FACTORS INFLUENCING THE AGGLUTINABILITY OF RED CELLS. II: THE AGGLUTINATION OF BOVINE RED CELLS PREVIOUSLY CLASSIFIED AS "INAGGLUTINABLE" BY THE BUILDING UP OF AN "ANTI-GLOBULIN: GLOBULIN LATTICE" ON THE SENSITIZED CELLS.

R. R. A. COOMBS, M. H. GLEESON-WHITE AND J. G. HALL.*

From the Departments of Animal Pathology and Pathology in the University of Cambridge.

Received for publication March 19, 1951.

IN a previous paper (Gleeson-White, Heard, Mynors and Coombs, 1950) we reported that random samples of bovine red cells sensitized with either the Paul-Bunnell antibody or with a rabbit or guinea-pig anti-ox red cell serum differed considerably in their susceptibility to agglutination by appropriate anti-globulin sera. The results obtained from the examination of samples of blood from sixteen oxen were such that those oxen could be divided arbitrarily into three groups on the basis of the susceptibility of their red cells to agglutination by that technique.

Although we used the terms "markedly" and "moderately agglutinable" and "inagglutinable" to describe those three classes of bovine red cell, it should be noted that even the "markedly agglutinable" cells were much less sensitive than sheep red cells to agglutination by the Paul-Bunnell antibody.

Simple absorption experiments showed no obvious difference in the amount of Paul-Bunnell antibody taken up by suspensions of the "markedly agglutinable" and "inagglutinable" class of bovine red cell. Similar results were obtained by Boursnell, Moyle and Coombs (unpublished data), who showed that these samples of bovine red cells sensitized with a guinea-pig anti-ox red cell serum adsorbed equal amounts of a rabbit anti-guinea-pig globulin serum marked with radio-active iodine.

Again, the haemolysin titre of any particular serum used for the sensitization of the bovine red cell suspensions was constant irrespective of the class of cell being tested.

There would appear to be no evidence from these experiments that this variation in susceptibility to agglutination was due to any appreciable difference in the number of antigen receptors present in the three classes of bovine red cell.

We came to the conclusion, therefore, that this phenomenon must be due to some variable character in the structure of the wall of bovine red cells which, although permitting an equal degree of sensitization of all three classes of cell, nevertheless inhibits to a greater or lesser extent the agglutination of these cells.

For instance, the majority of the antigen receptors might be situated at different levels in the cell wall of each class of cell. This would not necessarily prevent the easy access of the sensitizing antibody molecules to the corresponding antigen receptors, or the subsequent union of the former with anti-globulin

* Recipient of a grant from the Agricultural Research Council.

antibody molecules. If, however, these antigen receptors were situated at such a depth that even a sensitizing antibody molecule combined with an anti-globulin antibody molecule was of insufficient " length " for the latter to extend beyond the " outer effective limit of the cell wall," the agglutination of such cells might well be prevented.

If this were the case, it should be possible to obtain the agglutination of suspensions of even the so-called " inagglutinable " class of bovine red cell by repeated alternate treatment with anti-globulin serum and globulin. In other words, by the gradual building up of " anti-globulin : globulin lattices " at the sensitized antigen receptor sites, free globulin receptors would eventually appear at the cell surface. The subsequent addition of further anti-globulin serum should then bring about the agglutination of the cells. This hypothesis is presented in diagrammatic form in Fig. 1(*a*) and (*b*).

To test this hypothesis, suspensions of the three classes of bovine red cell were sensitized with the Paul-Bunnell antibody. These cells were then treated alternately with rabbit anti-human globulin serum and human γ-globulin. By this technique a degree of agglutination equal to the haemolytic titre of the sensitizing antibody was eventually obtained with each class of cell. Furthermore, as might be anticipated from the above hypothesis, the variation in susceptibility to agglutination observed in our previous experiments was reflected in the number of treatments required to obtain a similar degree of agglutination with each of the three classes of cell.

Similar results were also obtained when the cells were sensitized with a rabbit or guinea-pig anti-ox red cell serum and treated in the above manner with the appropriate anti-globulin serum and the corresponding γ-globulin fraction or whole serum.

MATERIALS.

The red cell suspensions and the various sera used in these experiments were prepared in the manner described in our previous paper (Gleeson-White *et al.*, 1950).

Oxen 5153, 5156 and 5157 were again used as the source of " markedly " and " moderately agglutinable " and " inagglutinable " cells respectively.

