Exhibit 31

to the Declaration of Cullen N. Pendleton in Support of Amgen's Opposition to Roche's Motion for Summary Judgment that Claim 7 of the '349 Patent is Invalid Under 35 USC §112 and is Not Infringed

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Erythropoietin and cord blood haemoglobin in the regulation of human fetal erythropoiesis

ROSLYN M. THOMAS,[1,2] CELIA E. CANNING,[3] P. MARY COTES,[3] DAVID C. LINCH,[1] C. H. RODECK,[4] C. E. ROSSITER,[5] & E. R. HUEHNS,[1] *Departments of [1]Haematology and [2]Paediatrics, The Medical School, University College, University Street, London WC1E 6JJ, [3]Division of Clinical Chemistry, Clinical Research Centre, Harrow, Middlesex, [4]Department of Obstetrics and Gynaecology, King's College Hospital, London and [5]Division of Computing and Statistics, Clinical Research Centre, Harrow, Middlesex, UK*

Summary. Erythropoietin was estimated by radioimmunoassay in serum from 78 cord blood samples, collected in the second and third trimesters in 72 pregnancies. In 43 samples obtained during or after normal pregnancy (from 19 to 42 weeks gestation) erythropoietin levels increased with gestation. Cord blood haemoglobin also increased with gestation, but the rate of increase was less during the last weeks of pregnancy. Erythropoietin levels were similar in the cord blood of infants of the same gestation, whether born vaginally or by caesarean section. The fetus can respond to severe anaemia or hypoxia with increased erythropoietin levels as early as 24 weeks gestation. Elevated erythropoietin levels were found in two out of eight infants born after labour in which there was 'acute' fetal distress, suggesting the presence of unrecognized chronic fetal hypoxia in these pregnancies.

The precise role of erythropoietin in the regulation of fetal erythropoiesis in man is uncertain. Fetal polycythaemia frequently occurs with intrauterine growth retardation (Humberg *et al.* 1969), and increased levels of erythropoietin have been reported in pathological pregnancies possibly associated with placental insufficiency (Finne 1966; Meberg 1980). This suggests that erythropoietin is important in the regulation of fetal red cell production, at least during the third trimester of pregnancy.

In the present study erythropoietin was estimated by radioimmunoassay to examine the relation of erythropoietin levels to advancing gestation in the second and third trimesters of pregnancy, and to determine the effects of anaemia and intrauterine transfusion on erythropoietin levels in early fetal life.

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Subjects and methods

Cord blood samples were obtained by venepuncture in 60 singleton infants and six pairs of twins immediately after birth. In addition, in five other pregnancies blood samples were obtained *in utero* by aspiration from an umbilical vessel under direct vision. Fetoscopy was performed in two patients to exclude β-thalassaemia and before intra-amniotic prostaglandin abortion in three patients. Gestational age (range 19–42 weeks) was calculated from the first day of the last menstrual period and was confirmed in most patients by early ultrasound examination. Serial cord blood samples were collected from two hydropic fetuses (24–30 weeks gestation) with severe rhesus haemolytic disease immediately before direct fetoscopic blood transfusion into an umbilical vessel as described previously (Rodeck *et al.* 1981). Samples of fetal blood for the present study were obtained after adequate diagnostic specimens had been taken and each fetoscopy

was performed with the written consent of the patient and the approval of the local ethical committee. A sample of maternal blood was collected at the time of each fetoscopy.

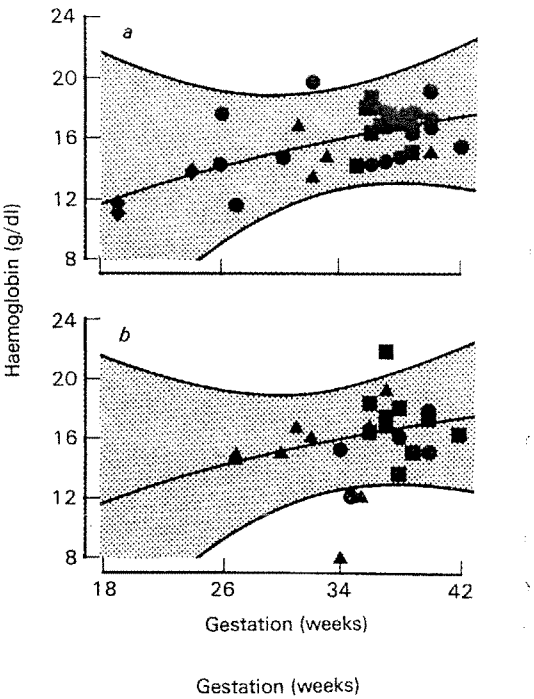
Blood samples for erythropoietin assay were allowed to clot at 20°C and the sera were then frozen in liquid nitrogen before storing at −40°C until thawed for assay. Erythropoietin was estimated in 100–800 µl samples by radioimmunoassay (Cotes 1982), with modification I (Cotes *et al.* 1983). All samples were tested as a batch in a single assay using the second International Reference Preparation of erythropoietin as standard (Annable *et al.* 1972).

