RED BLOOD-CELL ANTIGENS IN SOME LOWER VERTEBRATES

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INTRODUCTION

Following Landsteiner's discovery of the human ABO blood groups in 1900, many attempts were made to find blood groups in other animals. The investigations were confined mostly to mammals, but Fishbein (1913) made a brief mention of the fact that he had been unable to find blood groups, similar to the human ABO system, in frogs. This was confirmed by Skarzynska (1925), who found no evidence for blood groups in *Rana esculenta*, or in the fish, *Cyprinus carpio*. In addition, she was unable to induce the production of antibodies in either species by injecting red cells from another animal of the same species.

Antigens similar to the human A and B antigens are known to be of widespread occurrence among both plants and animals. Cushing & Sprague (1952) found an antigen, similar to the human B antigen in the fish *Genyonemus lineatus*, but not in *Cymatogaster aggregata*, which may however possess the Forssman antigen; species antibodies for both species were found in the human sera used for the tests. An antigen, identical with the human B antigen, is said to have been found in frogs by Japanese workers (Furuhata, 1947). Toads, but not frogs, have the Forssman antigen (Boyd, 1939).

The methods used in these early attempts to find blood groups in Amphibia and fish are rather dubious, since they are not fully described by the authors. It was decided to see if blood groups could be found among these animals using the human blood-grouping techniques. The reactions of the cells of several Amphibia with human sera were also investigated.

The notation for the human sera used in this paper is that proposed by Ford (1955).

METHODS

Taking the blood. (1) *Amphibia.* The most satisfactory method was to decapitate the animal and let the blood drip from the neck into a sterile bottle. The average amount so obtained from frogs was about 1 ml. If the red cells only were required, some anticoagulant (either acid citrate dextrose or heparin in the same concentrations as used for human cells) was put into the bottle before the blood was added. The blood was stored at 4° C. (2) *Fish.* The fish were much easier to bleed, due to the larger size of the heart. A heart puncture with a syringe usually yielded 1–3 ml.

of blood, depending on the size of the fish. The blood was treated in the same way as the amphibian blood.

Serum. When the serum was required, the whole blood was allowed to clot and was stored at 4° C. overnight to let the serum separate from the clot. The serum was then centrifuged to remove the free cells and stored at −15° C.

Cells. All the cells, whether obtained from a clot or from a sample of citrated or heparinized blood, were treated in the same way. They were washed three times in isotonic saline and then approximately 2 % suspensions of cells in saline were made.

Frog cells seemed to be preserved equally well in 0·9 % or 0·65 % saline, the former being the concentration used for human cells, and the latter being isotonic with their body fluids; either concentration was used for the suspensions. The fish cells were not so tolerant. 1 % saline was used; this seemed an average value since marine teleosts vary considerably in the salt content of their blood, e.g. between 0·10 M-NaCl and 0·26 M-NaCl (Green & Hoffman, 1953).

The cell suspensions were stored at 4° C. At this temperature they remained in a good condition for at least 4 days. Suspensions which had haemolysed were not used in the tests.

Tests. All the tests were done in precipitin tubes (2 by $\frac{1}{4}$ in.). Equal volumes (approximately 0·01 ml.) of cell suspension and serum were put into the tubes with a Pasteur pipette. After shaking the tubes to mix the cells and serum, they were left for 1–2 hr. The mixture was then placed on a slide and examined microscopically for agglutination.

In contrast to its effect on human antibodies, temperature seems to have little effect on the activity of animal antibodies. Therefore, as serum was scarce, the tests were incubated only at room temperature, which was 20 ± 4° C. The only exceptions were tests with anti-D(A), which were incubated at 37° C. for 2 hr.

Absorptions. The following method was used to remove an antibody from a sample of serum. Equal volumes of packed washed cells and serum were mixed and left for 1 hr. at an appropriate temperature, e.g. 4° C. for anti-G(A) and anti-G(B), etc. The mixture was centrifuged and the serum recovered. This was repeated with a fresh sample of the same cells, three or four times, until the antibody was completely removed.

Titrations. The amount of antibody agglutinating the cells may be determined by titration. Serial dilutions, in saline, from 1 in 2 to 1 in 1024, of the serum were made. The cells were then tested against the dilutions of serum in the way described for the other tests.

