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blood prothrombin
fibrinogen

Development of Blood Coagulation—A Fetal Lamb Model

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Summary

To study the normal development of blood coagulation factor activities in a growing fetus while avoiding the effects of labor and delivery, a chronic fetal lamb model was developed in which serial blood samples from 10 fetuses were studied during the third trimester of pregnancy and 24 hr after birth. Under operating room conditions with sterile technique, a polyethylene catheter to which heparin had been bound to both internal and external surfaces was inserted into the femoral artery of the fetus. The catheter was brought out through a skin pouch to the side of the ewe and enclosed in a zip lock bag. Blood samples were withdrawn from the catheter three times each week for measurement of coagulation factor activities. Levels of coagulation factor activities at birth in noncatheterized animals were not different from those found in catheterized animals except for factor IX activity which was 12% higher in the catheterized animals ($0.02 < P < 0.05$). The patterns of development for each of the coagulation factors were similar in all 10 animals studied. Fibrinogen, prothrombin, and factor VII show a decrease in activity early in the last trimester of pregnancy whereas other factors V, VIII, IX, X, XI, XII, and XIII show a gradual increase in activity throughout the last trimester of pregnancy. Both factors VIII and IX show a significant increase in activity (23% factor VIII and 12% factor IX) associated with the process of delivery. The levels of coagulation factor activities at birth in the lamb relative to adult sheep normals are similar to those found in humans with the exception of factor XIII. Factor XIII is at normal levels in the newborn lamb and is reported to be at levels approximately 50% of the adult level in human infants.

Speculation

The development of a chronic fetal lamb preparation has allowed the construction of developmental patterns for coagulation factor activities throughout the last trimester of pregnancy. Further studies into the biology of the changes in coagulation factor activities observed and the influences of such stresses as hypoxemia and premature delivery should enhance our understanding of the bleeding neonate.

Major hemorrhage or thrombosis was found in 40% of neonatal deaths in a recent survey (9), and intraventricular hemorrhage was detected in 26% of 101 autopsies of newborn infants in another survey (25). Thus, problems related to hemorrhage and/or thrombosis play a major role in the mortality of newborn infants, particularly premature infants. Deficiencies in various blood coagulation factor activities are reported to be present in the normal newborn with more severe deficiencies in the normal premature infant (10). The significance of coagulation factor deficiencies in relation to bleeding is often difficult to evaluate in the newborn infant because normal factor activity levels vary widely and are dependent upon gestational age (10). Blood coagulation factor activity measurements in premature infants are available by study of these infants after delivery or by study of fetuses of women undergoing abortion. The values obtained in these studies emphasize the wide variation in factor activities (2, 9, 10, 13, 15, 23). The variability may be normal or may also be variability in association with the stresses of hysterotomy, labor, and delivery.

To study the normal development of blood coagulation factor activities in a growing fetus while avoiding the effects of labor and delivery, a chronic fetal lamb model has been developed in which each fetus studied was followed longitudinally during the last third of gestation and 24 hr after birth. The model and measurements of coagulation factor activities in the model are the subject of this report.

MATERIALS AND METHODS

ANIMAL PREPARATION

Pregnant sheep were obtained from existing sources and were housed throughout the studies at the University of Iowa Animal Quarters. The gestational ages of the sheep fetuses were known based upon induced ovulation technique (1, 14). Pregnancy was confirmed 60 days later by monitoring the fetal heart. X-ray examination of each ewe was obtained as the fetus approached 100 days of gestation to confirm the appropriate gestational age.

Anesthesia of the ewe and surgery of the fetus were performed as described previously (22). Under operating room conditions with sterile technique, a polyethylene catheter (inside diameter, 0.86 mm, and outside diameter, 1.27 mm) to which heparin had been bound to both internal and external surfaces (CORMED, Inc., Middleport, NY) was inserted into the femoral artery of the fetus. The catheter was brought out through a skin pouch to the side of the ewe and enclosed in a zip lock bag. After surgery, the ewe was kept in a restricted area and fed a standard diet. Ampicillin (1 g) was given intramuscularly at the completion of surgery as prophylaxis for potential contamination of the mother or fetus.

