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Feedback inhibition of erythropoiesis induced in anaemic *Xenopus*

By VASSILIKI ALEPOROU[1] AND NORMAN MACLEAN[1]
From the Department of Biology, Southampton University

SUMMARY

Serum from normal *Xenopus*, when injected into anaemic *Xenopus*, causes reduction in both DNA and protein synthesis in erythroid cells as indicated by *in vitro* culture of the blood cells. Experiments with erythrocyte-conditioned medium, reveal that this inhibitory substance can be recovered from mature erythrocytes. Sephadex G-25 fractionation of normal serum and haemolysate demonstrates that the inhibitory factor consists of molecules of low molecular weight, and experiments with cells of *Xenopus* kidney reveal that the feedback inhibition may be tissue specific to erythroid cells.

INTRODUCTION

There is now considerable evidence which supports the view that, in mammals, mature red cells release factors which regulate erythropoiesis by feedback inhibition (Rytomaa & Kiviniemi, 1968; Kivilaasko & Rytomaa, 1970, 1971; Bateman, 1974; Lord *et al.* 1974; Cole & Regan, 1977).

Some of the experiments involved in these studies can be criticized either because extraneous cytotoxic effects had not been effectively excluded, or because contributions to thymidine pools had not always been taken into account in assessing the effects of [^{3}H]thymidine labelling. Lord *et al.* (1974) have employed a novel technique, originally devised by Cercek & Cercek (1972), to monitor the effects of erythrocyte extracts on the proliferation of erythroid precursor cells, but perhaps its very novelty raises some questions about the absolute authenticity of the data so derived. Even if some of the evidence is discounted however, there seems no doubt that some form of specific feedback control affects the numbers of erythroid cells in mammals.

We have employed the anaemic *Xenopus* as a model system for studies on the control of erythropoiesis. By injecting serum from normal animals into *Xenopus* rendered anaemic with phenylhydrazine the regulatory part of the technique is accomplished *in vivo*. This seems to us to have considerable advantages since this method enables us to study the effect in cells proliferating at their maximum rate, as happens in anaemic animals, and at the same time,

[1] *Author's address:* Department of Biology, Medical and Biological Sciences Building, Southampton University, U.K.

any possible cytotoxic effect of a tissue extract is minimized by *in vivo* injections due to the dilution that occurs in the body fluids.

The treated and control animals have been bled at various times during recovery and the number and behaviour of their erythroid cells determined in *in vitro* cultures. The characteristics of normal recovery from such anaemia in *Xenopus* have already been studied and published (Maclean & Jurd, 1971; Thomas & Maclean, 1974, 1975; Hilder, Thomas & Maclean, 1975). It is possible that *Xenopus* may mount an immune response to an injection of serum derived from another adult, but our assays at 4 days will, in any event, precede such a response.

MATERIALS AND METHODS

Animals

Mature adult female *Xenopus laevis* were obtained from Harris Biological Supplies, Weston-Super-Mare, and maintained as previously described (Maclean & Jurd, 1971).

Chemical reagents

All reagents were obtained from British Drug Houses Ltd., Poole, England, except where otherwise indicated. The radioactive reagents were obtained from the Radiochemical Centre, Amersham, England.

Induction of anaemia

Mature, female *Xenopus*, of body weight approximately 100 g, were made anaemic by phenylhydrazine injections as previously described (Thomas & Maclean, 1975). These test animals were injected either with 1 ml of complete serum from normal *Xenopus* or with aliquots from the chromatographic fractionation of serum or haemolysate on the 3rd day after the last phenylhydrazine injection.

Collection of serum from normal and anaemic animals

Normal animals were bled by cardiac puncture, following anaesthesia. The blood was kept at 4 °C overnight, then centrifuged at 1000 ***g*** for 5 min to pellet the cells and the supernatant serum collected. It was filtered (0·22 μm pore size Millipore filters) and injected intracardiacally into anaemic test animals, 1 ml per animal. In the same way, serum was prepared from anaemic animals, on the 4th day of anaemia. It was injected into anaemic animals, 1 ml per animal.