Temperature of frogs. It should be mentioned that before the frogs were bled, they were kept for at least 7 days at 20° C. The production of antibodies in a high titre in cold-blooded vertebrates is related to the external temperature. Frogs, injected with human red cells, and kept at 8–10° C. produced no antibody, while those kept at 22–27° C. produced a haemolytic antibody (Allen & McDaniel, 1937). The same is true of the production of antibodies to bacterial antigens in both fish and frogs (Bisset, 1947). After a week at 20° C., the titre of any iso-antibodies that may have been present should have increased.

RESULTS

The first experiments were to discover if the individuals within a species of Amphibia or fish can be divided into groups according to differences in their red-cell antigens and naturally occurring iso-antibodies. This involved testing the cells of each animal against the sera from as many other animals of the same species as possible. About sixty frogs (*Rana temporaria*), forty-three whiting (*Gadus merlangus*), thirteen pouting (*G. luscus*) and twelve plaice (*Pleuronectes platessa*) were used for these tests. No agglutination of red cells by the sera was observed, and so there was no evidence from these tests for the occurrence of blood groups in these species.

These results indicate that blood systems, comparable to the human ABO system, are not found in these species of Amphibia and fish. Since there were no reactions, there is no evidence for the presence of either antigens or iso-agglutinins. But the results do not exclude the existence of a blood-group system (similar to the human Rh system), in which antibodies are not present unless the animal has been injected with cells of another group. This possibility was investigated by injecting a series of frogs with red cells of other frogs.

Two batches of frogs, one from Cornwall and the other from near Oxford, were used. It was thought that there was a greater chance of finding antigenic differences if animals from different areas were used, since blood-group frequencies may vary considerably between different areas. A number of animals from each batch was bled and the cells made up into 2% suspensions in 0·65% saline. The surviving frogs were given injections of cell suspension into the dorsal lymph sac; each Cornish frog was injected with 1 ml. of suspension from an Oxford frog, and each Oxford frog with ½ ml. of suspension from a Cornish frog. A total of four injections on successive days were given. The animals were then left for 14 days after the last injection before being bled. Throughout the period of the injections, the frogs were kept at 21° C., for the reasons stated earlier.

Eleven samples of sera were collected from the injected frogs. These were tested against the cells of the same eleven animals and several more, making a total of twenty-four frogs. No agglutination was recorded, suggesting that no immune antibodies had been produced.

There remained the possibility that incomplete antibodies were present. Several tests have been devised for incomplete antibodies, but only the albumin method was used for the frog cells. The red cells were suspended in 20% bovine albumin and these suspensions tested against the sera in the same way as the saline suspensions. No reactions were recorded. This strongly suggested that no incomplete immune-antibodies were present in the serum of the frogs which had been injected, and also that there were no naturally occurring incomplete iso-antibodies, since these, too, would have agglutinated the cells under these conditions.

The cells of about eighty frogs (*Rana temporaria*) were tested with human anti-G (A), anti-G(B), anti-G(A+B) (i.e. anti-A, anti-B and anti-A+B) and AB serum. The cells from every animal were agglutinated by all the sera, but the reaction was noticeably stronger with the sera containing anti-G(B). This was verified by

titrating the sera against the cells. With anti-G(A) and AB serum, a reaction was recorded to dilutions of about 1 in 8 to 1 in 16, but with anti-G(B) and anti-G (A + B), the reactions continued to dilutions of 1 in 256. Since AB serum, which contains neither anti-G(A) nor anti-G(B), agglutinates the cells, it seems probable that a species antibody, i.e. a human anti-frog antibody, is causing the agglutination. The same explanation will apply to the agglutination with anti-G(A); the titrations show that the strength of the antibody concerned in the reaction with anti-G(A) and AB serum is similar. The reactions with anti-G(B) and anti-G(A + B) are probably due in part to the species antibody, since this should be present in all human sera, but also to anti-G(B) reacting with an antigen similar to the human B antigen.