BLOOD SAMPLING

Normal gestation in the lamb is 145 to 150 days. Sampling in the fetal lambs was begun 5 days after catheterization at which time the mean age of the fetal lambs was 107 days (range, 104 to 120 days). Blood samples were withdrawn from the catheter three times each week. After removing an initial one cm^3 of blood to clear the line of saline, two 3 ml aliquots of blood were obtained with each phlebotomy. Critical attention to aseptic technique was required in obtaining samples to prevent contamination of the fetus and subsequent abortion. New sterilized stopcocks were used with each sampling, and 1 g of Ampicillin was given intramuscularly to the ewe after each sampling. Within 24 hr after delivery of a full-term newborn lamb, another blood sample was obtained by direct venipuncture. Samples from 10 noncatheterized newborn lambs were also obtained within 24 hr of delivery for comparison with the samples from the catheterized animals. All samples were collected in plastic tubes. Samples for measurement of blood coagulation factor activities were diluted nine parts of blood to one part of 0.1 M sodium citrate (pH 5.0). Samples for measurement of the plasmin digestion products of fibrin/fibrinogen were collected and mixed nine parts blood to one part of 0.05 M

epsilonamino caproic acid:0.125 M calcium chloride:thrombin (10 units/ml). The quantities of blood withdrawn from the fetus each week (18 ml) are relatively small in relation to the total fetal blood volume, (135 ml/kg) which includes both the fetal and placental circulations (6) thus the hematocrits remained stable throughout. The mean hematocrit during gestation was 33.7% with a range of 30 to 43%. At birth, the mean hematocrit was 35.6% with a range of 27 to 54%. Blood samples were centrifuged at 1800 × *g* for 30 min and the supernatant platelet-poor plasma or serum was removed and stored at −70°C in aliquots and thawed just before measurement of clotting factor activities.

COAGULATION FACTOR ACTIVITY MEASUREMENTS

Coagulation factor activities were measured using standard techniques and included the prothrombin time (24) and the partial thromboplastin time (21). Specific coagulation factor activities II, V, VII, VIII, IX, X, XI, and XII were assayed using one-stage assays measuring the ability of the test plasma to correct human plasma known to be deficient in the factors to be tested (20). Fibrinogen concentration was measured as clottable protein (3). Factor XIII was assayed according to the method of Lorand *et al.* (17) as modified by Henriksson *et al.* (11). The activities measured are reported as percentages of a reference standard of sheep plasma obtained from 10 adult rams. Plasmin digestion products of fibrin/fibrinogen were measured according to the method of Meskey *et al.* (18) using rabbit anti-sheep fibrinogen antibody and sheep fibrinogen coated red blood cells. Fibrin monomer was measured by the immune precipitate method substituting anti-sheep fibrinogen antibody throughout (16).

ANALYSIS OF DATA

The levels of factor activities found at delivery in the 10 catheterized animals were compared with activities of these same factors in ten noncatheterized animals using an unpaired *t* test. The results of coagulation factor activity levels on the blood samples from each fetus before delivery were analyzed to determine if the pattern of emergence of factor activity was similar in all animals. Separate regression models were constructed for each of the coagulation factors during fetal life. It was assumed that although individual animals like humans might differ in the absolute level of factor activity, the shape of the response of a factor activity with time during gestation would be reproduced in all animals. To test this hypothesis, the regression model used was a model with separate intercepts for each individual animal but the same coefficients for the other terms of the polynomial. Step-down regression techniques were used to determine the degree of polynomial that most adequately described the data. Polynomial models of progressively smaller degrees were fit to the data and the fit compared to the fit of a fifth degree polynomial model. When significant loss in prediction was observed, the process was stopped. The adequacy of fit of data to the models was measured by the squared multiple correlation coefficient R^2. This statistic (R^2) can be interpreted as the proportion of the variability in the factor activity that is explained by the regression model with time. The polynomial model was expanded to predict the level of activity for each factor at birth. This predicted level was then compared with the observed level using a paired *t* test to determine whether there were significant changes associated with the process of labor and delivery and thereby not predicted by predelivery data. To simplify the regression curves for graphical presentation, curves were fit to the data without using separate intercepts to adjust for differences in absolute levels of factor activity between animals. These curves were the same shape as those using a separate intercept for each animal but represented an average curve for all the animals studied. There was usually a drop in R^2 values for these curves but the coefficients for terms other than the intercept term were nearly the same for the two methods. These curves along with 95% confidence regions are displayed on figures. All statistical analyses were done using the GLM procedure from the SAS statistical package.

