Regulation of pre-natal haemopoiesis: evidence for negative feedback control of erythropoiesis in the foetal mouse

By R. J. COLE¹ AND T. REGAN¹

From the School of Biological Sciences, University of Sussex

SUMMARY

The effects of medium conditioned by erythrocytes on in vitro differentiation of mouse prenatal erythroblasts were examined. Haem synthesis was inversely proportional to the concentration of erythrocyte-conditioned medium (ECM) in either presence or absence of erythropoietin (EP), but concentrations of ECM which limited haem synthesis to 50% of normal had no effect on changes in erythroblast numbers. ECM reduced uridine incorporation into RNA and limited the rise in incorporation due to stimulation by EP. ECM enhanced thymidine incorporation into DNA. This enhancement was additive with that due to EP, but appeared earlier. Thymidine labelling indices of liver cells were not changed by ECM in vitro. Serum from polycythaemic animals limited haem synthesis by EP stimulated foetal liver cells in vitro. These observations provide evidence for susceptibility of foetal liver erythroblasts to negative feedback control of differentiation, dependent on the concentration of circulating erythrocytes. Characteristic changes in kinetics of prenatal erythropoiesis in vivo can also be related to increasing erythrocyte concentration.

INTRODUCTION

Erythropoiesis in postnatal mammals is suppressed by polycythaemia, which is accompanied by the presence of erythrocyte-derived factors which can exert a direct, negative feedback control on erythrocyte production (Lindemann, 1976). Such factors occur in serum from naturally polycythaemic individuals (Reyna-farje, 1968; Skjaelaaen, Halvorsen & Seip, 1971), as well as in experimental polycythaemia (Whitcombe & Moore, 1968; Kivilaakso & Rytomaa, 1971; Bateman, 1974). An ‘erythropoiesis-inhibiting factor’ has also been isolated from human urine (Lindemann, 1971). During normal and stress erythropoiesis in postnatal mammals, production of erythrocytes is also regulated by the plasma level of erythropoietin, itself determined by regulatory mechanisms linked to oxygen tension in target tissues (Gordon, Zanjon, Gidara & Kuna, 1973). The major effect of erythropoietin in postnatal mammals is to determine the rate of proliferation of early erythroid cells. Exposure of mouse foetal liver erythroblasts to erythropoietin in vitro showed that prenatal blood-forming

¹ Author’s address: School of Biological Sciences, University of Sussex, Falmer, Brighton, Sussex.
cells are highly responsive to this hormone, exhibiting enhanced levels of haem synthesis in vitro during clearly defined developmental phases (Cole & Paul, 1966; Cole, Hunter & Paul, 1968; Paul, Conkie & Freshney, 1969; Tarbutt & Cole, 1970). Detailed studies have confirmed that in vitro, as in vivo, erythropoietin is predominantly effective on relatively early erythroid precursors, shortening the cell cycle time and enhancing their potential for haemoglobin production (Harrison, Conkie & Paul, 1973). The rate of erythropoiesis may therefore be under dual control, providing inherent stability with little oscillation.

In the present report the inhibitory effect of erythrocyte-derived material on erythropoiesis in prenatal erythroblasts in vitro has been investigated. In addition, the normal course of prenatal erythropoiesis has been re-examined to determine whether observed parameters are consistent with a physiological role for such factors in vivo.

**Materials and Methods**

*Animals.* Swiss albino mice (Porton strain) were used throughout. Foetal material was obtained from natural matings, timed from the appearance of mating plugs on day 0. Blood and serum were collected only from animals maintained on peat bedding, since our previous experience has shown that serum from animals kept on wood-shavings can inhibit the erythropoietin responsiveness of foetal liver cells in vitro, and limit iron uptake.

*Erythrocyte-conditioned medium.* Blood was collected from adult mice using heparin to a final concentration of 40 units/ml. Erythrocytes were sedimented by centrifugation and plasma and buffy coat removed. Erythrocytes were then washed twice in equal volumes of ice-cold medium. Washed packed red cells were resuspended in an equal volume (i.e. at a ‘haematocrit’ of 50%) of culture medium, Waymouth MB 752/1 supplemented with 10% foetal calf serum and 2% mouse serum, and incubated with gentle agitation for 1 h at 37 °C. Erythrocytes were removed by sedimentation, and the conditioned medium then filtered (0.2 μm Millipore) and stored at −40 °C. Batches of erythrocyte-conditioned medium were tested for their ability to depress haem synthesis by liver erythroblasts in vitro, in the presence and absence of erythropoietin. For subsequent experiments, 20% ECM, which depressed by 50% erythropoietin-stimulated haem synthesis 28 h after explantation, was generally used.