The human γ-globulin used was a Cohn fraction. This, and all anti-globulin sera and normal human, rabbit and guinea-pig sera employed were first absorbed free of antibodies to bovine red cells.

All sera were heated to 56° for half an hour before use.

METHODS.

Agglutination of Bovine Red Cells, Sensitized with the Paul-Bunnell Antibody, by the Building-up of an "Anti-globulin : Globulin Lattice."

The following is a detailed description of this procedure, which is summarized in Fig. 2.

A two-fold serial dilution of a human serum containing the Paul-Bunnell antibody was made in 0·4 ml. amounts of saline, and similar volumes of a 2 per cent suspension of the bovine red cells to be tested added to each tube. After incubation in a water-bath at 37° for half an hour the tubes were centrifuged

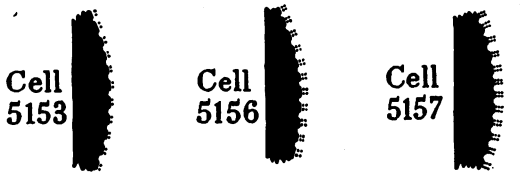


FIG. 1(*a*).— Disposition of antigen receptors beneath the outer effective limit of the cell wall in the three classes of bovine red cells.

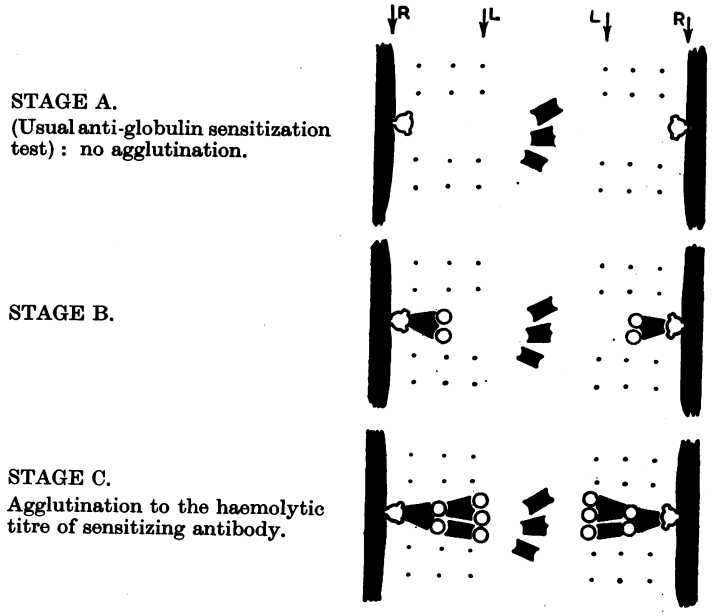
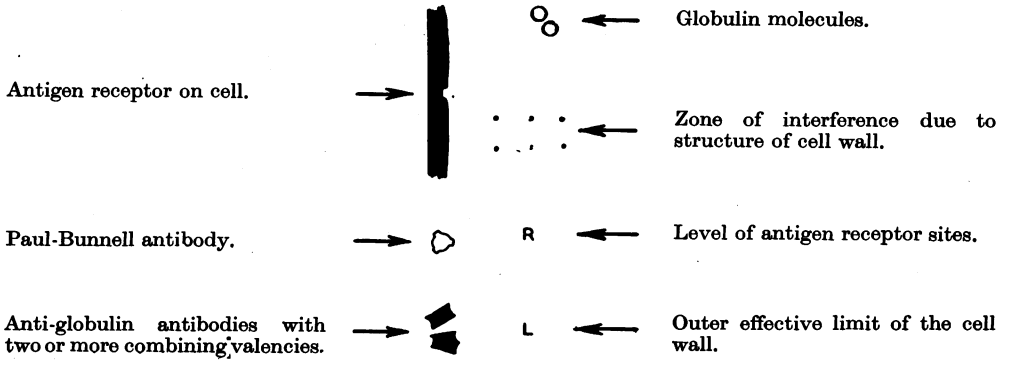


FIG. 1(*b*).—The building up of an " anti-globulin : globulin lattice " on sensitized " inagglutinable " bovine red cells (5157).



Key to figs. 1(*a*) and 1(*b*).

lightly and the supernatant fluid from each tube discarded. The cell deposits were then washed twice in saline and resuspended in 0·4 ml. amounts of saline.