RESULTS

In Table 1 are presented the means ± one standard deviation of values for coagulation factor activities measured on 10 adult rams and eight nonpregnant ewes in relation to a reference standard consisting of pooled plasma from 10 adult rams. Variability in the levels of coagulation factor activities between animals is similar to that seen in humans. There were no significant differences between the values obtained on rams and nonpregnant ewes.

In Table 2, the levels of activity at birth in the 10 catheterized animals is compared with levels of activity at birth in 10 noncatheterized animals. Factor IX activity was found to be lower in the noncatheterized animals at a level of significance ($0.02 < P < 0.05$). The values at birth for the other factors were similar in catheterized and noncatheterized animals.

The regression curve for the prothrombin time obtained on the 10 fetuses during the last 46 days of gestation is presented in Figure 1. The points on the graph represent the individual values, the middle solid line is the regression curve indicating the mean levels of activity, and the lines above and below the mean line define the 95% confidence limits. A horizontal line across the graph indicates 100% of adult activity. The mean activity ± 1 standard deviation at birth is designated by a bar at 147 days gestation. Figures 2 through 12 graphically display the values obtained for the partial thromboplastin time, factors I, II, V, VII, VIII, IX, X, XI, XII, and XIII during gestation and at birth. As is seen, all factor activities are below adult levels early in the third trimester of pregnancy and in general increase in activity before parturition. Fibrin/fibrinogen degradation products were unchanged throughout gestation and after delivery. The highest value for fibrin/fibrinogen degradation products during gestation was 2.4 μg/ml which is within 2 standard deviations of the mean for normal adult sheep. The values for fibrin/fibrinogen degradation products are therefore not graphically displayed. Fibrin monomer levels were done on 12 random samples from nine of the chronically catheterized animals (107 to 143 days gestation). The fibrin monomer levels in the fetal samples were 5.3 ± 3.1 μg/ml with a range of 1.5 to 10 μg/ml. These values are normal when compared with adult levels (means, 6.2 ± 2.7 μg/ml; range, 3.2 to 12.8 μg/ml).

The reproducibility of the developmental patterns of each of the coagulation factor activities during gestation is shown in Table 3. Factor activities were first adjusted to a common intercept. Then a comparison of the shape of the response of activity with time in all of the animals for each factor was made by analysis of the proportion of the variability of activity that is explained by the regression equation for that factor. In Table 3 is presented the R^2 values for the developmental patterns of each coagulation factor activity in all animals. The value R^2 indicates the proportion of variability in the data that is explained by the regression curve. An R^2 of 1 would indicate that all values precisely fit the regression equation. As is seen in Table 3, the developmental patterns in the

Table 1. *Coagulation factor activities in adult sheep*

Factor	Mean + 1 S.D. (18 adult sheep)
PT	11.4 ± 0.5 sec
PTT	41.5 ± 0.5 sec
Fibrinogen	191 ± 70 mg/dl
II	99 ± 18%
V	108 ± 28%
VII	84 ± 18%
VIII	91 ± 26.5%
IX	99 ± 19.2%
X	118 ± 26.3%
XI	95 ± 34%
XII	99 ± 16%
XIII	123 ± 20%
FDP	0.9 ± 1 μg/ml
Fibrin monomer	6.2 ± 2.7 μg/ml

Table 2. *Coagulation factor activities at birth*

Factor	Catheterized ($N = 10$)	Noncatheterized ($N = 10$)	P
PT	14.8 ± 1.8 sec	16.0 ± 1.1 sec	>0.05
PTT	44.7 ± 8.7 sec	47.6 ± 6.6	>0.05
Fibrinogen	117 ± 54 mg/dl	99 ± 36 mg/dl	>0.05
II	66 ± 22%	63 ± 26%	>0.05
V	108 ± 29%	93 ± 29%	>0.05
VII	80 ± 31%	69 ± 16%	>0.05
VIII	85 ± 29%	73 ± 26%	>0.05
IX	61 ± 13%	49 ± 12%	$0.02 < P < 0.05$
X	65 ± 20%	68 ± 16%	>0.05
XI	66 ± 13%	50 ± 18%	>0.05
XII	93 ± 25%	108 ± 20%	>0.05
XIII	92 ± 24%	106 ±36%	>0.05