Regression analysis was used to relate erythropoietin and haemoglobin levels in normal pregnancy to gestational age; for each, the 95% confidence band for single observations was calculated. For final analysis erythropoietin levels were logarithmically transformed on the basis of an analysis of residuals from fitting a linear relation with gestation age. Haemoglobin appeared to increase less in the last few weeks than earlier in pregnancy so the logarithm of gestational age was used as the independent variable.

Results

In normal pregnancies, there was a highly significant relation between haemoglobin levels and gestation (t=4·5, P<0·001), with haemoglobin increasing from about 12 g/dl at 20 weeks gestation to 17 g/dl at term (Fig. 1*a*). Erythropoietin concentration increased even more significantly with gestation (t=8·1, P≪0·001) using the approprite logarithmic scale for erythropoietin with a four-fold increase in erythropoietin between 20 weeks and term (Fig. 2*a*). The levels of cord haemoglobin and erythropoietin after a normal pregnancy were unaffected by the mode of birth, there being no significant difference at the same gestational age between the 20 infants born vaginally and the 12 infants born by planned elective caesarean section under general anaesthesia (Figs 1*a*, 2*a*) because of previous caesarean section, cephalopelvic disproportion, preterm breech presentation with ruptured membranes or previous intrauterine fetal death.

Table 1 details the 29 abnormal pregnancies. Cord blood samples from these infants have been excluded from the determinations of the normal variation of erythropoietin and haemoglobin concentration with gestation. Results from the twin



Gestation (weeks)

Fig. 1. Cord haemoglobin concentration and gestation in normal (*a*) and abnormal (*b*) pregnancy. ●, Vaginal delivery; ■, forceps; ▲, caesarean section; ✥, fetoscopy. The solid line shows the predicted value and the shaded area represents the 95% confidence band for single observations of haemoglobin in normal pregnancy.

pregnancies are shown in Table 2 and those from the two pregnancies complicated by rhesus-isoimmunization, in which intrauterine transfusions were given, are presented in Fig. 3. The abnormal pregnancies (excluding the two pregnancies complicated by rhesus-isoimmunization detailed in Fig. 3) are shown in Figs 1*b* and 2*b*. There were 14 complicated pregnancies and four of the eight singleton births in this group had erythropoietin levels above the upper limit (at 95% level) of normal variations for gestational age, seven of these eight singleton births were by elective caesarean section.

Table 2 shows the results of cord blood samples from the six pairs of twins. Erythropoietin levels in 10 of the 12 twin infants were greater than expected for those in singleton pregnancies of the same gestation, and in four of them, the level was above the upper 95% level of normal variation. In twin pair no. 6 there was a marked disparity of both cord haemoglobin and erythropoietin, possibly indicating that fetus-to-fetus transfusion had occurred some time before

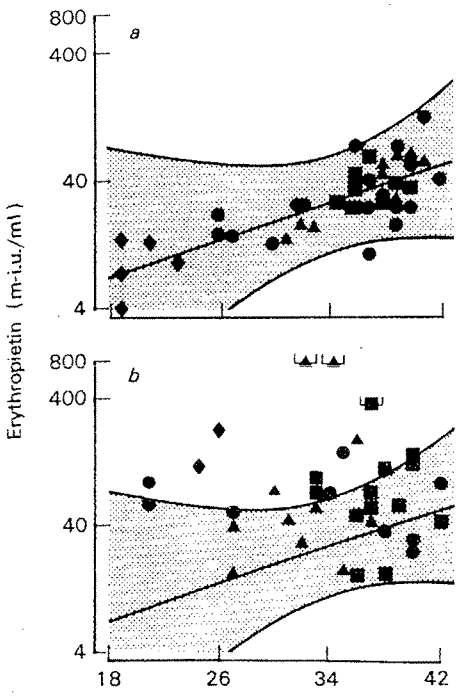


Fig. 2. Estimates of cord serum erythropoietin and gestation in normal (*a*) and abnormal (*b*) pregnancy. Symbols—as for Fig. 1. —▲— —■— Estimates ⩾ plotted value. The solid line shows the predicted value and the shaded area represents the 95% confidence band for single observations of erythropoietin in normal pregnancy.

