

## INHIBITION OF ERYTHROID PROGENITOR CELLS BY ANTI-KELL ANTIBODIES IN FETAL ALLOIMMUNE ANEMIA

JANET I. VAUGHAN, M.D., MONICA MANNING, B.Sc., RUTH M. WARWICK, M.D., ELIZABETH A. LETSKY, M.D.,  
NEIL A. MURRAY, M.D., AND IRENE A.G. ROBERTS, M.D.

### ABSTRACT

**Background** In alloimmune anemia of the newborn, the level of hemolysis caused by the presence of antibodies to antigens of the Kell blood-group system is less than that caused by antibodies to the D antigen of the Rh blood-group system, and the numbers of reticulocytes and normoblasts in the baby's circulation are inappropriately low for the degree of anemia. These findings suggest that sensitization to Kell antigens results in suppression of fetal erythropoiesis as well as hemolysis.

**Methods** We compared the growth in vitro of Kell-positive and Kell-negative hematopoietic progenitor cells from cord blood in the presence of human monoclonal anti-Kell antibodies and anti-D antibodies and serum from women with anti-Kell antibodies.

**Results** The growth of Kell-positive erythroid progenitor cells (erythroid burst-forming units and colony-forming units) from cord blood was markedly inhibited by monoclonal IgG and IgM anti-Kell antibodies in a dose-dependent fashion (range of concentrations, 0.2 to 20 percent), but monoclonal anti-D antibodies had no effect. The growth of these types of cells from Kell-negative cord blood was not affected by either type of antibody. Neither monoclonal anti-Kell antibodies nor monoclonal anti-D antibodies inhibited the growth of granulocyte or megakaryocyte progenitor cells from cord blood. Serum from 22 women with anti-Kell antibodies inhibited the growth of Kell-positive erythroid burst-forming units and colony-forming units but not of Kell-negative erythroid burst-forming units and colony-forming units ( $P < 0.001$  for the difference between groups). The maternal anti-Kell antibodies had no inhibitory effects on granulocyte-macrophage or megakaryocyte progenitor cells from cord blood.

**Conclusions** Anti-Kell antibodies specifically inhibit the growth of Kell-positive erythroid burst-forming units and colony-forming units, a finding that supports the hypothesis that these antibodies cause fetal anemia by suppressing erythropoiesis at the progenitor-cell level. (N Engl J Med 1998;338:798-803.)

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**T**HE Kell blood-group system is one of the major antigenic systems in human red cells. It consists of 23 known antigens that reside on one 93-kd transmembrane protein encoded by a single gene on chromosome 7 (7q33).<sup>1,2</sup> The Kell antigen is expressed only by erythroid progenitor cells and mature erythroid cells.<sup>1,3,4</sup> The Kell blood group is important in clinical medicine because antibodies to the principal antigen, K1, cause

both life-threatening transfusion reactions and severe alloimmune anemia in Kell-positive fetuses and newborn infants.<sup>5,6</sup> Alloimmunization occurs when Kell-negative women, who do not have the K1 antigen on their red cells, become sensitized by carrying a Kell-positive fetus to produce alloantibodies that may cross the placenta and cause fetal anemia in a Kell-positive, but not a Kell-negative, fetus.

Since the introduction of effective prophylaxis against alloimmunization to the D antigen of the Rh blood-group system, anti-Kell antibodies have accounted for 10 percent of the cases of antibody-mediated severe fetal anemia.<sup>7</sup> The mechanism of fetal anemia mediated by anti-Kell antibodies differs in several ways from that of classic hemolytic disease of the newborn associated with anti-D alloantibodies.<sup>7-10</sup> Affected Kell-alloimmunized fetuses have lower numbers of circulating reticulocytes and normoblasts than fetuses with hemolytic disease due to anti-D antibodies, and the levels are inappropriately low for the degree of fetal anemia.<sup>7,10</sup> In addition, the concentrations of bilirubin in amniotic fluid and in fetal or neonatal serum are lower than those in anti-D alloimmunized fetuses.<sup>7,9,11</sup> Also, the titer of anti-Kell antibodies in maternal serum correlates very poorly with the degree of fetal anemia.<sup>12</sup> These observations suggest that in Kell alloimmunization fetal anemia is caused by the suppression of erythropoiesis in addition to hemolysis.