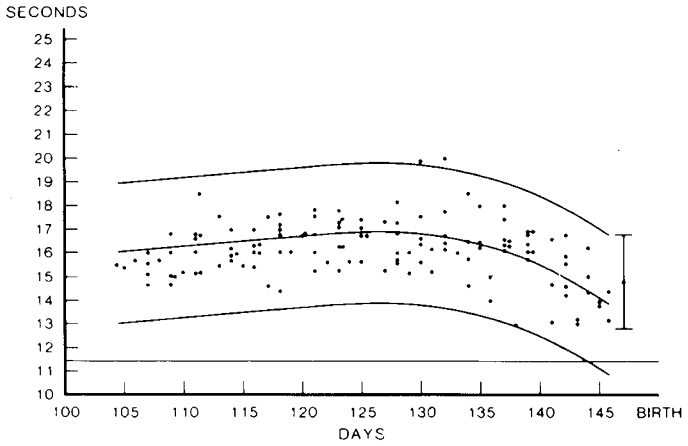

Fig. 1. Prothrombin time during last trimester of pregnancy in 10 lamb fetuses (mean ± 95% confidence regions). *Bar at birth* = mean ± 1 S.D. within 24 hr of delivery.

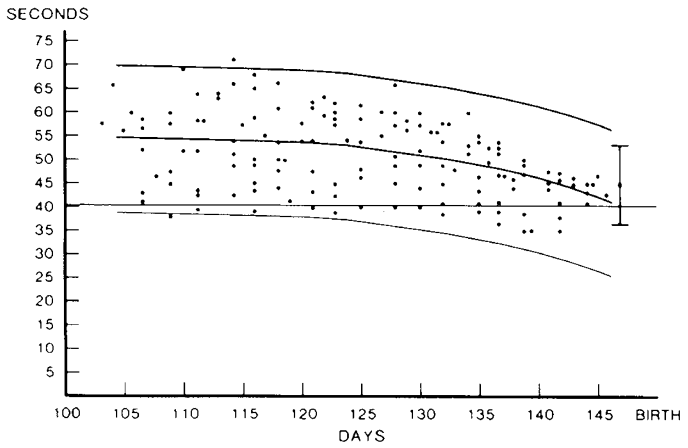

Fig. 2. Partial thromboplastin time during last trimester of pregnancy in 10 lamb fetuses (mean ± 95% confidence regions). *Bar at birth* = mean ± 1 S.D. within 24 hr of delivery.

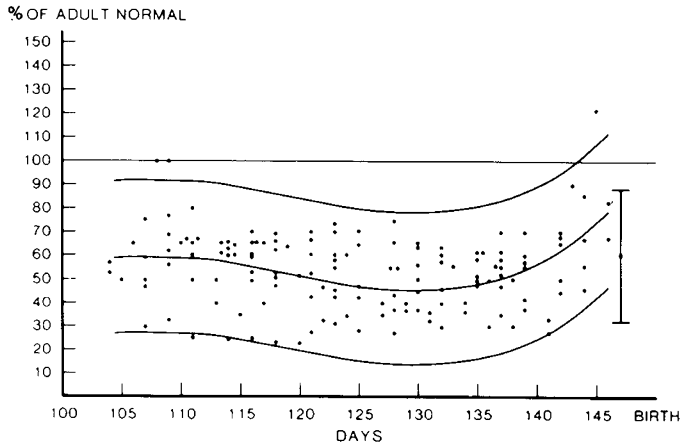

Fig. 3. Fibrinogen concentration during last trimester of pregnancy in 10 lamb fetuses (mean ± 95% confidence regions). *Bar at birth* = mean ± 1 S.D. within 24 hr of delivery.