At this point (Stage A in Fig. 2 and Table I) single drops of the washed cell suspensions were mixed on a tile with equal drops of a 1 in 20 dilution of a rabbit anti-human globulin serum. The tile was then rocked gently by hand at room temperature, and the degree of agglutination read at five-minute intervals over a period of half an hour.

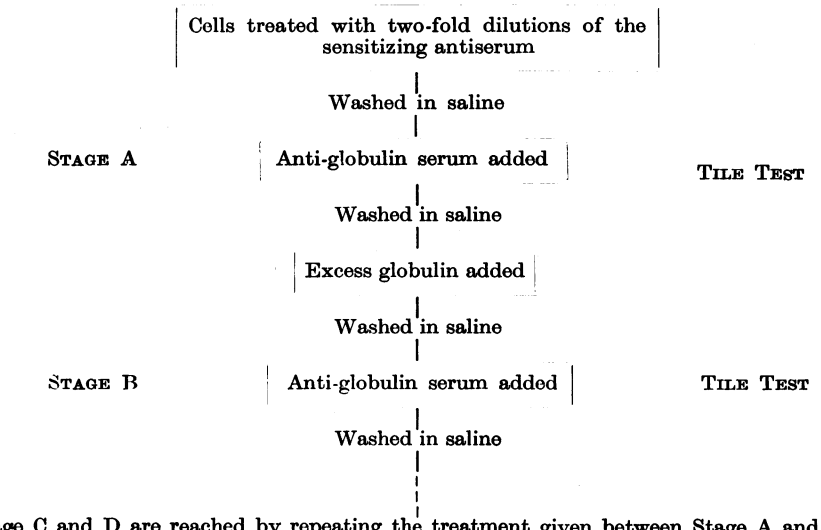
At the same time 0·4 ml. amounts of the same diluted rabbit anti-human globulin serum were added to each tube. After incubation in a water-bath at 37° for twenty minutes the tubes were again centrifuged lightly, the supernatant fluid discarded, and the cell deposits washed twice in saline and re-suspended in 0·4 ml. amounts of saline.

This procedure was then repeated using a 0·14 per cent solution of human γ-globulin instead of the rabbit anti-human globulin serum.

At the completion of this treatment with rabbit anti-human globulin serum and human γ-globulin, another tile test was carried out in the same manner as before (Stage B in Fig. 2 and Table I).

The cell suspensions were then given one or two more treatments with rabbit anti-human globulin serum followed by human γ-globulin, tile tests being carried out at the completion of each treatment (Stages C and D in Fig. 2 and Table I).

FIG. 2.—Summary of the procedure used for the building-up of " anti-globulin : globulin lattices " on sensitized bovine red cells.



Stage C and D are reached by repeating the treatment given between Stage A and B once or twice more respectively.

The only variation in technique as the experiment proceeded was the reduction by 0·05 ml. of the volume of reagents added to the tubes after each tile test to compensate for the volume of cell suspension removed for those tests.

The anti-globulin sensitization tests carried out on tiles at each stage of the experiment were controlled by a duplicate series of tests using a 1 in 20 dilution of normal rabbit serum instead of rabbit anti-human globulin serum.

TABLE I.—*The Agglutination of Suspensions of "Markedly" and "Moderately Agglutinable" and "Inagglutinable" Bovine Red Cells, Sensitized with the Paul-Bunnell Antibody by Repeated Alternate Treatments with a Rabbit Anti-human Globulin Serum and Human γ-globulin.*

