in addition to anti-A might have been neutralized by the A substance (e.g. the hypothetical anti-C, see discussion) an explanation for the recovery of true anti-B from A cells has to be found. A similar argument applies to anti-A antibody recovered from B cells.

Series 3 : Control experiments.

(*a*) Antibody solutions prepared from packed washed group O cells which had first been in contact with immune group O sera (Sparks case 4—Grayson case 2) were completely inactive, agglutinating neither A, B nor O cells.

(*b*) Antibody solutions obtained from A and B cells after treatment with immune sera of groups A and B did not cause any agglutination of heterologous red cells, i.e., antibody solutions from group B cells which had been treated with a serum containing only anti-A did not agglutinate cells of group A, while antibody solutions from group A cells which had been treated with a serum containing only anti-B did not agglutinate group B cells.

(*c*) Similarly when " artificial group O sera " were made by mixing equal parts of anti-A and anti-B immune sera, the antibody solutions prepared therefrom agglutinated either group A or group B cells but never both.

Comment.—The fact that group O cells are incapable of absorbing and releasing either anti-A or anti-B antibodies from group O sera refutes the possibility that the anti-A or anti-B recovered from heterologous cells in experiments in Series 1 is attached to those cells by means of a red cell receptor which is alike possessed by A, B and O cells. Thus the theory that linkage between anti-A and anti-B antibodies in immune group O sera exists is supported. Further confirmation for this linkage is afforded by the results of tests on antibody solutions prepared from monospecific sera containing one or other of the two antibodies, and by the results of experiments with " artificial " mixtures of anti-A and anti-B immune sera.

TABLE IV.—*Titres of Antibody Solutions Prepared from Non-immune and Random Group O Sera.*

Cases.	Immune antibody.	Titre of original serum.				Titre of antibody solution from A cells.				Titre of antibody solution from B cells			
		Anti-A.		Anti-B.		Anti-A.		Anti-B.		Anti-A.		Anti-B.	
		S	Co	S	Co	S	Co	S	Co	S	Co	S	Co
McRae pre-immunisation	—	128	512	64	512	16	128	1	2	1	16	8	64
Zeitlin pre-immunisation	—	128	256	32	64	8	256	—	2	1	32	16	32
Weir	—	128	512	64	256	4	8	—	1	—	8	1	2
Eeles	—	256	8000	64	128	8	32	—	1	—	—	2	4
019	—	128	2000	8	8	4	128	—	—	—	64	—	1
097558	—	128	1000	32	256	2	8	—	4	4	32	—	8
44786	—	512	1000	128	256	16	64	—	2	—	2	—	16
46434	—	256	256	128	128	64	128	—	1	1	2	4	16
44982	—	128	128	64	128	64	64	2	4	—	1	32	32

In all cases the antibody activity of the final washings of both A and B cells was *nil.*

Series 4 : Eluates made from A and B cells treated with non-immune and random group O sera.

Four group O sera known to be non-immune and five random group O sera were investigated in the same way (Table IV). (It should be noted that in order to fulfil the definition " non-immune " for the purpose of this investigation, a serum must be from an individual who, so far as can be ascertained, has never (*a*) carried a foetus of group A or B, (*b*) received an incompatible blood transfusion

of group A or B blood, or (*c*) received an injection of substances containing antigens A or B.)

Results.—The titration results of antibody released from A and B red cells after treatment with these sera show a lower range of values than the immune sera, and only small amounts of anti-A are obtained in the antibody solutions from B cells and also of anti-B in the solutions from A cells.

Comment.—If a comparison of the titres of antibody in the solutions prepared from both immune and non-immune group O sera is made, it is found that those for anti-A or anti-B recovered from heterologous cells are very much higher than the corresponding ones for non-immune sera. Of particular interest are those cases in which experiments have been carried out on serum samples taken both before and after the injection of either A or B antigen. Therefore it seems that immune sera are a richer source of antibody units having two different combining sites than non-immune sera.

Series 5 : Titration of group O sera after absorption with A and B red cells.

The portions of group O sera which had been absorbed by A and B red cells for the preparation of antibody solutions by elution were kept and their anti-A and anti-B titres estimated.

Results.—These revealed that in many instances, accompanying the expected reduction in titre of anti-A and anti-B when each has been absorbed with homologous cells, there was also a reduction in the titre of the heterologous antibody. Examples of this are shown in Table V and indicated by an asterisk. The non-immune sera tested did not show this.