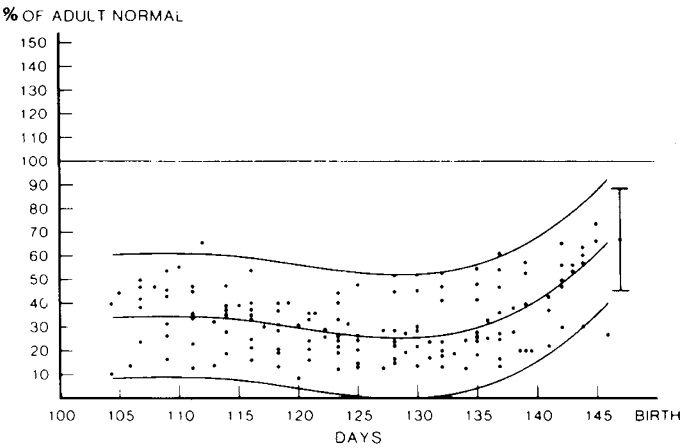

Fig. 4. Prothrombin activity during last trimester of pregnancy in 10 lamb fetuses (mean ± 95% confidence regions). *Bar at birth* = mean ± 1 S.D. within 24 hr of delivery.

animals are quite similar. For example, the regression equation explains 77% of the variability observed in factor VIII activity. Another regression equation explains 89% of the variability in factor XI activity.

DISCUSSION

The development of a chronic fetal lamb model has made possible the measurement of blood coagulation factor activities throughout the last trimester of pregnancy. Levels of activity at birth in noncatheterized animals were not different from those found in catheterized animals except that factor IX activity was significantly higher in the catheterized animals ($0.02 < P < 0.05$) (Table 2). The lack of differences between catheterized and noncatheterized animals for all other factors suggests that the process of catheterization does not influence the developmental pattern or activate the coagulation cascade. Additional evidence for the lack of activation of coagulation is the absence of increased levels of fibrin/fibrinogen degradation products in all samples and the absence of increased levels of fibrin monomer in 12 random samples from nine of the fetal lambs.

The patterns of development for each of the factors are similar

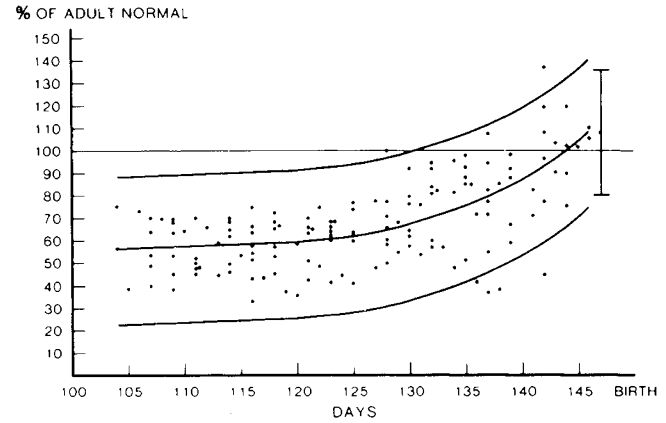

Fig. 5. Factor V activity during last trimester of pregnancy in 10 lamb fetuses (mean ± 95% confidence regions). *Bar at birth* = mean ± 1 S.D. within 24 hr of delivery.

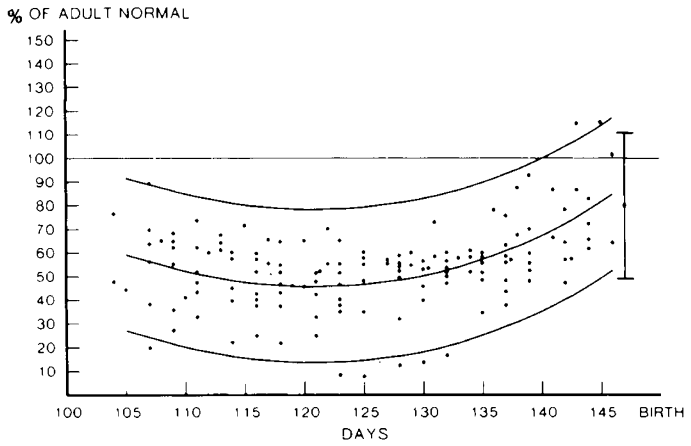

Fig. 6. Factor VII activity during last trimester of pregnancy in 10 lamb fetuses (mean ± 95% confidence regions). *Bar at birth* = mean ± 1 S.D. within 24 hr of delivery.