Preparation of normal haemolysate

One ml of blood was collected from normal toads by cardiac puncture. The blood cells were washed twice in 20 vols. of Rugh Ringer's solution (Rugh, 1962), and finally resuspended in 1 ml Ringer solution. The cells were lysed

with 1 mg saponin, the haemolysate was centrifuged at 1000 *g* to pellet the cell debris, and the supernatant was applied to a Sephadex G-25 column.

Collection of blood and culture of the erythroid cells

Four days after the injection of serum or chromatography fractions, the anaemic animals were anaesthetized with MS 222 (Sandoz Products Ltd., London) and blood was collected by cardiac puncture. An aliquot of the blood collected was used for determination of the blood cell count (Improved Neubauer haemocytometer). The remainder was washed twice in 20 vols. of Rugh Ringer's solution containing 8 mg/ml bovine serum albumin. The blood cells were resuspended in an incubation medium (Godsell & Balls, 1975) having the following composition: 60 % L-15 Medium (Flow Laboratories, Irvine, Scotland), 10 % foetal bovine serum (Flow Laboratories) and 30 % sterile distilled water, 100 i.u./ml penicillin (Sigma, U.S.A.) and 100 μg/ml streptomycin (Sigma) were also included. (6-[^{3}H]thymidine (sp. act. 25·6 Ci/mmol) and L-(4,5-[^{3}H]leucine (s p. act. 50 Ci/mmol) were added to separate cultures to give effective concentration of 20 μCi/ml. The cells were cultured in the radioactive medium for 6 h at 22 °C with agitation. The percentage of dead cells, both on the bleeding day and at the end of the culture period, was estimated by counting a sample stained by 0·5 % trypan blue (G. T. Gurr Ltd., London) in Ringer's solution in a haemocytometer. Scintillation counting and autoradiography were carried out as previously described (Maclean, Hilder & Baynes, 1973). Autoradiographic slides were normally developed after 6 days and stained with Giemsa (G. T. Gurr Ltd.) or with benzidine and counterstained with Giemsa (Bateman, 1974).

Erythrocyte conditioned medium

Blood was collected from normal animals as previously described. Washed blood cells were resuspended in Rugh Ringer's solution to a concentration in line with the actual haematocrit of the normal animal. The cells were kept in Ringer's solution with gentle agitation for 5 h at 22 °C. The cells were removed by centrifugation and the conditioned medium was filtered through a Millipore filter of 0·22 μm pore size and stored at −20 °C. Such conditioned medium was then supplemented with 8 % foetal bovine serum, penicillin and streptomycin at the usual concentrations and utilized as a culture medium. Erythroid cells of anaemic *Xenopus*, on the 7th day of anaemia, as in all cases, were resuspended in such media supplemented with [^{3}H]thymidine to give effective concentration 20 μCi/ml and cultured for 3 h at 22 °C. Control cultures were incubated in identical media made up with Ringer's solution not exposed to cells.

Column chromatography

Normal serum and haemolysate were applied to a Sephadex G-25 column ($1{\cdot}5 \times 30$ cm^2) and eluted with Rugh Ringer's solution, pH 7·4. Void volume was determined by the elution of blue dextran (Pharmacia, Uppsala). The elution speed was 1·3 ml/min for the haemolysate and 0·26 ml/min for the serum. The volume of each fraction collected was 1·3 ml. Approximately 10 adjacent fractions were pooled, the volume reduced to 2 ml by ultrafiltration and injected into anaemic toads on the 3rd day of anaemia, 1 ml per animal. Control animals were injected with Ringer's solution of appropriate volume. After 4 days the toads were bled and the cells cultured as previously described.

Kidney cell culture

A *Xenopus* kidney cell line was obtained from Harris Biological Supplies. The cells were cultured in identical conditions to those described by Balls & Godsell (1972). In half of the cultures the medium was supplemented with 5% normal serum, while in the other half, Ringer's solution was used as a supplement. After 4 days, [^{3}H]thymidine was added to give an effective concentration of 20 μCi/ml, and the cells cultured with the radioactive label for 6 h. Scintillation counting was carried out as previously described.