Table 1. Subjects studied

	Number
Total pregnancies	72
Total infants	78
Normal pregnancy and labour	43
Abnormal pregnancy or labour	29 (35 babies)
Complicated pregnancy	
Essential hypertension	2
Pre-eclampsia	3
Intrauterine growth retardation	2
Diabetic	1
Twin	6
Total	14
Acute fetal distress in labour	8
Fetal abnormality	
Down's syndrome	1
Cyanotic heart disease	2
Polycystic kidneys	1
Total	4
Rhesus haemolytic disease	
with intrauterine transfusions	2
without intrauterine transfusion	1
Total	3

delivery and that the resulting relative anaemia in one twin induced an increase in erythropoietin secretion in that twin. Although the difference in birthweight and haemoglobin level in this twin pair might be accounted for by intrauterine growth retardation in one twin this does not explain the high erythropoietin in the other twin.

All the labours had fetal monitoring and eight of them had signs of acute fetal distress [fetal bradycardia or tachycardia (<120 or >160

Table 2. Estimates of cord serum erythropoietin and cord haemoglobin in six pairs of twins

Twin pair	Gestation (weeks)	Birthweight (g)	Cord haemoglobin (g/dl)	Serum erythropoietin (m-i.u./ml)
1	21	—	15·1	88*
		—	14·2	60
2	27	1065	14·5	39
		1165	14·8	16
3	33	1660	—	76*
		1780	—	97*
4	36	2600	16·7	17
		2580	18·4	49
5	37	2980	17·0	73
		2880	17·3	56
6	38	2230	18·1	17
		2640	13·5	106*

* Greater than the upper 95% level of normal variation for gestational age.

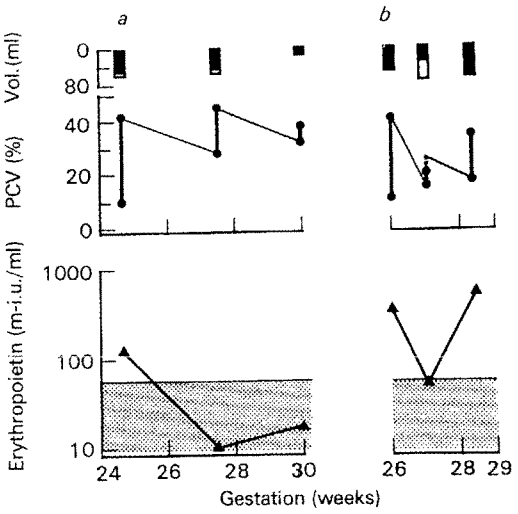


Fig. 3. Estimates of cord serum erythropoietin and packed cell volume (PCV) in two fetuses (*a* & *b*) before and after transfusions *in utero*. ■, Intravascular; □, intraperitoneal. The shaded area represents the 95% confidence band for single observations of erythropoietin in normal pregnancy.

Table 3. Estimates of erythropoietin in paired samples of maternal and fetal serum at the time of fetoscopy

Subject	Gestation (weeks)	Fetal erythropoietin (m-i.u./ml)	Maternal erythropoietin (m-i.u./ml)
1	19	14	18
2	19	4	54
3	19	8	18
4	21	14	27
5	24	10	33
6†	24	118*	59
	27	9	63
	30	20	69
7†	26	340*	44
	27	53	51
	28	536*	50

* Greater than the upper 95% level of normal variation for gestational age.

† Nos. 6 and 7 are the two fetuses with rhesus haemolytic disease who were transfused *in utero*, their results are shown in Figs 3*a* and 3*b* respectively.

beats/min) or meconium stained amniotic fluid]. Two of these infants had erythropoietin levels above the upper 95% confidence limit for gestational age. There was no significant difference in the mean duration of the first or second stage of labour between the 31 normal and 11 abnormal singleton pregnancies that ended in vaginal delivery.

High levels of erythropoietin were found in an infant with Down's syndrome (erythropoietin >400 m-i.u./ml, cord Hb 21·8 g/dl), in two infants with intrauterine growth retardation [erythropoietin >800 and 150 m-i.u./ml, and Hb (infant) 14·6 and (cord) 12·2 g/dl respectively], in an infant of a diabetic mother (erythropoietin 181 m-i.u./ml, cord Hb 16·8 g/dl) and in three infants with severe rhesus incompatibility and anaemia before transfusion (erythropoietin >800, 340 and 118 m-i.u./ml, cord Hb <8 g/dl in each infant). Two infants with cyanotic congenital heart disease and one infant with non-functioning polycystic kidneys had normal levels of erythropoietin in cord blood.