*Serum from polycythaemic animals.* Serum was obtained from post-hypoxic rebound mice. Animals were maintained for 12 days at half normal atmospheric pressure and then returned to normal pressure. Blood was collected after 4 days at normal pressure from animals with haematocrits above 65%.

*Culture media and methods*

Single-cell suspensions were prepared from prenatal livers by gentle pipetting in culture medium, without the use of trypsin or other enzymes. Cultures were set up at $1 \times 10^6$ cells/ml in Waymouth’s MB 752/1 medium supplemented with
Regulation of prenatal haemopoiesis

10% foetal calf serum (Flow laboratories) and 2% mouse serum, and maintained at 37°C and pH 7.4 in 5% CO₂ in air (Cole & Paul, 1966). This serum concentration allows maximum erythropoietin sensitivity to be expressed.

**Haem synthesis.** This was determined by exposing cultures to (⁵⁹Fe) ferric chloride previously equilibrated with mouse transferrin by incubation at 37°C with 50% mouse serum in Waymouth's medium. Cultures were labelled with 1-2 μCi/ml. Haem was extracted into acid ethyl methyl ketone and radioactivity determined by counting in a gas-flow counter. Total iron in medium was determined colorimetrically (Boehringer Test).

**DNA synthesis.** Cultures were labelled with [³H]thymidine (1 μCi/ml) for 60 min pulses, then washed with Hanks' saline and ice-cold 2 N perchloric acid. The cell pellet was extracted with 2N perchloric acid at 70°C for 20 min, neutralized, and radioactivity in the supernatant determined in a scintillation counter.

**RNA synthesis.** Cells were labelled with [³H]uridine for 60 min pulses, and radioactivity determined as above.

**Autoradiography.** Cytocentrifuge preparations of cells cultured in the presence of 1 μCi/ml [³H]thymidine for 60-min pulses were fixed in methanol, coated with Ilford K5 nuclear emulsion, and after exposure and development, stained with Lepehnese and Giemsa stains.

**Iron content of media** was determined colorimetrically (Boehringer Test 15947 TEAA) and utilization of ⁵⁹Fe for haem synthesis expressed on the basis of total iron in the medium during labelling. Exposure of medium to erythrocytes during the conditioning procedure raised the total iron content by less than 5%. No detectable increase in the haemoglobin content of conditioned medium (as assayed by absorption at 540 nm) was observed.

**Effects of erythrocyte-conditioned medium on non-erythroid cells**

Possible non-specific toxicity or medium depletion caused by erythrocytes was tested by examining the growth rate of two established cell lines in conditioned medium. Both cell types, mouse LS929 (skin fibroblasts) and mouse lymphoma (L5178 Y) cells, showed slightly enhanced growth in conditioned medium. Heparin in the concentrations used here had no effect on the parameters of erythroid differentiation in vitro examined during these experiments.

**Dose-response of 13-day foetal liver cells to erythropoietin**

Levels of haem synthesis achieved by 13-day foetal liver cells in response to varying doses of Step III sheep plasma erythropoietin (Lot ALO 336) (22 units/mg) and human urinary erythropoietin (Lot M₃TaLSL) (11 units/mg) were measured during the 23rd–25th hour of culture. The response to both types of erythropoietin was similar, with the maximum response at 0.2 units/ml. This concentration or less was used for subsequent experiments. Erythroblasts
Fig. 1. The effects of varying concentrations of erythrocyte-conditioned medium on haem synthesis in foetal liver cells. Cultures were labelled with $^{59}$Fe 27-29 h after explantation. Each point is the mean of four replicates shown ± standard deviation of mean. ●—●, + erythropoietin; ○—○, no added erythropoietin.

differentiating in vitro in this concentration appeared histologically normal with no observable increase in megaloblastosis (Harrison et al. 1973).