RESULTS

(A) In vivo *injection followed by* in vitro *assay*

Effect of normal serum on DNA and protein synthesis of erythroid cells in anaemic animals

Data in Tables 1 and 2 reveal that the normal serum exerts an inhibitory effect on the DNA and protein synthesis of the anaemic erythroid cells, an effect which is statistically significant, although in one case for reasons unknown, the serum had little or no inhibitory effect. Uridine incorporation into RNA, measured with tritiated uridine, differed slightly in the test animal cells compared to the control, but our data are not sufficient to indicate a positive effect. Control animals were injected either with Ringer's solution or anaemic serum. The use of the latter type of control confirms that serum from normal animals only inhibits DNA and protein synthesis in anaemic blood cells, while serum from anaemic ones has no effect (Table 3).

The results of leucine and thymidine incorporation are confirmed by the autoradiographic analysis. Autoradiographs were prepared at the end of the culture period. At least 1000 cells were scored in each slide and the percentage of labelled erythroid cells was determined.

An anaemic *Xenopus*, on the day of bleeding, possesses blood consisting of 33·23% erythroid cells, 55·41% damaged mature erythrocytes and 11·36% leucocytes, which consist of lymphocytes and granulocytes. The mature erythro-

Table 1. *Effect of normal serum on [^3H]leucine incorporation and [^3H]leucine labelling of anaemic blood cells*

The cells were cultured for 6 h with the radioactive precursor. Autoradiographs were developed for 6 days at 4 °C. 1000 erythroid cells were scored in each experiment. Figures represent means of four experiments.

Treatment	cpm/10^7 cells	Cells labelled (%)
Ringer	65306 ± 10091	27·31 ± 4·38
Normal serum	26801 ± 11084	8·95 ± 2·63
Difference in means ± S.E.	38505 ± 14989	18·36 ± 5·10
Significance Student's *t*-test	$P < 0.05$	$P < 0.02$

Table 2. *Effect of normal serum on [^3H]thymidine incorporation and [^3H]thymidine labelling of anaemic blood cells*

The cells were cultured with the radioactive precursor for 6 h. Autoradiographs were developed for 6 days at 4 °C. 1000 erythroid cells were scored in each experiment. Figures represent means of five experiments.

Treatment	cpm/10^7 cells	Cells labelled (%)
Ringer	1021514 ± 152463	17·21 ± 1·95
Normal serum	372462 ± 196529	8·06 ± 1·97
Difference in means ± S.E.	649052 ± 248734	9·15 ± 2·77
Significance Student's *t*-test	$P < 0.05$	$P < 0.02$

Table 3. *Effect of normal serum on [^3H]leucine and [^3H]thymidine incorporation of anaemic blood cells as compared to anaemic serum*

The cells were cultured with the radioactive precursors for 6 h. The figures of anaemic serum represent means of two experiments and the ones of normal serum of five experiments.

Treatment	[^{3}H]leucine cpm/10^7 cells ± S.E.	[^{3}H]thymidine cpm/10^7 cells ± S.E.
Anaemic serum	82018 ± 6886	1260117 ± 124898
Normal serum	26801 ± 11084	372462 ± 196529
Difference in means ± S.E.	55217 ± 17152	887655 ± 335431
Significance Student's *t*-test	$P < 0.05$	$P < 0.05$

cytes are cells affected by phenylhydrazine which have not yet been removed from the circulation. Such cells do not incorporate tritiated leucine, thymidine or uridine; they represent an inactive and probably dead population of cells, and do not interfere in our results. The leucocyte cell number does not vary significantly between anaemic animals, whether or not they have been injected