This study was undertaken to test the hypothesis that anti-Kell antibodies cause fetal anemia in part by inhibiting the growth of erythroid progenitor cells. We therefore compared the growth of Kell-positive and Kell-negative hematopoietic progenitor cells from cord blood in the presence of human monoclonal anti-Kell antibodies, anti-D antibodies, and serum from Kell-alloimmunized women.

### METHODS

The study was approved by the institutional review committee of Hammersmith and Queen Charlotte's hospitals, and all samples were collected with informed parental consent.

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From the Departments of Obstetrics and Haematology, Institute of Obstetrics and Gynaecology, Queen Charlotte's Hospital (J.I.V., R.M.W., E.A.L.), and the Department of Haematology, Imperial College School of Medicine at the Royal Postgraduate Medical School (M.M., N.A.M., I.A.G.R.) — both in London. Address reprint requests to Dr. Roberts at the Department of Haematology, Imperial College School of Medicine at the Royal Postgraduate Medical School, Du Cane Rd., London W12 0NN, United Kingdom.

### Cord-Blood Samples

Cells obtained from umbilical-cord blood from uncomplicated deliveries of normal infants at term were used for progenitor-cell assays. The cells were typed as Kell-positive or Kell-negative with monoclonal IgM anti-Kell antibodies (Biotec), and the Rh genotype was determined with antibodies against D, C, c, E, and e (Biotest). Mononuclear cells were isolated from cord blood by density centrifugation, depleted of adherent cells by allowing such cells to adhere to plastic tissue-culture dishes, and cultured in the presence and the absence of monoclonal anti-Kell antibodies, monoclonal anti-D antibodies, serum samples containing anti-Kell antibodies, and control serum samples from pregnant women who had no red-cell alloantibodies.

### Assays of Hematopoietic Progenitor Cells

#### *Erythroid Progenitor Cells*

Cord-blood mononuclear cells were plated at a density of 200,000 per milliliter (20,000 per well) in microtiter plates containing 1.2 percent methylcellulose supplemented with 20 percent fetal-calf serum, 2 U of erythropoietin per milliliter (Terry Fox Laboratories), 1 percent bovine serum albumin (Sigma), 2-mercaptoethanol, 5 percent 5637 conditioned medium,<sup>10</sup> penicillin-streptomycin, and glutamine. The cultures were established in the absence or the presence of serum containing anti-Kell antibodies (20 percent), control serum (20 percent), or serial dilutions of monoclonal anti-Kell or anti-D antibodies (20, 2, 0.2, 0.02, and 0.002 percent). The monoclonal IgM anti-Kell antibodies and anti-D antibodies (both from Biotest) contained azide and were therefore dialyzed to prevent nonspecific inhibition of progenitor-cell growth; the IgG anti-Kell antibodies (Biotec) did not contain azide and therefore were not dialyzed. The IgG monoclonal anti-Kell antibodies and anti-D antibodies were both IgG1. The cultures were incubated in 5 percent carbon dioxide at 37°C, and the number of erythroid colony-forming units was counted after 7 days and the number of erythroid burst-forming units after 14 days. Six wells were plated for each experiment, and the result was calculated as the mean number of colonies per well and expressed as the number of colonies per milliliter of cord blood.

#### *Granulocyte-Macrophage Progenitor Cells*

Cord-blood mononuclear cells were plated at a density of 100,000 per milliliter (10,000 per well) in microtiter plates in 0.9 percent methylcellulose supplemented with 10 percent fetal-calf serum, 10 percent 5637 conditioned medium, penicillin-streptomycin, and glutamine in the presence or the absence of serum containing anti-Kell antibodies (20 percent), control serum (20 percent), or serial dilutions of monoclonal anti-Kell or anti-D antibodies (20, 2, and 0.2 percent). The number of granulocyte-macrophage colony-forming units was counted after 14 days of incubation in 5 percent carbon dioxide at 37°C. The result was calculated as the mean number of colonies per well (six wells per experiment) and expressed as the number per milliliter of cord blood.

#### *Megakaryocyte Progenitor Cells*

Cord-blood mononuclear cells were cultured at a density of 200,000 per milliliter (20,000 per well) in agar in microtiter plates as previously described<sup>13</sup> in the presence or the absence of serum containing anti-Kell antibodies (20 percent), control serum (20 percent), or serial dilutions of monoclonal anti-Kell or anti-D antibodies (20, 2, and 0.2 percent). The number of megakaryocyte burst-forming units and colony-forming units was counted after 21 days, and the megakaryocytic lineage of the cells was confirmed by staining with monoclonal antibodies against platelet glycoprotein IIb/IIIa (CD61, Becton Dickinson) with the alkaline phosphatase-anti-alkaline phosphatase technique. Six wells were plated for each experiment, and the result was calculated as the mean number of colonies per well and expressed as the number per milliliter of cord blood.