Anti-G(A) and anti-G(B) were absorbed with human A and B cells respectively, and then titrated against frog cells. The results with absorbed anti-G(A) were similar to those with unabsorbed anti-G(A), but with anti-G(B), the reaction now ceased at dilutions of 1 in 8 to 1 in 16, compared to 1 in 256 with unabsorbed serum. The agglutination occurring with absorbed anti-G(B) was probably due entirely to the species antibody; this shows that frog cells are agglutinated by the anti-G(B) in the unabsorbed serum.

These results were confirmed by absorbing anti-G(A), anti-G(B) and anti-G (A + B) with frog cells, pooled from several animals. The absorbed sera were then tested against human A_1, A_2 and B cells. The anti-G(A) and anti-G(A + B) agglutinated the A_1 and A_2 cells, but the anti-G(B) and anti-G(A + B) no longer agglutinated the B cells. Therefore, frog cells remove anti-G(B) from the human serum and so must possess a B antigen. Since the frog cells have absorbed the anti-G(B) completely, the frog B antigen must be very similar to the human B antigen.

A similar series of tests was done with the cells from twenty-five toads (*Bufo bufo*), twenty-five tree frogs (*Hyla arborea*) and seventeen newts (*Triturus cristatus*). The results with the toad and tree frog cells were very similar to those with frog cells. When the sera were titrated against the cells, reactions were still apparent with anti-G(B) and anti-G(A + B) at much greater dilution, than with anti-G(A) or AB serum. Similarly, the reactions with anti-G(B) absorbed with human cells, were very like those with anti-G(A). Thus it appears that both toads and tree frogs have a B antigen.

It was not possible to verify the results for tree frogs by absorbing human sera with their cells, because the animals have so little blood. However, anti-G(A), anti-G(B) and anti-G(A + B) were absorbed with toad cells. When these absorbed sera were tested against human cells, the cells were still agglutinated by the appropriate serum. The sera were titrated and tested against the cells to see if the toad cells had reduced the titre of the antibodies. The results indicated that the titre of both anti-G(A) and anti-G(B) had been reduced from 1 in 256 to 1 in 8, so that neither antibody had been completely eliminated. The most probable explanation for the result with anti-G(B) is that the toad B antigen is not identical with the human B antigen, and so removes only part of the antibody. The result with anti-G(A) is more difficult to explain. It is known that toads possess the Forssman

antigen; if cells possessing this antigen are injected into a rabbit, lysins for sheep cells are produced. The Forssman antigen is similar to the A antigen and is known to reduce the titre of human anti-G(A). Thus, it is probably responsible for the reduction of the titre of anti-G(A) observed after absorbing with toad cells.

The results with the newt cells were rather different. The cells of each animal were agglutinated to the same extent by the serum of each of the four ABO groups. The reactions with anti-G(A) and anti-G(B), absorbed with human cells, were similar to those with unabsorbed anti-G(A) and anti-G(B). This suggests that newts have neither an A nor a B antigen, but that the cells are being agglutinated by a species antibody.

About forty suspensions of frog cells were tested against human anti-D(A), anti-Ag(M) and anti-Ag(N). With anti-D(A), an agglutination comparable to that with anti-G(A), was obtained; this was possibly due to the human anti-frog antibody. Anti-Ag(M) and anti-Ag(N) did not agglutinate the cells. These results are interesting because the anti-D(A) serum is human, but anti-Ag(M) and anti-Ag(N) sera are made in rabbits. Therefore, there is a human anti-frog antibody, but not a rabbit anti-frog antibody.

DISCUSSION

The results described agree with the results of previous workers that blood groups cannot be found in Amphibia and fish. Fishbein (1913) makes no mention of the species (which was probably *Rana pipiens*), number of frogs involved, or the methods used. Similarly, Skarzynska (1925) gives few details, except that the results were the same, whether the tests were done at room temperature or 6–7° C. She remarks that all the tests were performed in winter, which means that the titre of any antibodies present in the serum would be exceedingly low. By keeping the frogs at 20° C. for a week before bleeding them, the titre of any antibodies present in the serum should have increased sufficiently for a strong reaction to occur with the cells. However, no iso-agglutinins appear to be present in the frog serum. The number of frogs used is too small to exclude completely the possibility that blood groups occur in the Amphibia, especially since the frogs all came from Cornwall. But, if a dimorphism does occur among frogs, all those used must have belonged to one group, so the frequency of the other group must be low. However, if the less frequent group includes only 1 % of the population, this frequency is too high to be maintained in the population by recurrent mutation and so would indicate a true polymorphism.

