An immunofluorescent study of the haemoglobins in metamorphosing *Xenopus laevis*

By RICHARD D. JURD and NORMAN MACLEAN

From the Department of Zoology, The University, Southampton

It has long been known that in many vertebrates different haemoglobins exist in foetal and adult animals, and that during development the circulating foetal haemoglobin is gradually replaced by haemoglobin of the adult type, (reviewed by Gratzer & Allison, 1960). The phenomenon is well established in the bullfrog, *Rana catesbiana* (McCutcheon, 1936; Riggs, 1951; Baglioni & Sparks, 1963; Moss & Ingram, 1968a, b). Our studies on haemoglobin from the South African clawed toad, *Xenopus laevis*, employing column chromatography on carboxymethylcellulose, and polyacrylamide-gel disc electrophoresis, demonstrate a similar situation. Column chromatography reveals two tadpole haemoglobin peaks and three adult peaks, with no peaks common to the tadpole and the adult (Maclean, Brooks & Jurd, 1969; Jurd & Maclean, 1969).

It would be of great interest to know whether the changeover from tadpole to adult haemoglobin occurs within individual red blood cells, or whether separate populations of blood cells exist, each exclusively containing one or other of the two types of haemoglobin. If the first theory is correct, some cells would be expected to pass into circulation possessing haemoglobins of both foetal and adult types. If separate populations of cells are involved, the changeover will be due to a continuing release into the circulation of new blood cells containing only adult haemoglobin, which gradually replace the older cells containing only foetal haemoglobin.

This question has been posed by other workers investigating the replacement of foetal haemoglobin by adult, in man. Kleihauer, Braun & Betke (1957) removed adult haemoglobin from human cord blood cells by incubation in acid buffer, followed by eosin staining of the more acid-resistant foetal haemoglobin persisting in the cells. Erythrocytes staining with intermediate density were identified as those originally possessing both foetal and adult haemoglobins. Hosoi (1965) and Dan & Hagiwara (1967) have both employed immunofluorescent techniques to determine the type of haemoglobin possessed by individual human erythrocytes, both concluding that some cells contain both types of haemoglobin. However, the best evidence for two types of haemoglobin co-

1 Authors' address: The Department of Zoology, The University, Southampton, SO9 5NH, England.
existing in the same cell is provided by the observations of Schneider & Haggard (1955) on blood from babies carrying the gene for sickle-cell anaemia. In blood samples containing 20% foetal haemoglobin, they report that 'virtually all' cells can be induced to sickle, indicating that both the foetal haemoglobin and the sickle form of adult haemoglobin are present together in at least some of the cells.

In animals other than man, little evidence is available on this topic. Studies on amphibian metamorphosis by Jordan & Spiedel (1923) and by Moss & Ingram (1965) point to the possibility of exclusive production of tadpole and adult haemoglobins by separate cell lines derived from different erythropoietic sites.

To investigate this problem we prepared antibodies against *Xenopus* adult haemoglobin and *Xenopus* foetal (tadpole) haemoglobin, labelled them with fluorochromes, and used them to determine which type of haemoglobin was present within each blood cell, attempting to discover if any cells contained both types of haemoglobin. In our experiments the haemoglobins from tadpoles elute from our chromatography columns close together; they are grouped together and styled *Xenopus* foetal haemoglobin, ‘*Xenopus-HbF*’. The adult haemoglobins, also eluting close together, are similarly pooled and styled *Xenopus* adult haemoglobin, ‘*Xenopus-HbA*’.

**MATERIALS AND METHODS**

*Preparation of antibodies*

Antibody was prepared against *Xenopus-HbA* by modifying a technique successfully used by Dan & Hagiwara (1967) to raise antibodies against foetal and adult human haemoglobins.

Erythrocytes from mature adult *Xenopus* were lysed by osmotic shock in distilled water. The resulting haemoglobin solution was used as the antigen, its concentration being determined by comparison with cyanmethaemoglobin standards. 40 mg of this *Xenopus-HbA* in 2 ml water was emulsified with an equal volume of complete Freund’s Adjuvant (Difco Laboratories) and was injected subcutaneously into a female New Zealand White rabbit, weight *ca.* 3 kg. 10 days later 20 mg of *Xenopus-HbA*, precipitated with potassium aluminium sulphate (Proom, 1943) and resuspended in 1 ml 0.85% saline, was injected into the gluteus maximus muscle. This intramuscular dose of 20 mg alum-precipitated haemoglobin was repeated 11 times at 3-day intervals. 14 days after the last intramuscular injection 100 mg alum-precipitated *Xenopus-HbA* was injected intraperitoneally, and on each of the two succeeding days 10 mg of the antigen in aqueous solution, of a concentration of 20 mg/ml, were given intravenously. 7 days later the rabbit was bled by superficial venesection of the ear.