### Test and Control Serum Samples

Serum samples from 22 pregnant women found to have anti-Kell antibodies on routine screening during early pregnancy were collected at a median duration of gestation of 28 weeks (range, 23 to 30). These women represented the entire cohort of Kell-immunized pregnant women who were seen at Queen Charlotte's Hospital during the study period (1992 to 1996). Twelve of their fetuses were Kell-positive, with severe anemia detected by fetal-blood sampling, and required serial intrauterine transfusions. The remaining 10 fetuses underwent fetal-blood sampling for Kell grouping. Of these, three fetuses were Kell-positive and had mild anemia, neonatal phototherapy being the sole treatment required. The remaining seven women had a Kell-negative fetus and had not required intrauterine therapy in any previous Kell-positive pregnancies. Serum samples were collected serially during pregnancy from eight of the women whose fetuses had severe anemia. In all cases, clotted blood was separated within four hours after collection and the serum was stored at -20°C until tested. Anti-Kell antibodies were identified and measured with standard serologic methods.<sup>14</sup> Five of the women had other red-cell alloantibodies: two had anti-c antibodies and one each had anti-C, anti-D, and anti-Jk<sub>a</sub> antibodies (Kidd blood group). For these five women all assays used cord-blood progenitor cells that did not contain the antigen to which they had alloantibodies. As additional controls, 11 maternal serum samples containing no red-cell alloantibodies were tested for their effects on erythroid burst-forming units and colony-forming units, granulocyte-macrophage colony-forming units, and megakaryocyte burst-forming units and colony-forming units.

### Statistical Analysis

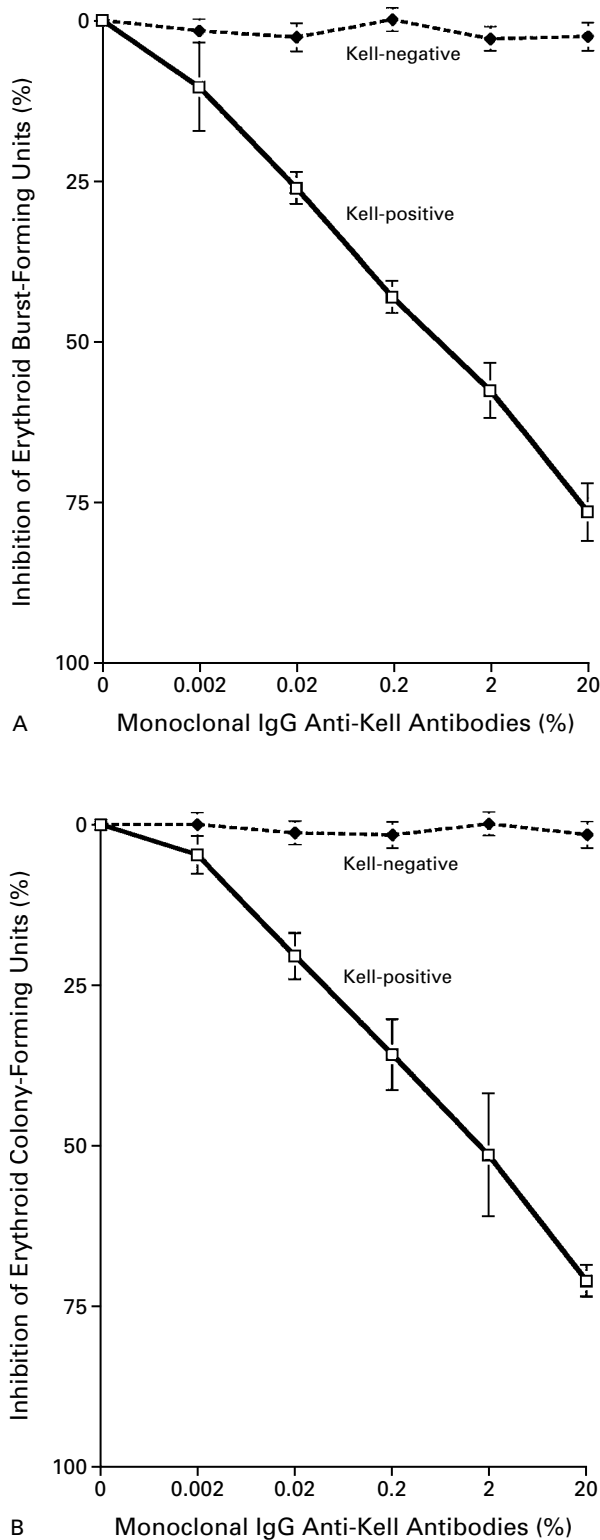
Data were analyzed by parametric or nonparametric methods as appropriate. Paired and unpaired comparisons were carried out with the two-sided Student's t-test or Wilcoxon test and the Mann-Whitney U test, respectively. Pearson's correlation coefficient was only used when the least-squares method supported a linear relation. For linear regression analysis of two separate groups, the slopes were compared with use of the test for the equality of regression coefficients and the positions by analysis of covariance.