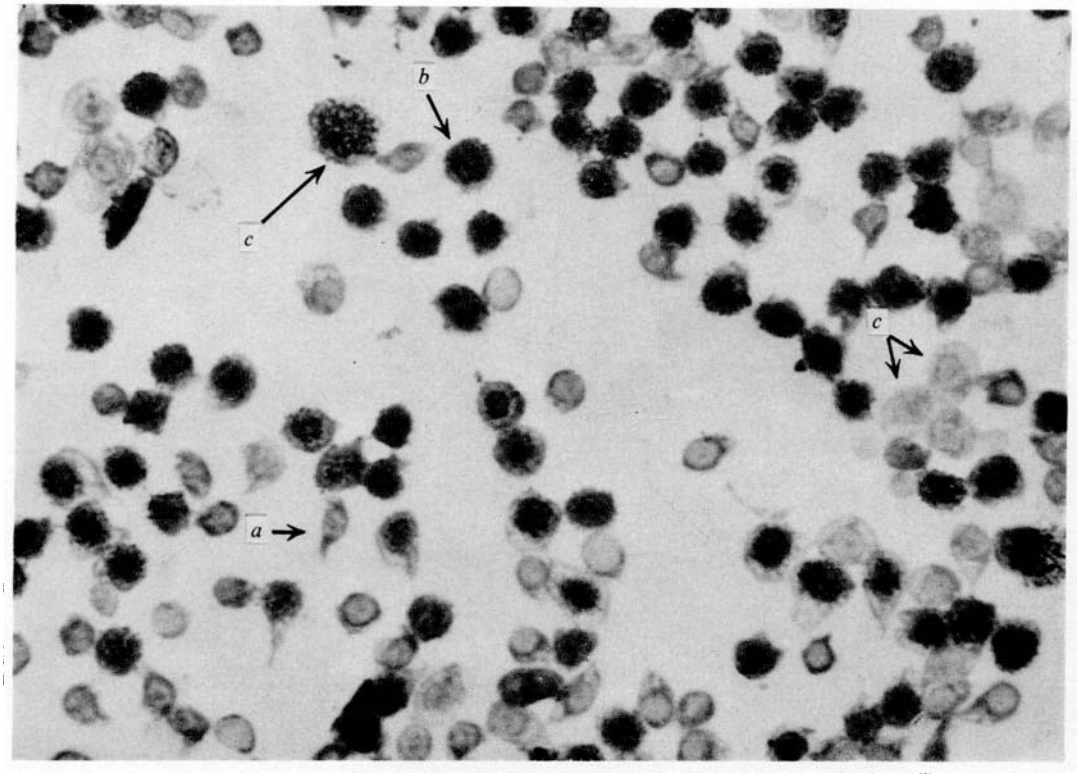

Fig. 1. Autoradiograph of blood cells from anaemic *Xenopus*, injected with Ringer's solution, labelled *in vitro* with [^{3}H]thymidine for 6 h. Developed for 6 days at 4 °C. Stained with benzidine–Giemsa. ×750. (*a*) = Damaged mature erythrocyte. (*b*) = Erythroid cells. (*c*) = Leucocytes.

with serum. Although the scintillation counting includes incorporation into leucocytes, the percentage of labelled cells scored from the autoradiographs represents erythroid cells only.

As shown in Tables 1 and 2, normal serum caused a marked decrease in the number of labelled erythroid cells of the test toads, compared to controls, implying a reduction in the synthesis of both DNA and protein in the culture. The autoradiographic analysis confirms the view that the normal serum has an inhibitory effect on the erythroid cells. It also indicates clearly that the reduction in the incorporation of both [^{3}H]thymidine and [^{3}H]leucine is chiefly attributable to a lower number of erythroid cells and not to a generalized fall in the amount of label incorporated by each cell (Figs. 1, 2).

The erythroid cells on the day of bleeding were invariably healthy, absence of dead cells being verified by a dye exclusion test (trypan blue). The damaged mature erythrocytes were the only ones stained.