Comment.—Since it has been shown that anti-A antibody can be recovered from group B cells and similarly anti-B antibody from group A cells, a reflection of this in the titration values of the absorbed sera is to be expected. The reduction in titre of the heterologous antibody seems therefore to be due to the removal from the group O serum of that part of the heterologous antibody which is situated on the same globulin molecule as the homologous antibody. It will be noted that it is more markedly the titre of anti-B which reduced in sera absorbed by A cells. It is likely that a reduction of heterologous anti-A antibody does occur, but is not apparent owing to the limitations of the technique. This is probably because the anti-A titration values are considerably greater on the whole than those of anti-B, thus a significant change in titre is more difficult to obtain.

Series 6 : Titration of group O sera after neutralization with A and B group specific substances.

Seventeen of the same sera were titrated after neutralization with purified A and B group specific substances. The necessary amount of substance to neutralize approximately the homologous antibody was added to a small quantity of serum, and after a half to one hour the serum was titrated against A and B red cells. Experiments to control the specificity of the A and B substances were also included (see Table V).

Results.—These findings are very similar to those of Series 5. In point of fact the reduction in titre of the heterologous antibody is even more pronounced than when cells are used for absorption.

TABLE V.—*Titre of Immune and Non-immune Group O Sera after Absorption with A and B Group Specific Substances and A and B Red Cells.*

Case.	Original serum titre.		Titre after absorption with A substance.		Titre after absorption with B substance.		Titre after absorption with A cells.		Titre after absorption with B cells.	
	Anti-A. S	Anti-B. S	Anti-A. S	Anti-B. S	Anti-A. S	Anti-B. S	Anti-A S	Anti-B. S	Anti-A. S	Anti-B. S
3. Dark (immune anti-A)	512	64	–	8*	1000	2	–	4*	512	–
5. Taylor („ „)	4000	32	–	2*	2000	1	2	4*	4000	–
9. McRae („ „)										
a. pre-immunisation	128	64	Not tested	Not tested	Not tested	Not tested	2	32	128	1
b. post-immunisation	4000	1000	16	64*	1000	1	8	64*	4000	2
15. Zeitlin (immune anti-B)										
a. pre-immunisation	128	32	Not tested	Not tested	Not tested	Not tested	2	32	128	2
b. Post-immunisation	1000	4000	1	512*	32*	1	4	4000	128*	8
20. Gore (immune anti-B)	1000	2000	1	2000	8*	2	4	2000	32*	256
21. Sterile („ „)	512	2000	8	2000	32*	4	4	1000	64*	2
44982 (non-immune)	128	128	4	64	128	–	1	64	128	1
46434 „	256	128	1	64	256	1	1	64	128	1
Controls.										
Mixture anti-A + anti-B serum	128	256	–	256	128	–				
Monospecific anti-A serum	64	–	–	–	64	–				
Monospecific anti-B serum	–	512	–	512	–	–				

* Titre of heterologous antibody also reduced (see text, p. 9).

Results of the control experiments show that neither A nor B substance reduces the titre of the heterologous anti-B or anti-A antibody when these occur singly in group A and group B sera. Moreover if a mixture of anti-A and anti-B sera is made, there is no reduction in titre of the anti-A antibodies when group B substance is added, nor a reduction in titre of anti-B antibodies when group A substance is added.

Comment.—It is of particular interest that neutralization of the group O sera with A and B group-specific substances has a similar effect to absorption of the sera with A and B red cells, for since neutralization with group-specific substances does not remove antibody from the serum, as do red cells, the reduction in titre of the heterologous antibody cannot in this instance be due to its removal from the serum in combination with the homologous antibody. It seems extremely likely therefore that the suppression of activity of one or other antibody after the addition of the heterologous group specific substance is a spatial effect, and is brought about by the fact that the anti-A and anti-B combining sites of an antibody molecule which possesses both these specific sites are in close proximity. Thus the polysaccharide residues induce an "overshadowing" or steric hindrance of the heterologous receptors. Hence, a molecule of a particular group-specific substance is able effectively to screen a nearby combining group so that the latter is unable to combine specifically with its homologous determinate group. If this is true, absorption with red cells, or neutralization with group-specific substances of a serum from which the antibody molecules possessing two kinds of combining group have been removed, should not cause a reduction in titre of the heterologous antibody.