## RESULTS

### Effect of Monoclonal Anti-Kell Antibodies on Cord-Blood Hematopoietic Progenitor Cells

#### *Erythroid Burst-Forming Units and Colony-Forming Units*

The monoclonal IgG anti-Kell antibodies caused dose-dependent inhibition of the growth of cord-blood erythroid progenitor cells from Kell-positive but not Kell-negative infants (Fig. 1). The growth of Kell-positive cells was inhibited at all but the lowest concentrations of IgG monoclonal anti-Kell antibodies. The monoclonal IgM anti-Kell antibodies also caused similar dose-dependent inhibition of the growth of Kell-positive cells and no inhibition of Kell-negative cells, even at the highest concentration of antibody (20 percent). The growth of the more immature erythroid progenitor cells, erythroid burst-forming units, was inhibited more than that of the mature erythroid progenitor cells, erythroid colony-forming units, for any given concentration of monoclonal IgG or monoclonal IgM anti-Kell antibodies ( $P=0.01$ ). Monoclonal anti-D antibodies caused no inhibition of cord-blood erythroid burst-forming units or colony-forming units from five Kell-positive, D-positive infants (data not shown). Because the



**Figure 1.** Effect of Monoclonal Anti-Kell Antibodies on the Growth of Cord-Blood Erythroid Progenitor Cells.

Monoclonal IgG anti-Kell antibodies caused dose-dependent inhibition of the growth of erythroid burst-forming units (Panel A) from Kell-positive cord blood ( $r = -0.98$ ,  $P < 0.001$ ) but not Kell-negative cord blood. Similarly, the anti-Kell antibodies inhibited the growth of erythroid colony-forming units (Panel B) from Kell-positive cord blood ( $r = -0.96$ ,  $P < 0.001$ ) but not Kell-negative cord blood. Results are expressed as the mean ( $\pm$ SE) percentage of inhibition in experiments with three Kell-positive and three Kell-negative samples of cord blood.

monoclonal anti-Kell antibodies and the monoclonal anti-D antibodies used in these experiments were both of the IgG1 subclass, the inhibitory effect on Kell-positive erythroid progenitor cells was specific for anti-Kell antibodies rather than representative of nonspecific inhibition by IgG1 antibodies.

**Granulocyte-Macrophage Progenitor Cells**

The monoclonal IgG anti-Kell antibodies did not inhibit the growth of Kell-positive or Kell-negative granulocyte-macrophage progenitor cells from cord blood (Table 1). Similarly, monoclonal IgG anti-D antibodies did not inhibit the growth of granulocyte-macrophage progenitor cells from cord blood from Kell-positive, D-positive infants.

**Megakaryocyte Progenitor Cells**

The monoclonal anti-Kell antibodies also did not inhibit the growth of Kell-positive or Kell-negative megakaryocyte progenitor cells from cord blood. The results of the studies with monoclonal IgG antibodies are shown in Table 1. Similarly, there was no significant difference between groups in the number of megakaryocyte burst-forming units and colony-forming units grown in the presence of monoclonal IgM anti-Kell antibodies. The monoclonal IgG anti-D antibodies did not inhibit the formation of megakaryocyte burst-forming units and colony-forming units in cord blood from Kell-positive, D-positive donors.