RESULTS

Can erythrocytes influence erythroblast function?—effects of erythrocyte-conditioned medium on haem synthesis and erythroblast numbers in cultured foetal liver

Levels of haem synthesis achieved in the presence of 0.15 units/ml erythropoietin, or without erythropoietin, when varying concentrations of medium conditioned by exposure to mature erythrocytes are present are shown in Fig. 1.

The assay is calculated in terms of the packed cell volume of erythrocytes from which material potentially affecting erythroblast development had been eluted, expressed as a percentage of the final culture volume. All culture media contained 12 % serum (the optimum concentration for haem synthesis in vitro), so that assuming a haematocrit of 50 % in the blood from which the sera were obtained control medium is equivalent to a value of 6 % erythrocytes. Medium conditioned in vitro with an equal volume of erythrocytes is equivalent to 56 % erythrocytes, 6 % coming from serum and 50 % from the further exposure to erythrocytes in vitro. This assay shows that utilization of exogenously supplied $^{59}$Fe for haem synthesis is reduced in linear proportion to the log of concentration of erythrocyte-conditioned medium in either the presence or absence of
Table 1. Effects of erythropoietin (EP) (0.15 u./ml) and erythrocyte-conditioned medium (ECM) (equivalent to 26 % erythrocyte volume) on haem synthesis in foetal liver cultures

<table>
<thead>
<tr>
<th></th>
<th>0–1 h</th>
<th>28–29 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ ECM/+ EP</td>
<td>360 ± 32 cpm</td>
<td>728 ± 56 cpm</td>
</tr>
<tr>
<td>– ECM/+ EP</td>
<td>353 ± 24 cpm</td>
<td>109 ± 6 cpm</td>
</tr>
<tr>
<td>+ EP/– ECM</td>
<td>480 ± 32 cpm</td>
<td>62 ± 5 cpm</td>
</tr>
<tr>
<td>– EP/– ECM</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Haem synthesis is expressed as $^{59}$Fe cpm/10$^6$ cells/h

Added erythropoietin. Since both mouse fibroblasts and mouse lymphoma cells (which have exacting growth requirements) show enhanced multiplication in erythrocyte-conditioned medium, this effect is unlikely to be due to medium depletion or non-specific toxicity.

Data in Table 1 show the effects of medium containing the products of 26 % erythrocytes on haem synthesis in vitro. Erythropoietin alone (0.15 µ./ml) caused a twofold increase in haem synthesis at 28 h over the initial rate, and a six- to sevenfold increase over the 28 h rate without erythropoietin, but in the presence of both erythropoietin and erythrocyte-conditioned medium the 28 h value was only 1.3 times that at explantation and 4 times the 28 h control level. In the absence of erythropoietin, erythrocyte-conditioned medium also caused a greater decline in the rate of haem synthesis by 28 h. Without erythropoietin the number of nucleated erythroblasts in the cultures after 88 h was similar to the initial number but almost doubled when erythropoietin was present. Erythrocyte-conditioned medium had negligible effects on this parameter of erythroid differentiation in vitro. Differential cell counts showed that erythropoietin promoted the continued production of pro- and basophilic erythroblasts, but erythrocyte-conditioned medium did not appear to affect this process.

**Effects of erythrocyte-conditioned medium on RNA synthesis**

Addition of erythrocyte-conditioned medium caused marked depression of uridine incorporation into RNA of differentiating foetal liver cell cultures labelled during 60-min pulses (Fig. 2). In the presence of erythropoietin alone, incorporation rose rapidly and after 10 h was approximately 1.5 times the rate at explantation. In the presence of erythrocyte-conditioned medium (26 % packed cell volume, as above) without erythropoietin, uridine incorporation fell during the 2nd hour of culture to one-third the level of control cultures at explantation. This was followed by a steady return towards the level seen in control cultures which was reached after 10–12 h. Erythropoietin slightly reduced the depression due to erythrocyte-conditioned medium, and incorporation rose above the
effects of erythrocyte-conditioned medium on uridine incorporation into RNA. Data expressed as differences from control cultures without EP or ECM at equivalent times. ECM was used at a concentration which halved the 27–29 h rate of erythropoietin-stimulated haem synthesis. In the first hour of culture uridine uptake was 15000 dpm without ECM and 9000 in the presence of ECM. With erythropoietin, •—•; with erythropoietin and erythrocyte-conditioned medium, •—••. Each point is the difference between pairs of triplicate samples shown ± standard deviation of difference of means. Cultures were labelled for 60-min pulses.

equivalent controls during the 6th hour of culture, but the total increment in uridine incorporation was very small.