The following experiment was designed to test this point:

Series 7 : Neutralization of a serum with group specific substance A after the serum had been absorbed with group A red cells.

Serum McRae was selected for this experiment, and was first absorbed with packed washed group A red cells in the proportion of 2 ml. of serum to 10 drops of packed cells. A part of this absorbed serum was then subjected to a further neutralization with 0·25 ml. of group specific substance A. Anti-A and anti-B titrations were carried out by direct agglutination and Coombs' techniques both on the serum after absorption with A cells and after the further absorption with group-specific substance.

Results.—After absorption with A cells the anti-A titre was reduced from 4000 to 1 by direct agglutination methods and 4000 to 2 by Coombs' technique, and was accompanied by a reduction in titre of the heterologous anti-B agglutinin from 512 to 32 by direct agglutination method and from 2000 to 64 by Coombs' technique. The same serum sample after the addition of 0·25 ml. of A substance showed complete neutralization of anti-A agglutinin but no further reduction in titre of the anti-B agglutinin.

Comment.—The first absorption with A cells removed almost all the anti-A agglutinins, including those which were linked to anti-B. The removal of antibody molecules with both anti-A and anti-B combining groups is reflected in the considerable reduction in titre of anti-B. The further absorption with group A specific substance, while neutralizing the small amount of anti-A still remaining. caused no further reduction in titre of the anti-B, presumably because all antibody

molecules possessing both anti-A and anti-B combining groups had already been removed from the serum.

Series 8 : Haemolysin experiments.

Most of the immune sera lysed A cells and/or B cells to some extent. The non-immune sera caused slight lysis usually of only one kind of red cell. Eight of the immune sera lysing both A and B cells were titrated before and after absorption by A and B red cells.

Results.—The titres range from 2 to 16, but most are not higher than 4. Although every serum lysed both A and B cells, none lysed group O. Absorption of the sera by A and B cells produced an almost constantly different effect (Table VI). Thus if anti-A was the immune* antibody, absorption of the serum by A cells destroyed the lytic activity for both A and B cells, whereas absorption by B cells destroyed the anti-B lytic activity only.

TABLE VI.—*Haemolysin Titres of Immune Group O Sera Compared with those after Absorption with A and B Red Cells.*

Cases.	Stimulating antigen.	Titre of haemolysin.						
		Against A cells.	Against B cells.	Against O cells.	After absorption with A cells.		After absorption with B cells.	
					Against A.	Against B.	Against A.	Against B.
2. Grayson	B	8	16	—	—	4	2	—
3. Dark	A	4	2	—	—	—	1	—
4. Sparkes	A	16	4	—	1	—	16	—
9. McRae	A	4	4	—	—	—	4	—
11. Pearson	A	2	2	—	—	—	2	—
12. Young	B	2	8	—	—	8	—	—
14. Tysson	B	2	4	—	—	4	1	—
15. Zeitlin	B	2	2	—	—	2	—	—

Conversely, if anti-B is the immune antibody, absorption with A cells removes all trace of anti-A haemolysin but the anti-B haemolysin remains while absorption with B cells leaves a serum which lyses neither A nor B cells.

Comment.—Increased lytic activity in immune sera is almost constant. It is also well known that immune group O sera, whether the anti-A or the anti-B is the immune agglutinin, will often lyse both A and B cells, although never group O cells. Hitherto this apparently non-specific lysis of heterologous cells has been difficult to explain, but these experiments suggest that they are lysed by antibodies which have both anti-A and anti-B combining sites. If the lysin is associated with the immune antibody and therefore with those immune antibodies which have both anti-A and anti-B combining sites, then it would be possible for a lysin associated with anti-A to lyse group B cells on account of close proximity with these cells, and conversely a lysin associated with anti-B to lyse group A cells for the same reason. Moreover this hypothesis would also account for the particular pattern of results obtained after the sera are absorbed with A and

* It should be explained that the term " immune " applies to that antibody in a group O serum which corresponds in specificity to the antigen which was responsible for the immunisation. The term " non-immune " is used to describe the antibody which is heterologous to the immunising antigen, e.g., if the immunising antigen is A (from red cells or group-specific substances) then anti-A is the immune antibody and anti-B is the non-immune antibody.

B red cells ; for if both anti-A and anti-B lysins are formed in the serum as entities which are separate from each other, then in all cases absorption with A cells should result in a serum which would haemolyse B cells only and *vice versa.* Alternatively, if a non-specific lysin had developed in the immune serum, absorption with either A or B cells should completely remove all lytic activity from the serum. It will be seen from a study of Table VI that neither of the two last possibilities fits the experimental findings.