As we have suggested earlier, the *in vivo* systems can neutralize and eliminate

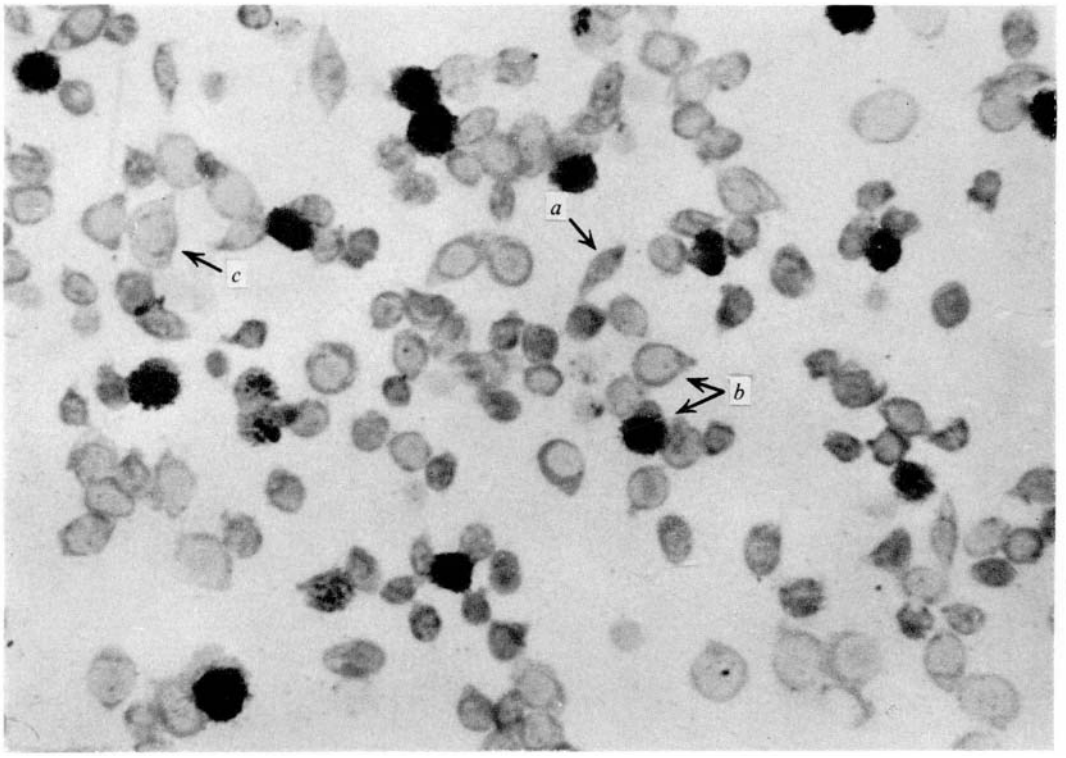

Fig. 2. Autoradiograph of blood cells from anaemic *Xenopus*, injected with normal serum, labelled *in vitro* with [^{3}H]thymidine for 6 h. Developed for 6 days at 4 °C. Stained with benzidine–Giemsa. ×750. (*a*) = Damaged mature erythrocyte. (*b*) = Erythroid cells. (*c*) = Leucocyte.

toxic factors better than *in vitro* systems by diluting them via the body fluids and metabolism.

Effect of normal serum on the blood cell count of anaemic animals

Anaemic animals injected with normal serum exhibited, in most cases, a reduced blood cell count compared to the controls, but in only a few cases was this significant. This is no doubt because the variability in normal red cell count seriously affects the result.

Fractionation of serum on Sephadex G-25

Information (Kivilaasko & Rytomaa, 1971; Bateman, 1974) about the molecular weight of erythrocyte inhibitory factors led us to the use of Sephadex G-25 for its partial purification from serum. Rugh Ringer's solution was used as eluant and the sample volume was 1 ml.

The results are presented in Fig. 3(*a*). It is evident that the pooled fractions with the relative elution volume V_e/V_o: 2·76 and fractions with relative elution