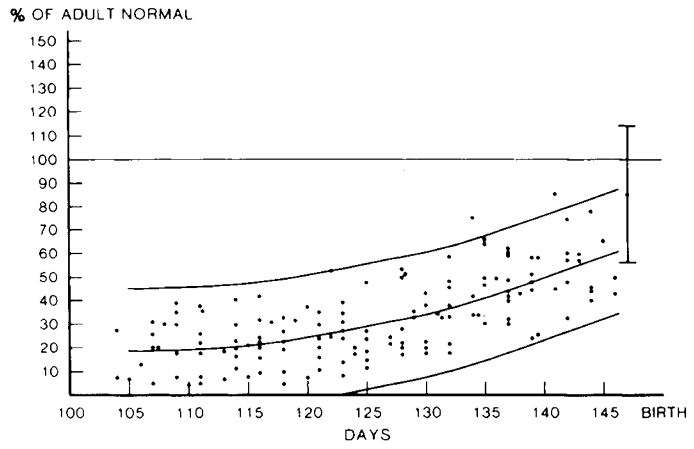

Fig. 7. Factor VIII activity during last trimester of pregnancy in 10 lamb fetuses (mean ± 95% confidence regions). *Bar at birth* = mean ± 1 S.D. within 24 hr of delivery.

in all animals. The shape of the activity over time in different animals is remarkably constant as shown by the ability of single regression curves to explain most of the changes in activity that occur throughout the last trimester of development (Table 3). The chronic fetal lamb model can therefore provide insight into both similarities and differences in developmental patterns of the coagulation factors.

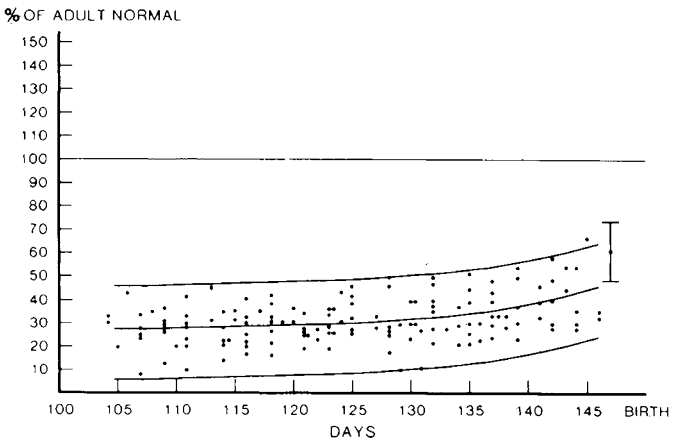

Fig. 8. Factor IX activity during last trimester of pregnancy in 10 lamb fetuses (mean ± 95% confidence regions). *Bar at birth* = mean ± 1 S.D. within 24 hr of delivery.

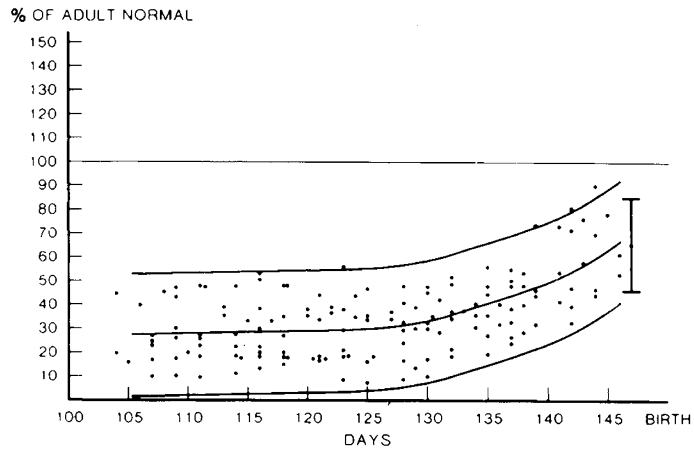

Fig. 9. Factor X activity during last trimester of pregnancy in 10 lamb fetuses (mean ± 95% confidence regions). *Bar at birth* = mean ± 1 S.D. within 24 hr of delivery.