DISCUSSION.

Before discussing these results, it is necessary to define what they prove. They show that :

(1) Antibody solutions prepared from immune group O sera (Series 1) agglutinate both A and B red cells but not O cells.

(2) Antibodies responsible for this agglutination are in firm combination with the A and B red cells and only released on warming ; this is shown by the almost complete absence of antibody in the saline extracts obtained from the final washing of the agglutinated red cells before the warming takes place.

(3) Anti-B is present in antibody solutions prepared from group A cells, and anti-A is present in solutions prepared from group B cells (Series 2).

(4) Neither anti-A nor anti-B agglutinins are found in antibody solutions prepared from group O cells which have been in contact with immune group O sera (Series 3).

(5) It is not possible to recover heterologous anti-A or anti-B agglutinins from B or A cells respectively when these cells are allowed to absorb agglutinins from mono-specific anti-A (group B) or anti-B (group A) sera, or from mixtures of these agglutinins made by mixing together group A and group B sera (Series 3).

(6) Hence it can only be through the existence of antibody molecules in group O sera with combining groups of more than one specificity that the presence of anti-B in antibody solutions prepared from group A cells, or conversely anti-A in antibody solutions prepared from group B cells, can be explained satisfactorily.

(7) A comparison between results of experiments in Series 1 and 4 shows that immune group O sera contain more antibody molecules of the special type than non-immune group O sera. This is true irrespective of whether the immunisation is caused by a foetus *in utero*, incompatible red cells or injection of group-specific substances.

(8) The removal of heterologous antibody from an immune group O serum is often demonstrable by reduction in titre of that antibody after absorption of the serum with appropriate red cells (Series 5).

(9) When neutralization of either anti-A or anti-B agglutinins in group O immune sera by means of the homologous group-specific substances take place there is also a reduction in titre of the heterologous agglutinin (Series 6, Table V). If the specificity of the A and B substances is controlled (Table V) by testing them against anti-A and anti-B sera, both singly and as a mixture, no reduction in titre of heterologous antibody is detected.

(10) A group O serum which is absorbed with group A cells followed by treatment with group-specific substance A shows no further reduction in the titre of the anti-B after the addition of the group-specific substance.

(11) When the titre of *immune* antibody recovered from heterologous cells is compared with the titre of the non-immune antibody recovered from heterologous cells, a distinct pattern of results is formed. This is discussed later.

(12) Haemolysis of both A and B cells but not group O cells is caused by some immune group O sera.

(13) The haemolysin titres of these sera after absorption with A and B cells are shown to conform to a definite pattern as mentioned above.

So far the points enumerated have been borne out by experimental findings, and it is tempting to proceed further and suggest a theory to fit these findings. As mentioned previously, the presence of anti-A in antibody solutions prepared from group B cells and anti-B in antibody solutions from group A cells indicates

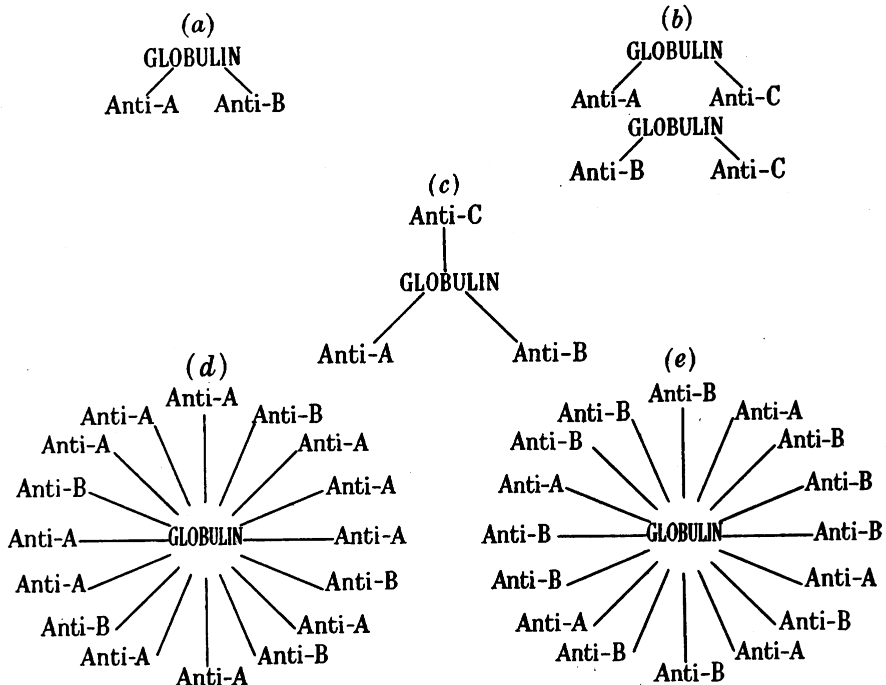

FIG. 1.