Dilutions of sensitizing Paul Bunnell antibody (haemolytic titre (1280).	Cells 5153 "markedly agglutinable." 1:20.	1:40.	1:80.	1:160.	1:320.	1:640.	1:1280	1:2560	1:5120	Saline.	Cells 5156 "moderately agglutinable." 1:20.	1:40.	1:80.	1:160.	1:320.	1:640.	1:1280.	1:2560.	1:5120.	Saline.	Cells 5157 "inagglutinable." 1:20.	1:40.	1:80.	1:160.	1:320.	1:640.	1:1280.	1:2560.	1:5120.	Saline.	Time of reading on tile.
Direct agglutination by sensitizing Antibody	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	5 min.
	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	15 ,,
	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	30 ,,
Agglutination by rabbit anti-human globulin serum following treatment to Stage A (Fig. 2)	++	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	5 ,,
	++	++	+	—	—	—	—	—	—	—											—	—	—	—	—	—	—	—	—	—	15 ,,
	++	++	++	++	—	—	—	—	—	—	++	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	30 ,,
Agglutination by rabbit anti-human globulin serum following treatment to Stage B (Fig. 2)	++	++	++	+	—	—	—	—	—	—	++	++	+	—	—	—	—	—	—	—	+	—	—	—	—	—	—	—	—	—	5 ,,
	++	++	++	++	++	+	w	—	—	—	++	++	++	++	w	—	—	—	—	—	++	++	w	—	—	—	—	—	—	—	15 ,,
	++	++	++	++	++	++	+	w	—	—	++	++	++	++	+	—	—	—	—	—	++	++	++	+	w	—	—	—	—	—	30 ,,
Agglutination by rabbit anti-human globulin serum following treatment to Stage C (Fig. 2)											++	++	++	++	+	w	—	—	—	—	++	++	++	++	w	—	—	—	—	—	5 ,,
											++	++	++	++	++	++	+	—	—	—	++	++	++	++	++	+	w	—	—	—	15 ,,
											++	++	++	++	++	++	++	w	—	—	++	++	++	++	++	++	+	—	—	—	30 ,,
Agglutination by rabbit anti-human globulin serum following treatment to Stage D (Fig. 2)																					++	++	++	++	++	++	+	—	—	—	5 ,,
																					++	++	++	++	++	++	++	w	—	—	15 ,,
																					++	++	++	++	++	++	++	w	—	—	30 ,,
Controls for the above tests in which normal rabbit serum was used in the tile tests instead of rabbit anti-human globulin serum.																															
Agglutination in normal rabbit serum following treatment to Stage B (Fig. 2)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	5 min.
	++	++	w	—	—	—	—	—	—	—	w	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	30 ,,
Agglutination in normal rabbit serum following treatment to Stage C (Fig. 2)											—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	5 ,,
											+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	30 ,,
Agglutination in normal rabbit serum following treatment to Stage D (Fig. 2)																					—	—	—	—	—	—	—	—	—	—	5 ,,
																					+	—	—	—	—	—	—	—	—	—	30 ,,

Key. ++ = very strong agglutination; + = agglutination; w = weak agglutination; — = no agglutination.

RESULTS.

In the first series of experiments suspensions of the three classes of bovine red cell, sensitized with two-fold dilutions of the Paul-Bunnell antibody, were treated alternately with a rabbit anti-human globulin serum and human γ-globulin. The results of these experiments are given in Table I.

It will be seen that, as far as Stage A, this procedure was similar in every respect to the usual anti-globulin sensitization test. No agglutination of cells 5157 was observed at Stage A, but as the experiment proceeded agglutination began to appear, and by the time Stage C was reached it extended well up to the haemolytic titre of the sensitizing Paul-Bunnell antibody.

As observed in our previous paper (Gleeson-White *et al.*, 1950) the sensitization of cells 5153 was detected by the first treatment with anti-human globulin serum. However, the end-point of agglutination of those cells was greatly enhanced by carrying the experiment to Stage B. These experiments again showed that cells 5156 lie between cells 5153 and 5157 in their susceptibility to agglutination.

Similar experiments to those shown in Table I were also carried out with cells 5153, 5156 and 5157 sensitized with either a rabbit anti-ox red cell serum or a guinea-pig anti-ox red cell serum. For the experiments with the rabbit sensitizing antibody a goat anti-rabbit globulin serum and normal rabbit serum were used instead of a rabbit anti-human globulin serum and human γ-globulin. Similarly, with the guinea-pig sensitizing antibody, a rabbit anti-guinea-pig serum and normal guinea-pig serum were used for the building up of the "anti-globulin : globulin lattices." The results of these experiments were similar to those obtained with the Paul-Bunnell antibody. By carrying the experiment to Stage C or D, cells 5157 were again agglutinated to the haemolytic titre of the serum used for sensitization.

A point of interest observed throughout these experiments was that at each stage of the test the agglutination obtained with the anti-globulin serum was greatly reduced or even inhibited by the subsequent treatment with globulin. This inhibition of agglutination was not unforeseen, since, from our hypothesis, the addition of excess globulin after the re-suspension of the cells would be expected to saturate all free receptors of the anti-globulin antibody molecules, and so reduce the degree of agglutination obtained by the previous treatment with anti-globulin serum.

We were also able to demonstrate that the agglutination obtained in these experiments was not due simply to a non-specific adsorption by the sensitized cells of an immune complex of anti-globulin serum and globulin formed during the course of the test. Cells 5157, sensitized with a *rabbit* anti-ox red cell serum, were given repeated alternate treatments with a rabbit anti-*human globulin* serum and *human globulin*; no agglutination was observed. This showed that the anti-globulin serum must combine specifically with the sensitizing antibody molecules on the cells before agglutination can occur.