**Effect of Maternal Serum Containing Anti-Kell Antibodies on Cord-Blood Hematopoietic Progenitor Cells**

Serum samples from 22 women with anti-Kell antibodies were tested for their ability to inhibit the growth of Kell-positive and Kell-negative erythroid burst-forming units or colony-forming units in cord blood. All 22 samples significantly inhibited the growth of erythroid burst-forming units from Kell-positive samples (median degree of inhibition, 59 percent; range, 16 to 85;  $P < 0.001$ ) but not Kell-negative samples (median degree of inhibition, 4 percent; range, 0 to 30) (Fig. 2A). Maternal serum also inhibited the growth of erythroid colony-forming units from Kell-positive samples of cord blood (median degree of inhibition, 26 percent; range, 7 to

**TABLE 1.** EFFECT OF MONOCLONAL IgG ANTI-KELL ANTIBODIES ON THE GROWTH OF KELL-POSITIVE AND KELL-NEGATIVE GRANULOCYTE-MACROPHAGE PROGENITOR CELLS AND MEGAKARYOCYTE PROGENITOR CELLS FROM CORD BLOOD.\*

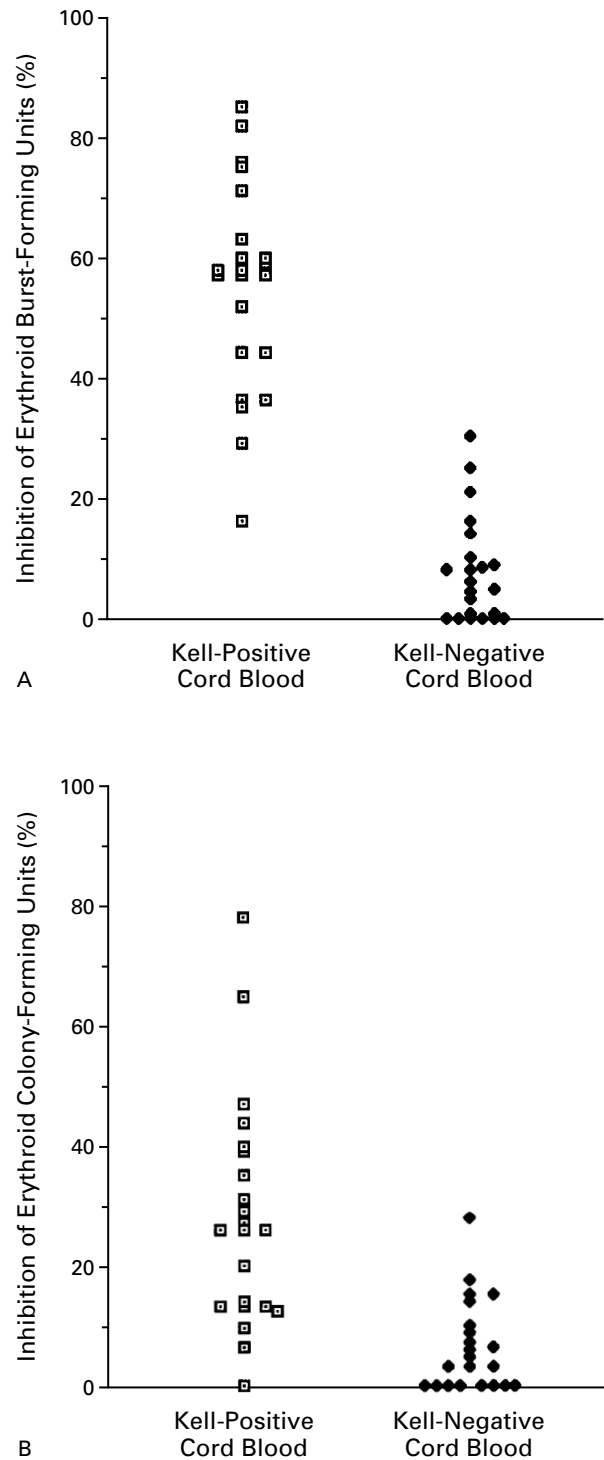
IgG ANTI-KELL ANTIBODIES	GRANULOCYTE-MACROPHAGE CFU		MEGAKARYOCYTE BFU AND CFU	
	KELL-POSITIVE	KELL-NEGATIVE	KELL-POSITIVE	KELL-NEGATIVE
	no. of colonies/ml of cord blood			
None	583±242	493±240	517±158	349±166
0.2%	569±235	502±246	529±159	364±172
2%	570±225	494±247	516±154	344±156
20%	581±248	499±236	517±153	347±166

\*Plus-minus values are means ±SE. The experiments were carried out on six samples of cord blood (three Kell-positive and three Kell-negative), with six replicate wells for each colony assay. CFU denotes colony-forming units, and BFU burst-forming units.