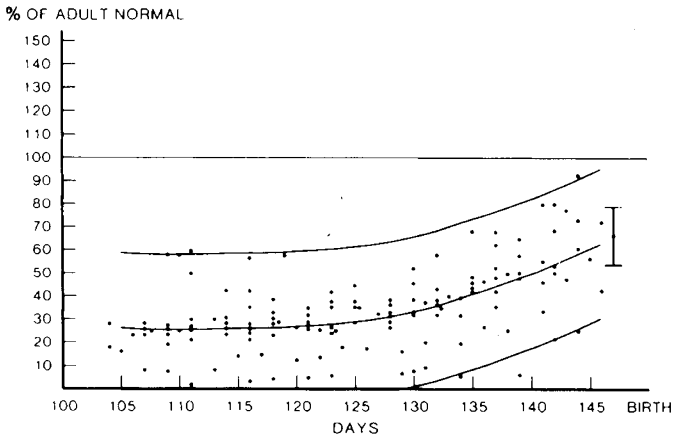

Fig. 10. Factor XI activity during last trimester of pregnancy in 10 lamb fetuses (mean ± 95% confidence regions). *Bar at birth* = mean ± 1 S.D. within 24 hr of delivery.

Fibrinogen concentration in the fetal lamb during the last trimester of pregnancy is the least consistent of all of the factors ($R^2 = 0.49$). The average concentration throughout the last trimester is 53% of the adult normal level. The changes in concentration appear to be an initial decrease during the early part of the last trimester followed by an increase beginning at about 130 to 135

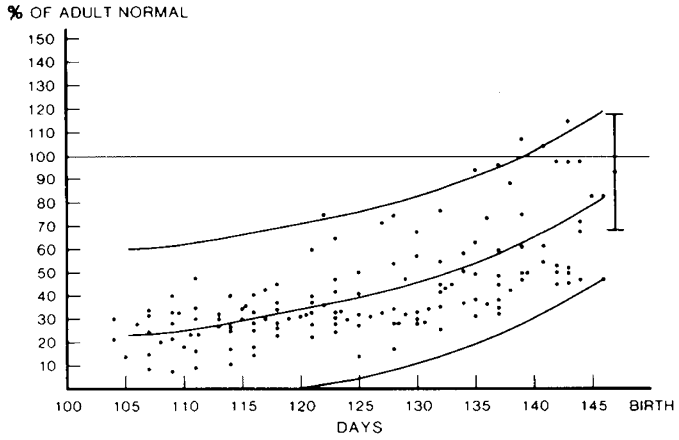

Fig. 11. Factor XII activity during last trimester of pregnancy in 10 lamb fetuses (mean ± 95% confidence regions). *Bar at birth* = mean ± 1 S.D. within 24 hr of delivery.

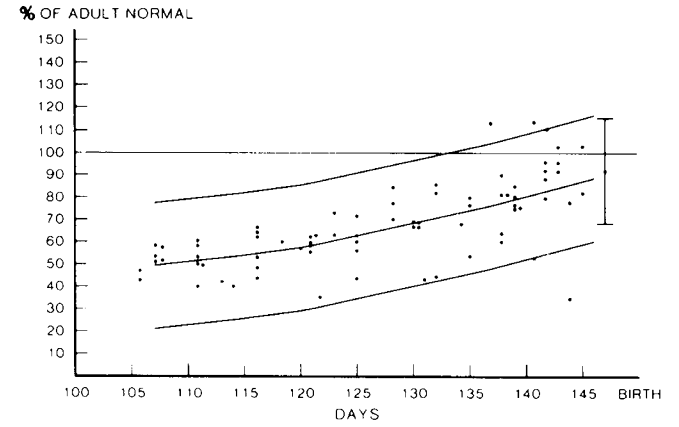

Fig. 12. Factor XIII activity during last trimester of pregnancy in 10 lamb fetuses (mean ± 95% confidence regions). *Bar at birth* = mean ± 1 S.D. within 24 hr of delivery.

Table 3. R^2 *values during gestation* ($N = 10$)

Factor	R^2
PT	0.69
PTT	0.80
I	0.49
II	0.74
V	0.67
VII	0.77
VIII	0.77
IX	0.60
X	0.85
XI	0.89
XII	0.76
XIII	0.84

days gestation. At birth, the mean concentration of fibrinogen averages 117 mg % or 60% of the adult normal level. Human studies have shown that the concentration of fibrinogen in the full-term infant is 75% of that for healthly older children and preterm infants have concentrations approximating that of the full term (23). Although the mean value at birth appears to be somewhat lower than expected, the level is within the range predicted by expanding the regression curve generated by analysis of fetal samples. Whether the increase beginning at 130 to 135 days of gestation is a result of an increase in synthesis, a decreased catabolism, a change in blood volume, or perhaps a change in the relative concentrations of fetal and adult fibrinogens is unknown and will require further study.