Effects of erythrocyte-conditioned medium on DNA synthesis

The effects of erythrocyte-conditioned medium (26 % packed cell volume, as above) on incorporation of [3H]thymidine into DNA are shown in Fig. 3. In the presence of erythropoietin alone, incorporation reached a peak during the 6th hour of culture, at about 1.6 times the rate at explantation. When erythrocyte-conditioned medium was present in addition to erythropoietin, there was a rapid biphasic rise in incorporation with peaks of incorporation more than twice the rate at explantation. Erythrocyte-conditioned medium without erythropoietin also promoted increased thymidine uptake into DNA. Relative to total incorporation over 28 h by control cultures, erythropoietin alone increased incorporation twofold, erythrocyte-conditioned medium alone 1.7-fold, and erythropoietin in the presence of erythrocyte-conditioned medium threefold.
Fig. 3. Effects of erythrocyte-conditioned medium on thymidine uptake into DNA. Data expressed as differences in incorporation from control cultures without EP or ECM at equivalent times. ECM was used at a concentration which halved the 27–29 h rate of erythropoietin-stimulated haem synthesis. In the first hour of culture with erythropoietin, thymidine uptake was 12,000 dpm without ECM and 17,000 dpm in the presence of ECM. With erythropoietin, •—•; with erythropoietin and erythrocyte-conditioned medium, ■—■; with erythrocyte-conditioned medium, □—□. Each point is the difference between pairs of triplicate samples shown ± standard deviation of difference of means. Cultures were labelled for 60-min pulses.

Effects of erythrocyte-conditioned medium on thymidine-labelling indices of foetal liver cells in vitro

At explantation, 3-day foetal liver cultures contain a large proportion of early erythroblasts (Fig. 4). These have a high labelling index early in the culture period which is only maintained in the presence of erythropoietin. Erythrocyte-conditioned medium did not have any detectable effect on changes in labelling index in spite of its effect on [3H]thymidine labelling of DNA, and no change occurred in the labelling index of non-erythroid cell populations, suggesting that the effect of ECM is to potentiate thymidine uptake, or to accelerate DNA synthesis, rather than to recruit cells into the cycle. Some non-erythroblast cell types label during a 1-h pulse with [3H]thymidine in vitro, but none represent more than 5% of cells in 'cytocentrifuge' preparations of 13-day foetal liver. Only one non-erythroblast cell type showed increased labelling index in response to erythropoietin: it made up 5% of the total in 13-day foetal liver at explantation and resembled the 'undifferentiated' cell described by Lucarelli et al. (1968) in rat foetal liver and early bone marrow, which they considered to be an erythroid precursor characteristic of erythropoiesis early in development. It also resembled the 'undifferentiated' cells which characterize the putative 'suppressed erythroid colonies' formed by haemopoietic stem cells in the spleens of
irradiated polycythaemic mice (Trentin, 1970). No effect of erythrocyte-conditioned medium on the labelling index of this cell type was observed.

The effects of serum from polycythaemic animals on haem synthesis in prenatal erythroblasts in vitro

If erythroid differentiation is affected by factors which can be eluted from erythrocytes in vitro, then such factors might be present in raised concentration in serum from polycythaemic animals. Data in Table 2 show a comparison between cultures exposed to 4-day rebound serum and 15% erythrocyte-conditioned medium, in addition to the normal serum component of culture medium which was standardized throughout. In the presence of both materials, haem synthesis immediately after explantation was reduced relative to controls and there was a marked fall in the 28-h value after prolonged absence of erythropoietin. When erythropoietin was present, the 28-h haem synthesis rate showed reductions to 59% of control in the presence of 15% serum from polycythaemic animals and 54% in the presence of 15% erythrocyte-conditioned medium.

Are changes in prenatal erythropoiesis consistent with a physiological role for inhibitory factors in vivo?