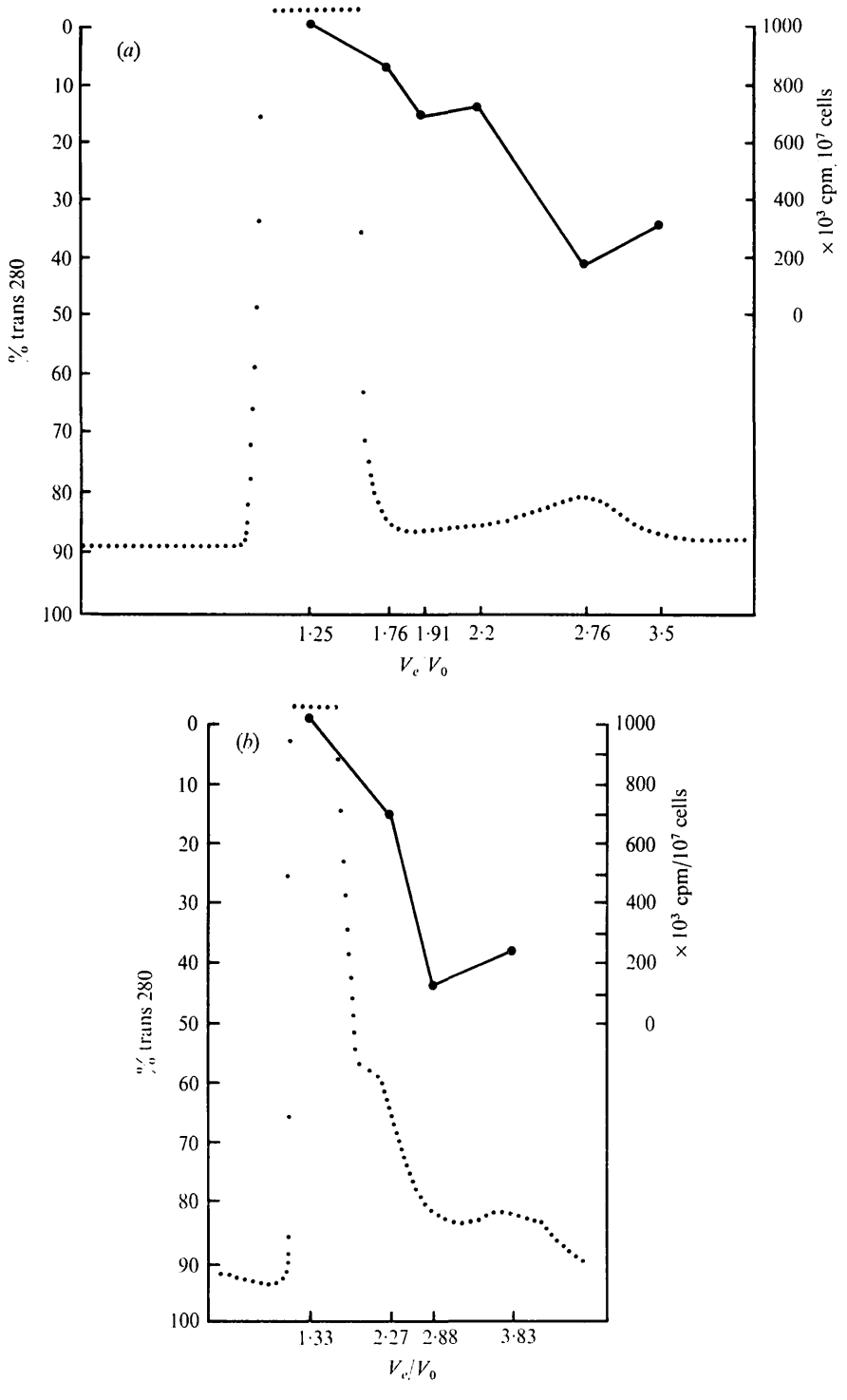

Fig. 3. Column chromatography of molecules which inhibit thymidine incorporation into erythroid cells on Sephadex G-25. (*a*) Normal serum. (*b*) Normal haemolysate.

In the upper curve, ● represents [^{3}H]thymidine incorporation of pooled fractions.

In the lower curve, • represents % transmittance of each fraction of V_e/V_o indicated on abscissa. Abscissa: Relative elution volume V_e/V_o. Left ordinate (applies to lower curve): % transmittance at 280 nm; right ordinate (applies to upper curve): [^{3}H]thymidine incorporation *in vitro*, in $\times 10^3$ cpm/10^7 cells.

Table 4. *Effect of erythrocyte conditioned medium on* [^3H]*thymidine incorporation of anaemic blood cells* in vitro

The cells were cultured in the radioactive medium for 3 h. Figures represent means of two experiments.