The serum was separated from the blood cells by allowing it to stand overnight at 2°C, and the gamma-globulin component was then isolated by adsorbing
the unwanted plasma proteins on to Whatman 'Chromedia DE52' diethylaminoethyl cellulose (Stanworth, 1960). Some of the gamma G-globulin was conjugated with fluorescein iso-thiocyanate (FITC), and the rest with the sulphophenyl chloride of lissamine rhodamine B (RB200SC), following the methods recommended by Nairn (1964). In an endeavour further to purify the conjugates and reduce the risk of non-specific fluorescence, each ml of conjugate was treated with 60 mg acetone-dried pig-liver powder (Burroughs Wellcome) for 30 min (Curtain, 1958). Sodium azide (0·15 %) was added to the conjugates as a bacteriocidal preservative before they were stored in small batches at −20 °C.

A similar scheme to that outlined above was used to prepare antibody against Xenopus-HbF, but as the amount of haemoglobin which can be obtained from tadpoles is very much less than that obtainable from adult toads, a number of modifications were necessary.

Haemolysates of Xenopus-HbF were prepared by lysing erythrocytes from young (legless) tadpoles in distilled water. The concentrations of the haemolysates were again determined by comparison with cyanmethaemoglobin standards. Antibody was prepared in a small male Hartley albino guinea-pig, weight ca. 400 g. The immunization schedule exactly followed that previously described for the rabbit against Xenopus-HbA, with the exceptions that only one-tenth of the amount of antigen was given for each injection, and the final two intravenous injections were omitted. 9 days after the last, intraperitoneal, injection of 10 mg alum-precipitated Xenopus-HbF the guinea-pig was anaesthetized with diethyl ether and sodium pentobarbitone, and was bled by heart puncture. 22 ml of blood were obtained.

The gamma G-globulin from the blood of the guinea-pig was separated and purified, and was then conjugated with FITC, all as described above for the rabbit. Possible impurities were again adsorbed on pig-liver powder before storage.

Tests for the specificity of the antisera

Precipitin-ring tests were carried out using the fluorochrome-conjugated gamma G-globulin from the rabbit and the Xenopus-HbA antigen. Precipitates were formed within 2 min with the antigen at a dilution of 1/16 of its original concentration of 20 mg/ml. No precipitates were formed when Xenopus-HbF was used. Precipitin-ring tests with the FITC-conjugated gamma G-globulin from the guinea-pig gave the reverse results: precipitates appeared with Xenopus-HbF at 1/16 its original concentration of 2 mg/ml, but no precipitates were formed with Xenopus-HbA.

This apparent specificity of the antibodies was confirmed using Ouchterlony agar-gel diffusion plates (Ouchterlony, 1949). Two precipitin lines appeared between the rabbit gamma G-globulin and the Xenopus-HbA, and between the guinea-pig gamma G-globulin and the Xenopus-HbF in all the plates made;
these lines were not detectable between the rabbit antiserum and the Xenopus-HbF, or the guinea-pig antiserum and the Xenopus-HbA.

Tests were now conducted using the antiserum on Xenopus blood cells. Blood cells from tadpoles and mature adult toads respectively were washed in Rugh's amphibian Ringer solution (Rugh, 1962), and were then smeared on to slides. After drying, the slides were soaked for 1 h in 0.85 % saline buffered with 0.01 M-sodium phosphate at pH 7.1 (phosphate-buffered saline). One drop of the fluorescent gamma-globulin solution was placed over the cells and was allowed to interact with them and their contents for 30 min. The smears were then thoroughly washed for at least 2 h in phosphate-buffered saline to remove surplus fluorescent protein before mounting in glycerol buffered at pH 7.1.

The antibody-treated blood smears were viewed through a Wild M 20 microscope under bright and dark field ultraviolet illumination at wavelength 360 nm from a Wotan HBO 200 W mercury lamp.

As reported previously (Jurd & Maclean, 1969), when cells taken from adult Xenopus were treated with the anti-Xenopus-HbA antibody prepared in the rabbit and conjugated with FITC, they were seen to fluoresce very brightly. The fluorescent antibody was taken into the cytoplasm where it was visible as a bright green ring surrounding a dark nucleus (Fig. 1 A). Similarly treated cells from young tadpoles showed only a very dim fluorescence over their whole surfaces, and their nuclei were almost indistinguishable (Fig. 1 B). If artificial mixtures were prepared containing known proportions of adult and tadpole blood cells respectively, treatment with the rabbit anti-Xenopus-HbA conjugate resulted in a proportion of cells fluorescing which was identical to the known proportion of adult cells present. Antibody conjugated with RB200SC gave similar results to the FITC conjugate, although the orange fluorescence was much dimmer.