Marked changes in several features of foetal erythropoiesis occur on day 15–16 (Fig. 5), e.g. alterations in length of cell cycle in early and late
Table 2. Effects of serum from polycythaemic adults and erythrocyte-conditioned medium on haem synthesis in foetal liver cell cultures

The rate at explantation in normal medium is expressed as 100%. 'Medium' contains 10% of calf serum and 2% mouse serum in each case.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0–2 h</th>
<th>27–29 h</th>
<th>27–29 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>227 ± 13 (100 %)</td>
<td>1253 ± 57 (553 %)</td>
<td>209 ± 18 (92 %)</td>
</tr>
<tr>
<td>Medium + 15% serum from 30-day rebound animals with normal haematocrit</td>
<td>176 ± 8 (78 %)</td>
<td>1107 ± 26 (488 %)</td>
<td>133 ± 12 (59 %)</td>
</tr>
<tr>
<td>Medium + 15% serum from 4-day rebound animals with 65% haematocrit</td>
<td>110 ± 9 (48 %)</td>
<td>746 ± 74 (329 %)</td>
<td>84 ± 2 (37 %)</td>
</tr>
<tr>
<td>Medium + erythrocyte-conditioned medium (21% p.c.v.)</td>
<td>198 ± 19 (87 %)</td>
<td>681 ± 52 (300 %)</td>
<td>55 ± 5 (24 %)</td>
</tr>
</tbody>
</table>

Fig. 5. Developmental changes in prenatal blood formation. (1) Daily increment in haemoglobin content of foetus relative to number of haemoglobin synthesizing cells, □—□. (2) Rate of haem synthesis in newly explanted foetal liver cells, relative to haemoglobin synthesizing cells, ○—○. (3) Haemoglobin content of foetus relative to body weight, ●—●. (4) Blood haemoglobin concentration, △—△. (5) Blood erythrocyte concentration, ■—■. (Data from Paul, Conkie & Freshney, 1969 and Tarbutt & Cole, 1970.)
Fig. 6. Characteristics of the prenatal mouse circulation relative to normal adult values. Oxygen transport (as blood flow \( \times \) \( \text{O}_2 \) capacity), \( \bigcirc \)–\( \bigcirc \); viscosity (in vitro), \( \square \)–\( \square \); haematocrit, \( \triangle \)–\( \triangle \); ratio of total haemoglobin content to body weight (\( \mu g \) Hb/mg body weight), \( \bullet \)–\( \bullet \); erythrocyte numbers/unit volume of blood, \( \blacksquare \)–\( \blacksquare \).

erthroblasts, a change from erythropoietin responsiveness to non-responsiveness in erythroblasts in vitro, and a five- or sixfold increase in the population doubling time of circulating erythrocytes (Paul, Conkie & Freshney, 1969; Tarbutt & Cole, 1970). The concentration of circulating erythrocytes rises rapidly from day 13 to day 16, when a steady value is maintained until birth. The foetal content of haemoglobin per unit of body weight reaches a peak on day 17, at 85% of the adult value. The daily increment in haemoglobin relative to the total number of haemoglobin synthesizing cells (i.e. erythroblasts and reticulocytes) per foetus and the hourly rate of haem synthesis in newly explanted foetal liver cells both show marked reduction from days 15–16.

Direct observations of effective oxygen tension in the prenatal circulation and tissues have been restricted to late gestational stages of large mammals. However, it is possible to predict the state of tissue oxygenation at different gestational stages, relative to the adult, from peripheral blood values (Fig. 6), although these cannot take account of changes in efficiency of oxygen binding and release mediated by, for example, chemical modification of oxygen binding capacity of haemoglobin by 2,3-diphosphoglycerate (Bellingham & Grimes, 1973). In spite of the reduced haemoglobin content of foetal tissue relative to adult, oxygen transport through foetal capillaries may not be severely restricted, More than 90% of the maximum rate of oxygen transport can be achieved between haematocrits of 30 and 60 (Stone, Thompson & Schmidt-Nielson,
Regulation of prenatal haemopoiesis

The foetal circulation may therefore reach over 90% of the adult capacity for oxygen transport into the tissues by day 16 of gestation, with a haematocrit apparently little over half the adult value.