Treatment	cpm/10^7 cells	S.E.
Ringer	470245	45065
Erythrocyte conditioned medium	59164	5983
Difference in means	411081	45460
Significance Student's *t*-test	$P < 0{\cdot}02$	

volume V_e/V_o: 3·5 show an inhibitory effect and that the first shows the stronger effect. This suggests a molecular weight of less than 5000.

The effect appears to be a genuine inhibition of cell proliferation and not a general cytotoxicity, as judged by the criteria mentioned earlier.

Fractionation of haemolysate on Sephadex G-25

The results given in Fig. 3(*b*) show that the pooled fractions of mean relative elution volume of V_e/V_o: 2·88 and V_e/V_o: 3·83 have an inhibitory action. These results are in accordance with those of the normal serum, suggesting that the inhibitory substance which exists in the serum comes from the mature erythrocytes and inhibits proliferation of the erythroid cells.

(B) *Wholly* in vitro *technique*

In vitro *inhibitory effect of erythrocyte conditioned medium*

The effect of erythrocyte conditioned medium was measured in terms of [^{3}H]thymidine incorporation in erythroid cells of anaemic toads in *in vitro* cultures. This also inhibits DNA synthesis (Table 4) and confirms, together with the haemolysate inhibition, that the factors which reduce DNA synthesis come from the erythrocytes.

Specificity of action – effect of Xenopus *kidney cells*

The erythropoiesis inhibitory substance was tested for its specificity of action, by examining the effect of normal serum on DNA synthesis of another cell line, namely *Xenopus* kidney cells.

Eight parallel cultures of kidney cells in Petri dishes were prepared. In half of them normal serum was added while the others served as control receiving only Ringer's solution. The cells seemed to be healthy and dividing on microscopic examination. After 4 days, [^{3}H]thymidine was added to all cultures and incubation with the radioactive precursor continued for 6 h.

The data shown in Table 5 are the mean [^{3}H]thymidine incorporation values of the four separate cultures. The results of serum-treated and control cells

Table 5. *Effect of normal serum on [^3H]thymidine incorporation of kidney cell cultures*

The cells were cultured with the radioactive precursor after a 4-day incubation with normal serum or Ringer for 6 h. The figures represent means of four cultures.

Treatment	cpm/10^7 cells	S.E.
Ringer	26865	599
Normal serum	15684	5887
Difference in means	11217	5918
Significance Student's *t*-test	Insignificant	

reveal that the effect of the serum is statistically insignificant, which means that the normal serum has no effect on the DNA synthesis of kidney cells, that is in another cell line of the same animal species. A negative feedback mechanism has been previously demonstrated to operate in *Xenopus* kidney (Chopra & Simnett, 1971).

DISCUSSION

Our data support the theory that factors released from mature erythrocytes inhibit proliferation of erythroid cells, probably in a tissue specific way. The action of these molecules involves a reduction in protein and DNA synthesis in our system. A similar action has been reported for lymphocyte (Houck, refer to Nakai, 1976) Ehrlich Ascites Tumour (Nakai, 1976) and melanoma (Seiji, Nakamo, Akiba & Kato 1974), proliferation inhibitors in mammals.

We believe that this is the first reported evidence for feedback inhibition of erythroid cell proliferation in amphibians.

During recent years the presence of specific inhibitors of erythropoiesis has been reported in plasma from hypertransfused mammals (Krzymowski & Krzymowska, 1962; Whitcombe & Moore, 1965) in plasma from high altitude natives brought to sea level (Reynafarje, Raimos, Faura & Villavicencio, 1964) and in human urine (Lindemann, 1971). A direct connexion between such inhibitory factors and those revealed in our work is not clear however, since in most cases their origin has not been investigated.

Recent work by Cole & Regan (1977), on the other hand, showed that erythrocyte conditioned medium exerts a negative feedback control in mammalian foetal liver erythroblasts through inhibition of RNA and haem synthesis, but not DNA synthesis. In our system, effects on RNA synthesis are very variable but the effect on DNA synthesis is striking.

It appears the erythropoiesis is regulated by at least two control mechanisms in vertebrates. Erythropoietic stimulation is exerted by erythropoietin in response to hypoxia, while inhibition of overproduction of erythroid cells may be effected by factors originating from mature erythrocytes.

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