Results which were exactly the reverse of those given above were obtained when the FITC-conjugated anti-Xenopus-HbF antibody, prepared in the guinea-pig, was used. Tadpole cells fluoresced brightly (Fig. 1 C), but not adult cells, (Fig. 1 D), while the percentage of cells fluorescing in adult-tadpole artificial mixtures corresponded with the percentage of tadpole cells present.

From these results we concluded that we had raised an antibody in the rabbit

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**Figure 1**

(A) Red blood cells from a mature adult *Xenopus*, treated with anti-*Xenopus*-HbA-FITC, showing fluorescence.

(B) Red blood cells from a young *Xenopus* tadpole treated with anti-*Xenopus*-HbA-FITC; fluorescence is absent.

(C) Red blood cells from a young tadpole treated with anti-*Xenopus*-HbF-FITC, showing fluorescence.

(D) Red blood cells from a mature adult treated with anti-*Xenopus*-HbF-FITC; fluorescence is absent.
which was specific against *Xenopus-HbA*. This is called anti-*Xenopus-HbA-FITC*, or anti-*Xenopus-HbA-RB200SC*, according to whether it is conjugated with FITC or RB200SC respectively. Similarly the antibody prepared in the guinea-pig and conjugated with FITC, which was shown to be specific against *Xenopus-HbF*, was styled anti-*Xenopus-HbF-FITC*.

**Use of the antibodies on blood cells from metamorphosing Xenopus**

(i) ‘*Indirect method*’. *Xenopus* in various stages of metamorphosis were measured mouth to anus, bled, and pairs of blood smears were made from each animal. One smear from each pair was treated with anti-*Xenopus-HbF-FITC*, the other with anti-*Xenopus-HbA-FITC*. The smears were viewed by u.v. light and the proportion of fluorescing cells on each slide was determined.

(ii) ‘*Direct method*’. Attempts were also made to ‘double label’ blood smears with both antibodies. A smear of blood cells from a metamorphosing toad was dried and then soaked in phosphate-buffered saline for 1 h. The cells were first treated with anti-*Xenopus-HbF-FITC* for 30 min and were then rewashed in phosphate-buffered saline for 2 h. This was followed by treatment with anti-*Xenopus-HbA-RB200SC* for 45 min before a final 2 h wash in phosphate-buffered saline and mounting.

![Graph](image)

**Fig. 2.** Percentages of red blood cells fluorescing from toads in various stages of metamorphosis, when two identical blood smears from each animal were treated with anti-*Xenopus-HbF-FITC* and anti-*Xenopus-HbA-FITC* respectively. Animals were standardized by size. The animals’ ages at a given size vary according to their feeding regime and the time of the year: thus the ages on the figure are only approximate.
FIGURE 3

(A) Blood cell smear from a metamorphosing toad, 18 mm mouth to anus, treated with anti-Xenopus-HbF-FITC. Note the contrast between fluorescing and non-fluorescing cells.

(B) Smear of blood cells from a metamorphosing toad, 18 mm mouth to anus, treated with anti-Xenopus-HbA-FITC. Note the contrast between fluorescing and non-fluorescing cells.
RESULTS

When blood smears from metamorphosing toads were treated with the fluorescent antibodies it was found that some cells fluoresced very strongly, whilst fluorescence was absent in others. A small proportion of cells exhibited fluorescence of intermediate brilliance.

The percentages of cells fluorescing when two identical blood cell smears from various toads of known length were treated with anti-Xenopus-HbF-FITC and anti-Xenopus-HbA-FITC respectively are shown in Fig. 2. It will be seen that before the toad reaches 12 mm mouth to anus length, at least 99 % of the cells fluoresce with anti-Xenopus-HbF FITC, but less than 1 % with anti-Xenopus-HbA FITC. This suggests that almost all the cells contain tadpole haemoglobin, and almost none adult haemoglobin at this stage. In a mature adult animal of 60 mm mouth to anus length 100 % of the cells show fluorescence specific for adult haemoglobin, but only 1 % for tadpole haemoglobin. During metamorphosis the proportion of cells fluorescing with anti-Xenopus-HbA-FITC gradually increases from 5 % in 12 mm toads which have just lost their tails, to reach 98 % at 24 mm length. Meanwhile the number of cells fluorescing with anti-Xenopus-HbF-FITC decreases from 95 to 2 %.

During the early stages of metamorphosis there is a considerable overlap in the percentages of cells staining with each antibody; the overlap becomes less marked as metamorphosis proceeds. Thus during early metamorphosis some cells must be capable of being stained with both fluorescent antibodies, suggesting that they contain both Xenopus-HbA and Xenopus-HbF.

Fig. 3 illustrates blood smears from a toad of 18 mm length. The cells in Fig. 3A are treated with anti-Xenopus-HbF-FITC, those in Fig. 3B with anti-Xenopus-HbA-FITC. The presence of fluorescing and non-fluorescing cells can be clearly seen.