Up to day 15–16 of gestation the mouse erythropoietic system is exhibiting characteristics which, in an adult mammal, would be consistent with a high demand for erythrocytes, mediated by significantly raised plasma erythropoietin levels. The rapid changes on day 16 are consistent with transition to a state where tissue oxygenation is more adequate, where erythropoietin levels fall (with a consequent drop in both numbers of liver erythroblasts and proportion of cells in cycle) and where, since there are now high concentrations of erythrocytes in the circulation, erythropoietic inhibitory factors released from erythrocytes could reach effective levels.

**DISCUSSION**

Several studies have indicated the presence of erythropoiesis-inhibiting factors in plasma of individuals with suppressed erythropoiesis, e.g. high altitude dwellers brought to sea level (Reynafarje, 1968) and newborn babies (Skjælaaen et al. 1971). Some workers have suggested that such factors are produced by mature erythrocytes and involve a concentration-dependent negative feedback regulation of erythropoiesis mediated via a control of cell proliferation, consistent with the ‘chalone theory’ (Kivilaakso & Rytomaa, 1971; Bateman, 1974).

The data reported here do not indicate a ‘chalone-like’ effect of unfractionated material eluted from mature erythrocytes on prenatal erythroblasts. There is no evidence for suppression of DNA synthesis or cell proliferation in vitro; on the contrary, thymidine uptake is increased. The rise observed in thymidine uptake is similar to the effects of erythrocyte-derived material on other haemopoietic cells (Darzynkiewicz & Ballazs, 1971; Bradley, Telfer & Fry, 1971).

However, erythrocyte-conditioned medium reduces both RNA and haem synthesis in erythroblasts maturing in vitro, which is consistent with a restriction on differentiation of erythroblasts. Such effects are comparable to the results of previous in vivo tests of ‘erythropoiesis-inhibitory factors’ which take the form of reduced iron uptake by marrow and spleen. In our cultures, RNA and haem synthesis are reduced whether or not erythropoietin is present, suggesting that factors present in erythrocyte-conditioned medium and in serum from polycythaemic animals can interact with the effects of erythropoietin at the cellular level and are not merely effective in its absence.

Studies of the effects of erythropoietin on erythroid cells in vitro have established that rises in RNA and DNA synthesis are early responses to this hormone. Increased RNA synthesis is directed towards enhanced proliferation and enhanced haemoglobin synthesis per responsive cell, and increased haemoglobin synthesis may occur even when DNA synthesis and cell replication are prevented (Gross & Goldwasser, 1970).
Prenatal erythroblasts may therefore be subject to negative feedback control of differentiation in vivo mediated via the concentration of circulating erythrocytes. Some previously unexplained aspects of the kinetics of prenatal erythropoiesis may result from such effects, e.g. the marked changes in hepatic erythropoiesis which occur on days 15–16, coincident with achievement of high erythrocyte concentrations, seen as alterations in the length of cell cycle in early and late erythroblasts, a change from erythropoietin responsiveness to non-responsiveness in erythroblasts in vitro, and a five- to sixfold increase in the population doubling time of circulating erythrocytes (Tarbutt & Cole, 1970; Paul et al. 1969).

Both the rate of transport and the relative concentration changes achieved during transplacental maternal-foetal exchanges are critically dependent on the rate of blood flow in the foetal placenta (Bartels, 1970). Rapid reduction in the production and release of new erythrocytes late in gestation may therefore be important to maintain optimal viscosity in the foetal blood. Depression of the rate of differentiation of erythroblasts may be as effective in limiting numbers of erythrocytes in the circulation as restrictions on cell proliferation. The release of reticulocytes from erythropoietic tissue is dependent on their state of maturity and the number of sites in the late foetal liver capable of supporting erythropoiesis is probably limited (Cole, 1975). The experimental observations reported here provide evidence for a directly acting and rapidly responding control mechanism which could prevent over-production of erythrocytes by the foetal liver late in gestation, and which can interact with the control exerted by erythropoietin earlier in gestation.

This work was supported by grants from the Medical Research Council. Erythropoietin was made available by the Blood Resource Programme, U.S.D.H. E.W. Human erythropoietin was obtained by the University of the Northeast, Corrientes, Argentina, and processed by the Haematology Section, Children's Hospital, Los Angeles.

REFERENCES

Regulation of prenatal haemopoiesis


(Received 13 June 1975, revised 17 September 1976)