the existence of certain antibody molecules in group O sera which possess not only more than one combining receptor but receptors of different specificity. Fig. 1 (*a*), which is an attempt to depict this type of globulin molecule in a purely diagrammatic form, shows a bivalent structure with two receptors, one (anti-A) specific for antigen A and the other (anti-B) specific for antigen B. Thus a red cell of either group A or group B would be capable of taking up this type of antibody and afterwards releasing it on warming. Antibody solutions containing these antibodies would be capable of agglutinating both A and B red cells.

Although a direct linkage between anti-A and anti-B receptors or combining sites after this manner is most likely to be the correct explanation of the experi-

mental findings, the possibility of the existence of a third type of receptor (anti-C) in combination with anti-A and anti-B must be mentioned. The possible existence of a third type of antibody in group O sera was suggested by some early workers on blood groups, who postulated the presence of another antibody, anti-C, in addition to anti-A and anti-B, for which there was a specific antigen C which was alike possessed by A, B and AB red cells. The work of Hooker and Anderson (1921) seems to support this idea. They noticed that when certain anti-B immune rabbit sera were absorbed with group O blood, they still strongly agglutinated not only B cells but also A cells. Similar results were obtained by Landsteiner and Witt (1926) with immune anti-A sera.

However, the results of the absorption experiments of Koeckert (1920) and Schutze (1921) failed to indicate the presence of anti-C in group O sera, and most workers do not favour the existence of C and anti-C in the ABO blood-group system.

Diagrams introducing the hypothetical anti-C are shown in Fig. 1 (*b*) and (*c*). Fig. 1 (*b*) is a diagrammatic representation of globulin molecules of two kinds, one having combining sites anti-C and anti-A, and the other combining sites anti-B and anti-C. Fig. 1 (*c*) is an alternative to this arrangement in which anti-A, anti-B and anti-C combining sites are all situated on the same molecule. In either case A or B cells, each of which according to the theory possess antigen C, would be able to take up and release molecules of the types shown. Moreover antibody solutions which contained them would agglutinate both A and B red cells. Nevertheless the possible presence of this third antibody in group O sera, while difficult to disprove, does not remove the need for assuming the existence of molecules with combining sites of different specificities ; the anti-A which is recovered from B cells must be attached to anti-B or anti-C, and similarly the anti-B recovered from A cells must be attached either to anti-A or anti-C (see series 2) as the A receptors of red cells do not take up anti-B and *vice versa*. Thus if the choice lies, as it does, between anti-A and anti-B, each being the vehicle by which the other becomes attached to heterologous cells, or anti-C being responsible for this attachment, then the theory which need not include a hypothetical C and anti-C is much to be preferred. Experimental proof for a direct linkage between anti-A and anti-B might be forthcoming if blocking of the A or B receptors of red cells of groups A and B could be effected before these cells were allowed to absorb antibody from group O sera. Thus if the A receptors of group A cells could be saturated with anti-A antibody and then exposed to an immune group O serum, it might be possible to show that the antibody solution afterwards prepared only agglutinated cells of group A and not group B. This would show that blocking the A receptors of the cells so that they were no longer able to take up anti-A from the group O serum also prevented them from taking up and releasing anti-B. So it could be concluded that it is by means of linkage to anti-A that anti-B can be recovered from A cells. A similar experiment might be performed by blocking group B receptors with anti-B serum. Unfortunately preliminary experiments along these lines, although suggestive, have not been conclusive, owing to the technical difficulty of completely blocking the red cell receptors. Experiments in which the anti-A combining sites are blocked by neutralisation with group-specific substance A before the preparation of antibody solutions meet with greater success, for if this is done, anti-B is completely absent from anti-body solutions from A cells. However, it is conceivable that A and B group-specific substances might also contain the substance C, so for this reason

these experiments are not valid as a means of proving direct linkage between anti-A and anti-B.