Similarly, only small numbers of fish were used, and so again the possibility of blood groups is not excluded. But, as in the case of the frogs, the other groups would have a low frequency.

Unfortunately, it was only possible to inject a few frogs and so there remains a chance that red-cell antigen differences may still be found by this method. It was hoped that under the experimental conditions any incompatibility between the cells of the donor and those of the injected frogs would produce a reaction. The conditions of the earlier attempt to induce the production of antibodies in frogs and fish (Skarzynska, 1925) were not so favourable, as each frog had a single injection of

0·5 ml. of a 10% suspension of cells and the experiment was performed in winter, which suggests that the animals were not at a sufficiently high temperature to produce antibodies.

Skarzynska concludes that frogs and fish are unable to produce antibodies, but there is evidence that frogs (*R. pipiens* and *R. catesbeiana*) will produce a haemolytic antibody to human red cells, although only if kept at a high temperature (Allen & McDaniel, 1937). The production of antibodies to bacterial antigens is a similar process; it was found that both fish and frogs would produce antibodies to bacterial antigens if kept at 20° C. (Bisset, 1947). So, in Amphibia or fish, if there are antigenic differences between the red cells of the different animals of one species, antibodies should be produced after injection provided that the animals are kept under the appropriate conditions. The only conclusion to be drawn from the experiments here described is that all the frogs used had the same antigens on their red cells.

The tests with human sera should be mentioned. Frogs, toads and tree frogs appear to have a B antigen, whilst newts probably do not. This fact is interesting, since the frogs and toads belong to the Anura and the newts to the Urodela. These two groups diverged very early in the history of the Amphibia. Similarly, in determining the relationships of the Amphibia as shown by the serum antigens, Boyden & Noble (1933) found a closer relationship among the members of the Anura, than between the Anura and Urodela.

The B antigens possessed by the frog and toad are not identical. The human B antigen has three components, B_1, B_2 and B_3, and animal B antigens may possess one, two or all of these components, e.g. the rabbit has B_2 and B_3 (Friedenreich & With, 1933). Frog cells absorb all the anti-G(B) from the human serum, and so must contain the same components as the human B antigen; this agrees with the results of the Japanese workers (Furuhata, 1947) but no mention is made of the species of frog used for their experiments. On the other hand, toad cells do not absorb the antibody completely and probably lack one or more of the components. Anti-G(B) was not absorbed with tree frog cells and the similarity of their antigen to the human antigen cannot be assessed.

The only example of blood groups in cold-blooded animals appears to be in two species of turtle, *Chrysemys picta belli* and *Chelydra serpentina* (Bond, 1940). Forty-seven *Chrysemys* were tested and these formed three groups; only five *Chelydra* were used. The tests were repeated with several other species of turtle and also with snakes (Bond, 1939), but there was no evidence for blood groups. It may appear strange that the groups are confined to only two species of reptile among those studied, but as yet there is no evidence that *all* the species within a single vertebrate class either have, or have not, blood groups, that all species have blood groups, or that no species have blood groups. For example, whilst most mammalian species possess blood groups, there is no evidence, so far, that they occur in the guinea-pig (Boyd & Walker, 1934).

The evidence at present suggests that blood groups may have started to occur in the reptiles and so their possession is confined mainly to the warm-blooded animals, i.e. birds and mammals. Although the significance of the blood group antigens is

unknown, they are important in producing haemolytic disease of the newborn. The naturally occurring disease is confined to mammals, but it can be induced artificially in chicks (Briles, 1948). The reason why blood groups should be confined to these classes of animals is not at present clear.

SUMMARY

1. It was not possible to find evidence for blood groups in frogs (one species) or in fish (three species).

2. Frogs could not be induced to make antibodies after injection with red cells from another frog.

3. Frogs, toads and tree frogs have a B antigen; newts probably have not.

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