78;  $P < 0.001$ ) but not from Kell-negative samples (median degree of inhibition, 4 percent; range, 0 to 28) (Fig. 2B). The degree of inhibition was more variable than with the monoclonal anti-Kell antibodies. There was significantly greater inhibition of the more immature progenitor cells, erythroid burst-forming units, than of erythroid colony-forming units ( $P < 0.001$ ). The serum samples containing anti-Kell antibodies caused no significant inhibition of Kell-positive or Kell-negative granulocyte-macrophage colony-forming units, megakaryocyte burst-forming units, or megakaryocyte colony-forming units (Table 2). For the eight women for whom serial samples were analyzed, there was no effect of duration of gestation on the degree of inhibition of erythroid burst-forming units or colony-forming units (data not shown). Maternal serum samples without red-cell alloantibodies had no effect on Kell-positive or Kell-negative erythroid burst-forming units or colony-forming units (Table 3).

**Effect of Severity of Disease**

Maternal serum samples containing anti-Kell antibodies from the fetuses with severe anemia inhibited Kell-positive erythroid burst-forming units to a greater extent than serum samples from women whose fetuses had mild anemia (Table 4). There was no difference between groups in the degree of inhibition of erythroid colony-forming units, and there was no correlation between the anti-Kell antibody titer and the degree of inhibition of either erythroid burst-forming units or colony-forming units. The median anti-Kell antibody titer was significantly higher in the women whose fetuses had severe anemia than in the women whose fetuses had mild anemia.



**Figure 2.** Effect of Maternal Serum Containing Anti-Kell Antibodies on the Growth of Cord-Blood Erythroid Progenitor Cells. Maternal serum (20 percent vol/vol) added to cultures of erythroid progenitor cells from cord blood from normal infants at term inhibited the growth of erythroid burst-forming units from Kell-positive samples of cord blood but not Kell-negative samples ( $P < 0.001$ ) (Panel A). It also inhibited the growth of erythroid colony-forming units from Kell-positive samples of cord blood but not Kell-negative samples ( $P < 0.001$ ) (Panel B).

**TABLE 2.** EFFECT OF MATERNAL SERUM CONTAINING ANTI-KELL ANTIBODIES OR NO RED-CELL ALLOANTIBODIES ON GRANULOCYTE-MACROPHAGE AND MEGAKARYOCYTE PROGENITOR CELLS.\*

MATERNAL SERUM	GRANULOCYTE-MACROPHAGE CFU		MEGAKARYOCYTE BFU AND CFU	
	KELL-POSITIVE	KELL-NEGATIVE	KELL-POSITIVE	KELL-NEGATIVE
	no. of colonies/ml of cord blood			
None	528±76	408±95	475±118	479±123
Serum containing no red-cell alloantibodies (20%)	514±132	633±161	661±164	474±112
Serum containing anti-Kell antibodies (20%)	656±144	413±122	508±137	539±134

\*Plus-minus values are means ±SE. The experiments were carried out on 22 samples of cord blood (11 Kell-positive and 11 Kell-negative), with six replicate wells for each colony assay. CFU denotes colony-forming units, and BFU burst-forming units.

**TABLE 3.** EFFECT OF MATERNAL SERUM CONTAINING ANTI-KELL ANTIBODIES OR NO RED-CELL ALLOANTIBODIES ON ERYTHROID PROGENITOR CELLS FROM CORD BLOOD.\*

MATERNAL SERUM	ERYTHROID BFU		ERYTHROID CFU	
	KELL-POSITIVE	KELL-NEGATIVE	KELL-POSITIVE	KELL-NEGATIVE
	no. of colonies/ml of cord blood			
None	676±84	451±45	199±29	196±25
Serum containing no red-cell alloantibodies (20%)	645±167	529±109	260±65	212±16
Serum containing anti-Kell antibodies (20%)	297±63	425±45	134±17	185±23

\*Plus-minus values are means ±SE. The experiments were carried out on 44 samples of cord blood (22 Kell-positive and 22 Kell-negative), with six replicate wells for each assay. BFU denotes burst-forming units, and CFU colony-forming units.