‘Double labelling’ treatment of one blood smear with anti-Xenopus-HbF-FITC and anti-Xenopus-HbA-RB200SC in turn shows that some cells exhibit orange fluorescence, indicating the presence of Xenopus-HbA, some cells have green fluorescence indicating the presence of Xenopus-HbF, while some cells show both colours, indicating the presence of both haemoglobins within one cell. In examining double-labelled cells, we endeavoured to separate the two colours by the use of appropriate filters, but frequently suspected that the brilliance of the fluorescein staining masked the rather weak rhodamine fluorescence. The proportions of cells found to contain each type, or both types, of haemoglobin respectively agree fairly well with the proportions found using the alternative ‘indirect method’ described above, and shown in Fig. 2. However, we believe that the so-called ‘indirect method’ is a much more reliable way of analysing the situation.
DISCUSSION

The successful preparation of fluorochrome-labelled antibodies against *Xenopus* adult and tadpole haemoglobins has provided a powerful tool for scrutinizing the changeover from tadpole to adult haemoglobin during metamorphosis. It had previously been noted (Jurd & Maclean, 1969) that the number of cells containing *Xenopus*-HbA during metamorphosis was rather greater than might be expected from the proportions of *Xenopus*-HbA actually present in the haemolysates as revealed by chromatography, assuming that any one cell contained adult or tadpole haemoglobin, but not both. At that time an anti-*Xenopus*-HbF antibody had not been prepared. Our observations suggested that some cells contained less than their full complement of *Xenopus*-HbA, either because they were young, or because they contained *Xenopus*-HbF as well. The latter explanation appears now to be the correct one.

The presence of cells with both *Xenopus*-HbA and *Xenopus*-HbF, in circulation during metamorphosis, renders very improbable any explanation of the haemoglobin switch in terms of different cell populations. That the main site of erythropoiesis does shift from one organ to another during development seems beyond dispute (Jordan & Spiedel, 1923; Moss & Ingram, 1965), but our experiments suggest that this shift of site of cell production may not be the mechanism affecting the change from foetal to adult haemoglobin in circulation.

Apart from the question of whether the control is at the transcriptional or translational level, the following possibilities would appear to exist for a cell ultimately possessing both *Xenopus*-HbF and *Xenopus*-HbA. (a) Both *Xenopus*-HbF and *Xenopus*-HbA were synthesized together from the inception of synthesis; (b) synthesis of *Xenopus*-HbF preceded, and has been replaced by, synthesis of *Xenopus*-HbA; (c) synthesis of *Xenopus*-HbF preceded synthesis of *Xenopus*-HbA, but latterly both are made together.

It is pertinent to mention that we have recently artificially induced adult *Xenopus* to recommence synthesis of an apparently foetal haemoglobin (N. Maclean & R. D. Jurd, in preparation), suggesting that the multipotential character of the blood cell line during metamorphosis persists into adult life.

SUMMARY

1. Separate haemoglobins, detectable by column chromatography and disc electrophoresis, are found in *Xenopus laevis* adults and tadpoles respectively.
2. During metamorphosis a progressive changeover from tadpole to adult haemoglobin occurs in the blood.
3. Fluorochrome-labelled immunoglobulins were prepared against the adult and the tadpole haemoglobins respectively; both were shown to act specifically against their antigens.
4. The fluorescent antibodies were used on smears of red blood cells taken...
from metamorphosing toads. This showed that up to 25% of the blood cells contained both adult and tadpole haemoglobins.

5. The number of cells containing both types of haemoglobin decreased as metamorphosis proceeded.

6. The results suggest that the changeover from tadpole to adult haemoglobin occurs within the one cell line, and does not result from two populations of cells exclusively synthesizing each of the two haemoglobins.

RÉSUMÉ

Une étude par immunofluorescence des hémoglobines au cours de la métamorphose de Xenopus laevis

1. Des hémoglobines distinctes et discernables par chromatographie sur colonne et par électrophorèse en disque, ont été trouvées respectivement dans des adultes et des têtards de Xenopus laevis.

2. Au cours de la métamorphose on assiste à un virage progressif de l’hémoglobine du têtard à l’hémoglobine adulte dans le sang.

3. Des immunohémoglobines marquées par fluorochrome ont été préparées respectivement vis à vis des hémoglobines de l’adulte et du têtard; les deux ont montré une action spécifique vis à vis de leur antigène.


5. Le nombre de cellules contenant les deux types d’hémoglobines a décru au fur et à mesure de la progression de la métamorphose.

6. Les résultats suggèrent que le changement de l’hémoglobine du têtard à celle de l’adulte procède dans la même lignée cellulaire, et n’est pas dû à l’existence de deux populations dont chacune synthétiseraient exclusivement l’un des deux types d’hémoglobine.

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REFERENCES


Haemoglobins and metamorphosis


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