Fig. 3 shows the erythropoietin levels and packed cell volume (PCV) before and after transfusion plotted against gestational age in the two fetuses who were given direct intravascular and intraperitoneal transfusions *in utero*. In one of them (Fig. 3*a*) the initial high erythropoietin fell to within the normal variation for gestation after the first transfusion. A live infant was born at 33 weeks gestation after three successful intrauterine transfusions. On the first two occasions fetal ascitic fluid was aspirated and transfusion was intraperitoneal as well as intravascular. On the third occasion resolution of hydrops was apparent on ultrasound. In the other (Fig. 3*b*), the pre-transfusion PCV was initially 10% and the erythropoietin level was high (340 m-i.u./ml). The erythropoietin level fell following the first transfusion and rose again after the less successful second transfusion. Despite a successful third transfusion and some improvement in the hydrops, gross hydramnios persisted and the fetus died during preterm labour at 30 weeks gestation.

Estimates of immunoreactive erythropoietin in corresponding maternal and fetal serum samples collected at the time of fetoscopy (Table 3) showed no relation between maternal and fetal levels. Maternal erythropoietin was greater than the corresponding fetal level except in subjects 6 and 7 (the two fetuses depicted in Figs 3*a* and 3*b* respectively with rhesus haemolytic disease). In these, high fetal levels were not associated with abnormally high maternal levels (Cotes *et al.* 1983*b*) of erythropoietin.

...in paired samples
...time of fetoscopy

	Maternal serum erythropoietin (m-i.u./ml)
	18
	54
	18
	27
	33
	59
	63
	69
	44
	51
	50

...of normal variation

...with rhesus haemo-
...*in utero*, their
...respectively.

...thropoietin fell to
...ation after the
...was born at 33
...successful intra-
...two occasions
...and transfusion
...vascular. On
...hydrops was
...(Fig. 3*b*), the
...10% and the
...m-i.u./ml). The
...the first trans-
...less successful
...successful third
...ment in the
...ated and the
...at 30 weeks

...thropoietin in
...serum samples
...(Table 3)
...ternal and fetal
...greater than
...in subjects 6
...Figs 3*a* and 3*b*
...disease). In
...sociated with
...Cotes *et al.*

Discussion

Erythropoietin was found in all cord blood samples tested, even as early as at 19 weeks gestation. In addition, physiological responses to severe anaemia and to intrauterine transfusion were seen from 24 weeks gestation.

Estimation of erythropoietin by radioimmunoassay has permitted us to study the normal variation of serum erythropoietin in fetal life. We found a steady increase in erythropoietin levels from 19 weeks gestation until term (Fig. 2*a*). Using different methods which may be less specific than radioimmunoassay, other workers have reported elevation of erythropoietin levels with increasing gestation and a marked increase, but wide variation in values at or near term (Meberg 1980; Halvorsen 1963; Finne 1964; Mann *et al.* 1965; Halvorsen & Finne 1968). We have shown previously that fetal progenitor cells from peripheral blood are more sensitive *in vitro* to erythropoietin than adult progenitor cells (Linch *et al.* 1982) and we suggest that the increase in fetal erythropoietin production during the third trimester, when the rate of increase in haemoglobin concentration slows down, is related to the gradual switch from fetal to adult type of progenitor cells.

Maternal erythropoietin levels also increase during pregnancy (Cotes *et al.* 1983*b*). However, evidence from other species suggests that transplacental transfer of erythropoietin does not occur (Zanjani & Gordon 1971; Zanjani *et al.* 1974) and our results (Table 3) suggest that this is also true in man.

The present study demonstrates that the mode of delivery does not influence erythropoietin levels, as similar levels were found in the cord blood of infants of the same gestation whether born vaginally or by caesarean section. Two out of the eight infants born after labour in which there was acute fetal distress had elevated erythropoietin levels, suggesting unrecognized chronic fetal hypoxia during these pregnancies or a rapid response to the fetal distress with increased erythropoietin secretion.

Increased fetal erythropoietin levels in the presence of placental insufficiency have been reported (Finne 1966; Meberg 1980) and we have confirmed this observation. We have also found a high level of erythropoietin in the cord blood of one infant born to a mother with insulin-dependent diabetes. In such infants erythrocytosis is often present and may be induced by increased secretion of erythropoietin found to occur in association with fetal hyperinsulinism (Widness *et al.* 1981). These authors suggest that erythropoietin production may be stimulated by insulin; an alternative explanation is the possibility of placental insufficiency in diabetics. We found high levels of erythropoietin and haemoglobin in the cord blood of an infant with Down's syndrome. Polycythaemia occurs more frequently in infants with Down's syndrome than in normal infants; it may occur as an isolated haematological abnormality or as part of a more generalized disorder of haemopoiesis (Weinberger & Oleinick 1970).

The present study provides information about secretion of erythropoietin during normal gestation and suggest that estimates of cord serum erythropoietin may provide an indicator of unrecognized fetal hypoxia.

Acknowledgments

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