The activities of the vitamin K dependent cogulation factors (II, VII, IX, and X) are below adult levels throughout the last trimester of pregnancy and are respectively 66 ± 22, 80 ± 31, 61 ± 13, and 65 ± 20% of normal at birth. The values in human infants, although widely variable, are also reported to be lower than normal adult levels at birth: factor II, 58% (8); factor VII, 63% (8); factor IX, 27% (19); and factor X, 64% (4). The developmental pattern seen with prothrombin is almost identical to that of fibrinogen and a similar pattern is also seen with factor VII. The pattern shows a higher initial concentration of factor activity early in the third trimester which subsequently falls at the midpoint of the third trimester and then increases just before delivery. The early fall in activity does not appear to be related to the surgical procedure of catheter placement. The day of surgery varied in the animals ranging from 99 to 115 days of gestation and thus provided the opportunity to examine early changes in activity in relation to the day of surgery. Regression equations using the days after surgery as the dependent variable did not explain the variability in activities whereas as shown in Table 3 equations based on gestational age did explain the majority of the variability. Whether the initial fall in fibrinogen concentration and factors II and VII activities represents an actual decrease in synthesis, an increased catabolism, or a constant turnover with an expanding blood volume is unknown. The values at birth attained for prothrombin and factor VII and X are as predicted based upon the developmental patterns. There does, however, appear to be a significant increase in factor IX activity associated with birth. The mean level at birth (61%) is greater than predicted (49%) based upon expanding the regression curve ($P = 0.015$). Factor IX activity was also slightly higher at birth in the catheterized animals; 61% vs. 49% (Table 2). The mechanism of the increase in activity at birth and the association if any to catheterization is unexplained.

Factor V activity is normal at birth in the newborn lamb (108%). A finding also true in human newborns (23). In contrast to fibrinogen, factor II, and factor VII, there is no initial decrease in factor V activity early in the third trimester. The activity of factor V increases gradually throughout the third trimester, accelerating in the mid to late third trimester. The levels observed at birth are predicted from the predelivery developmental pattern.

Factor VIII follows a developmental pattern not unlike that of factor V but starting at a lower initial concentration approximating 20% of the normal adult level. A significant increase in factor VIII coagulant activity appears in association with delivery as the observed post delivery activity mean (85%) is significantly above that expected (mean, 62%) based upon the predelivery developmental pattern ($P = 0.028$). As in the lamb model, factor VIII coagulant activity in human newborns shows an increase with increasing gestational age and is reported to be normal or to exceed normal adult levels at birth (7, 23). Factor VIII antigen and von Willebrand activity was not measured in these studies.

Factor XI remains below adult levels at term (66%). As in the lamb, the activity of factor XI in humans is decreased at birth, 65% in one study (4) and 36% in another study (12). The mean level of factor XII in the newborn lamb is normal (93%). Factor XII in human infants has been reported to be 84% of normal in one study (4) and 47% in another (5).

Factor XIII activity in the fetal lamb begins at approximately 50% of normal early in the third trimester and follows a gradual almost linear increase to 92% of normal at term. The value at term is as predicted from the prenatal developmental pattern but is above the 50% level reported for the human infant (11).

The measurement of blood coagulation factor activities in the chronic fetal lamb preparation has allowed the construction of developmental patterns for coagulation factor activities throughout the last trimester of pregnancy. Differences in the developmental patterns suggest there may be multiple factors controlling the activity levels. Certain subsets of factors follow very similar patterns of development. Fibrinogen, prothrombin, and factor VII show a decrease in activity early in the last trimester whereas

other factors V, VIII, IX, X, XI, XII, and XIII show a gradual increase activity throughout the last trimester of pregnancy. Both factors VIII and IX show significant increases in activity associated with the process of delivery. The levels of the coagulation factor activities relative to adults at birth in the lamb are similar to those found in humans with the exception that factor XIII is at normal adult levels in the newborn lamb and is reported to be at levels approximately 50% of adult levels in the human infant.

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