### DISCUSSION

Kell-alloimmunized fetuses have fewer circulating reticulocytes and normoblasts than fetuses affected by anti-D antibodies, suggesting that erythroid suppression is the mechanism responsible for this type of anemia.<sup>7,10</sup> Our data confirm this hypothesis by demonstrating that both human monoclonal anti-Kell antibodies and serum samples from women with anti-Kell antibodies specifically inhibit erythroid progenitor cells from Kell-positive but not Kell-negative cord blood and that the inhibition is dose dependent, specific for cells of the erythroid lineage, and specific for anti-Kell antibodies.

The greater inhibitory effect of both monoclonal

**TABLE 4.** RELATION BETWEEN THE SEVERITY OF FETAL ANEMIA AND MATERNAL ANTI-KELL ANTIBODY TITERS AND THE DEGREE OF INHIBITION OF ERYTHROID BURST-FORMING UNITS AND COLONY-FORMING UNITS IN CORD BLOOD.\*

GROUP	NO. OF WOMEN	ANTI-KELL ANTIBODY TITER IN MATERNAL SERUM	INHIBITION IN CORD BLOOD†	
			BFU-E	CFU-E
			%	
Women with fetuses with severe anemia	12	512 (512-2048)‡	57 (44-71)§	20 (12-31)
Women with fetuses with mild anemia	10	16 (4-128)	47 (25-53)	13 (6-30)
All women	22	384	53	14

\*Median values are given, with 95 percent confidence intervals in parentheses.

†The degree of inhibition was calculated by subtracting values from Kell-negative samples of cord blood from values from Kell-positive samples of cord blood. BFU-E denotes erythroid blast-forming units, and CFU-E erythroid colony-forming units.

‡P<0.001 for the comparison with women with fetuses with mild anemia.

§P=0.03 for the comparison with women with fetuses with mild anemia.

anti-Kell antibodies and maternal serum on the more immature erythroid cells (erythroid burst-forming units) was also reflected in the clinical severity of the fetal anemia and may indicate the importance of the Kell antigen at a specific stage in erythroid differentiation. Alternatively, the apparently greater inhibition of erythroid burst-forming units by maternal serum may represent the wide variation in inhibition of erythroid progenitors by human serum, which contains many stimulatory and inhibitory cytokines.<sup>15</sup> In addition, we did not concentrate or purify the serum samples, which may have reduced the variability and increased the correlation between the inhibition of erythroid burst-forming units and colony-forming units, the maternal anti-Kell antibody titer, and the severity of disease.

The Kell antigen is known to be expressed early in fetal life<sup>16</sup> and on immature erythroid cells. It is therefore not surprising that erythroid burst-forming units and colony-forming units were inhibited by anti-Kell antibodies. That granulocyte-macrophage and megakaryocyte progenitors from the same Kell-positive fetuses were not inhibited by anti-Kell antibodies is consistent with previous findings of the restriction of Kell messenger RNA and protein to cells of erythroid lineage.<sup>3,4</sup>

Little is known about the function of the Kell protein. It is a transmembrane protein with sequence and structural similarities to endopeptidases that process peptide hormones and may be expressed on early hematopoietic cells.<sup>17-21</sup> The puta-

tive endopeptidase region of the Kell gene has 59 percent homology with the common acute lymphoblastic leukemia antigen,<sup>21,22</sup> which is a functional endopeptidase. Therefore, the common acute lymphoblastic leukemia antigen and Kell may have a role in regulating the growth and differentiation of the cells on which they are expressed, perhaps by modulating peptide growth factors on the cell surface.<sup>23</sup>

Our data have implications for the care of pregnancies in which Kell alloimmunization is identified either by the detection of anti-Kell antibodies at routine pregnancy screening or on the basis of a history of a previous fetus with anemia. Detecting hemolysis indirectly by measuring the concentration of bilirubin in amniotic fluid is unlikely to be helpful, since bilirubin concentrations in Kell-alloimmunized fetuses do not reflect the severity of fetal anemia.<sup>7,10</sup> Management of pregnancies involving Kell alloimmunization should focus on Kell genotyping of fetal DNA<sup>24</sup> when the father is heterozygous for K1 and monitoring the Kell-positive fetus for the development of anemia by cordocentesis and serial ultrasonography.

In conclusion, both monoclonal and naturally occurring anti-Kell antibodies inhibit the growth of Kell-positive erythroid progenitor cells. These findings suggest that suppression of erythropoiesis at the progenitor-cell level is an important mechanism of fetal anemia due to anti-Kell antibodies.

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