DISCUSSION.

Unlike the red cells of most animals, bovine red cells are not agglutinated readily by specific antisera. Even when fully sensitized only certain samples can be agglutinated by anti-globulin sera, and then only to a degree far below

that which might be expected from the haemolytic titre of the sensitizing serum.

However, the results of the experiments reported in this paper show that sensitized bovine red cells can be agglutinated by repeated alternate treatment with anti-globulin serum and the appropriate species of globulin or whole serum. Even the class of bovine red cell previously found by us to be inagglutinable in the usual anti-globulin sensitization test is agglutinated up to or beyond the haemolytic titre of the sensitizing serum by this technique.

The mechanism by which these cells are agglutinated is considered to be, *ex hypothesi*, a building up from the sensitizing antibody molecules of " lattices " composed of alternate layers of anti-globulin antibody and globulin.

It should not be assumed, however, that an " anti-globulin : globulin lattice " is necessarily built up in a simple linear fashion from the sensitizing antibody molecule ; this would only be the case if all components were strictly bi-valent. On the contrary, it is far more likely that multi-valency is the general rule, and that the building up of these lattices takes place in the manner suggested in Fig. 1 (*b*).

From this it might be argued that the agglutination of sensitized bovine red cells by this technique is due solely to an increase in the number of free globulin receptors attached to the cells. If, however, this view were correct, then the application of this technique to any sensitized red cell system should enhance the end-point of agglutination by anti-globulin sera. Nevertheless, no such enhancing effect was observed in preliminary trials with rhesus-positive human red cells sensitized with an incomplete anti-D serum.

In our hypothesis we have suggested that the variation in susceptibility of sensitized bovine red cells to agglutination by anti-globulin sera was due to the antigen receptors being situated at different levels in the cell wall of each class of cell. Whether, in fact, these receptors lie within the actual structure of the cell wall or are situated superficially and masked by an unusually powerful field of repellent forces at the cell surface does not disturb this hypothesis. In either case one can still regard these receptors as lying at varying depths beneath the " outer effective limit of the cell wall," which, although permitting the free access of the specific antibody molecules to the corresponding receptors, renders the cells inagglutinable by ordinary methods.

This conception is supported by the results of these experiments, which show that the " anti-globulin : globulin lattices " have to be built up further on cells 5157 than on cells 5153 before the same degree of agglutination is obtained. Cells 5156 were again shown to occupy an intermediate position between the other two classes of cell.

Furthermore, we have confirmed by repeated tests that the degree of susceptibility to agglutination is a constant property of the red cells of individual oxen. An investigation is now being carried out to discover whether this property of bovine red cells is determined genetically or developed at random.

In due course this " anti-globulin : globulin lattice " technique may also prove to be of value in the investigation of many problems in the field of human blood groups.

In our opinion the results of these experiments with sensitized bovine red cells are also of interest since they, in common with the observations of van Loghem, Kresner, Coombs and Roberts (1950) on the prozone phenomenon

exhibited by certain anti-globulin sera, provide fresh, though indirect, evidence in support of the theory of the multivalency of antibodies and the lattice hypothesis of agglutination.

Summary.

Sensitized suspensions of the class of bovine red cell previously classified as " inagglutinable " can be made to agglutinate by repeated alternate treatments with anti-globulin serum and globulin. The other two classes of bovine red cell behave in a similar manner, but fewer such treatments are required to obtain the same degree of agglutination.

These results support our hypothesis that the antigen receptors of the three classes of bovine red cell are situated at different levels in the cell wall, and that the building up of " anti-globulin : globulin lattices " from the sensitizing antibody molecules to the " outer effective limit of the cell wall " would bring about the agglutination of these cells.

We wish to express our thanks to Mr. A. E. Harding of the Department of Animal Pathology for the numerous samples of blood used in these experiments, and to Mr. H. P. Hudson, Mr. S. W. Patman and Miss M. H. Redfarn for preparing and photographing the diagrams in this paper.

REFERENCES.

Gleeson-White, M. H., Heard, D. H., Mynors, L. S., and Coombs, R. R. A.—(1950) *Brit. J. exp. Path.*, **31**, 321.

van Loghem, J. J., Kresner, M., Coombs, R. R. A., and Roberts, G. F.—(1950) *Lancet*, ii, 729.