A further question is whether the antibody molecules which have been shown to have at least two combining sites have in reality many more. An indication that the antibody molecules may indeed have more than two combining sites is gained from a comparison between the titres of immune and non-immune antibody recovered from heterologous cells.

Although in cases 8, 9, 10, 11, 14, 16 and 18 the titre of anti-A recovered from group B cells in each instance is approximately equal to the anti-B recovered from group A cells (Table II), in most of the remaining cases there is a definite, sometimes extremely marked difference between them, the titre of immune antibody recovered from heterologous cells being higher than the titre of non-immune antibody from heterologous cells. This is well shown by serum specimens 1, 4, 5, 7, 12 and 13, in which the immune antibody, anti-A, is present in unequivocally higher titre when recovered from group B cells than the non-immune anti-B recovered from A cells. In fact, in cases 1, 4 and 5 the titre of anti-A antibody recovered from group B cells far exceeds not only the titre of anti-B recovered from A cells but the original anti-B titre of the group O serum itself. Similarly, serum specimens 12, 15, 20, 21 and 22, in which the immune antibody is anti-B, conform to the same pattern. If anti-B is the vehicle by which anti-A is carried into the antibody solution prepared from B cells, it would seem to be impossible for the titre of anti-A from B cells to be greater than the original anti-B titre. It could be explained, however, by postulating multi-receptor antibodies (Fig. 1 (*d*) and (*e*)) with more immune than non-immune receptors. Fig. 1 (*d*) depicts the kind of antibody produced when A is the stimulating antigen, and Fig. 1 (*e*) the antibody produced to antigen B. Antibodies of the first type might give higher titration values with A cells, while antibodies of the second type might give higher titration values with B cells.

If the diagrams in Fig. 1 are representative of the kind of antibody molecules which exist in immune group O sera, it is easy to visualise the " overshadowing" or steric hindrance which would be caused when antibodies of this type, particularly those depicted in Fig. 1 (*d*) and (*e*), became attached to the relatively very large molecules of A or B group-specific substances which have been shown to have a particle size in excess of 250,000 (Aminoff, Morgan and Watkins, 1950). Here also it is interesting (Table V) that the non-immune antibody is much more readily overshadowed by its heterologous group-specific substance than the immune antibody. This finding supports the theory of multivalent antibodies of the type shown (Fig. 1 (*d*) and (*e*)). In Fig. 1 (*d*), for instance, the attachment between the anti-A receptors and molecules of group-specific substance A would readily cause overshadowing of the relatively few anti-B receptors, whereas an attachment between the anti-B receptors and molecules of group-specific substance B would cause less overshadowing of the larger number of anti-A receptors.

Whether a non-immune group O serum possesses antibodies with receptors or combining sites of two kinds is debatable, because it is impossible to be certain that the anti-A and anti-B agglutinins in a group O serum are non-immune. Substances containing antigens A and B or closely related substances are probably ubiquitous, and may easily gain access to the human body through the intake of food or by inhalation through the nasal mucous membrane. It is possible that droplets of saliva of group A, B or AB individuals which contain

potent group-specific substances are inhaled by group O individuals, resulting in at least a slight degree of immunization.

SUMMARY.

Antibody solutions prepared by warming either group A or group B red cells, after these cells have absorbed antibody from group O immune sera, are found to agglutinate both A and B red cells. This is due to the presence of both anti-A and anti-B antibodies in the solutions irrespective of whether these have been prepared from A or B cells.

These and other findings suggest that the immune group O sera tested contained some antibody molecules which possessed both anti-A and anti-B combining sites and which therefore could be taken up and subsequently released on warming by either A or B red cells. Solutions containing molecules of this type would be able to agglutinate both A and B cells.

Immune sera are a richer source of antibody molecules possessing both anti-A and anti-B combining sites than non-immune sera.

Neutralization of the anti-A antibody in immune group O sera by purified A group specific substance markedly reduced the titre of the heterologous antibody anti-B, and *vice versa.*

A series of haemolysin experiments demonstrated that immune O sera lysed both A and B cells whether the stimulating antigen was A or B. No lysis of group O cells took place. Non-immune sera showed very little lytic activity. The possibility that the haemolytic activity of antibody molecules possessing both anti-A and anti-B combining sites was responsible for the haemolysis of heterologous cells is suggested.

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