EXPERIMENTAL CELL RESEARCH 319 (2013) 2700–2707

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Research Article

Unique monoclonal antibodies specifically bind surface structures on human fetal erythroid blood cells

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ARTICLE INFORMATION

Article Chronology:
Received 6 March 2013
Received in revised form
22 June 2013
Accepted 24 June 2013
Available online 29 June 2013

Keywords:
Monoclonal antibody
Immunophenotyping
Fetal nucleated red blood cells
Prenatal diagnostics

ABSTRACT

Background: Continuing efforts in development of non-invasive prenatal genetic tests have focused on the isolation of fetal nucleated red blood cells (NRBCs) from maternal blood for decades. Because no fetal cell-specific antibody has been described so far, the present study focused on the development of monoclonal antibodies (mAbs) to antigens that are expressed exclusively on fetal NRBCs.

Methods: Mice were immunized with fetal erythroid cell membranes and hybridomas screened for Abs using a multi-parameter fluorescence-activated cell sorting (FACS). Selected mAbs were evaluated by comparative FACS analysis involving Abs known to bind erythroid cell surface markers (CD71, CD36, CD34), antigen-i, galactose, or glycophorin-A (GPA). Specificity was further confirmed by extensive immunohistological and immunocytological analyses of NRBCs from umbilical cord blood and fetal and adult cells from liver, bone marrow, peripheral blood, and lymphoid tissues.

Results: Screening of 690 hybridomas yielded three clones of which Abs from 4B8 and 4B9 clones demonstrated the desired specificity for a novel antigenic structure expressed on fetal erythroblast cell membranes. The antigenic structure identified is different from known surface markers (CD36, CD71, GPA, antigen-i, and galactose), and is not present on circulating adult erythroid cells, except for occasional detectability in adult bone marrow cells.

Abbreviations, in order cited: NRBC, Nucleated red blood cell; mAb, Monoclonal antibody; FACS, Fluorescence-activated cell sorter; CD, Cluster of differentiation; GPA, Glycophorin A; BFU–E, Burst-forming unit–erythoid; SBA, Soybean agglutinin; PCR, Polymerase chain reaction; FISH, Fluorescence in situ hybridization; DNA, Deoxyribonucleic acid; STR, Short tandem repeats; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures); MNCs, Mononuclear cells; FCS, Fetal calf serum; DMSO, Dimethylsulfoxide; FSC, Forward scattered light; SSC, Side scattered light; APC, Allophycocyanine; PE, Phycoerythrin; FITC, Fluoresceinthiocyanate; PBS, Phosphate-buffered saline; FcR, FC receptor; RT, Room temperature; APAAP, Alkaline phosphatase anti-alkaline phosphatase; Tris, Tris(hydroxymethyl)aminomethane; TBS, Tris-buffered saline; HRPO, Horseradish peroxidase; BSA, Bovine serum albumin; DAPI, 4′-6-Diamidino-2-phenylindol; GalNAc, Terminal α- or β-*N*-acetylgalactosamine.

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http://dx.doi.org/10.1016/j.yexcr.2013.06.015

*Conclusions:*The new mAbs specifically bind the same or highly overlapping epitopes of a surface antigen that is almost exclusively expressed on fetal erythroid cells. The high specificity of the mAbs should facilitate development of simple methods for reliable isolation of fetal NRBCs and their use in non-invasive prenatal diagnosis of fetal genetic status.

Introduction

Development of a reliable non-invasive alternative to prenatal diagnostic procedures such as amniocentesis and chorionic villous sampling used to detect fetal aneuploidies has been the goal of numerous scientific groups. Aside from fetal nucleated red blood cells (NRBC, also known as erythroblasts), trophoblasts [1,2] or fetal cell-free DNA [3,4] have also been considered as objects for prenatal diagnostics out of maternal blood. Methods used to enrich the rare fetal NRBCs (about 1–8 fetal erythroblasts in 2 mL maternal blood) are amongst others combinations of cell sorting with magnetic particles or flow cytometry [5,6], density gradient centrifugation [7], selective cell lysis [8], or depletion of unwanted cell populations (5). Cells from very early developmental stages such as the colony forming definitive erythroid progenitors (BFU-E) can be isolated from umbilical cord blood using CD34 [9]. To enrich and identify erythroid cells from later developmental stages, surface markers such as CD71 [10], glycophorin A [11], CD36 [12] and intracellularly expressed hemoglobins have been used [13,14].

Since Bianchi et al. [15] first used antibodies to CD71 to isolate fetal erythroid cells from maternal peripheral blood, the transferrin receptor has become one of the most frequently used selection markers [16]. Soybean agglutinin (SBA) has also been used to isolate fetal erythroid precursors from blood of pregnant women because the formation of membrane galactose is closely linked to the development and maturation of erythroid precursor cells [17].

The fetal origin of the isolated cells can be proved by PCR-amplification of Y-chromosome-specific sequences [18], by fluorescence in situ hybridization (FISH), by detecting ε- and γ-globin, or by comparing DNA-polymorphisms with STR (short tandem repeats)-markers from mother and child [19]. However, the Y-chromosome detection method excludes the examination of pregnancies with a female fetus, fetal globins are also formed in adults that have hematologic diseases, and STR-markers do not always detect a DNA-polymorphism between mother and child [20].

The identification and isolation of fetal cells is still difficult because no surface antigens are known that are exclusively expressed on these cells. Therefore, the aim of this study was the development of a fetal cell-specific monoclonal antibody which enables the characterization of fetal erythroid cells as well as their differentiation and isolation from adult erythroid blood cells and leucocytes.

Materials and methods

Samples

To test the specificity of the new mAbs for fetal erythroid cells, cytospin preparations with mononuclear cells from term umbilical cord blood, blood smears with fetal blood, fetal and adult bone marrow, and frozen sections of yolk sac tissue, fetal and adult liver, fetal and adult lymphatic tissue were investigated (Table 1). The samples were provided, stained and analyzed by Professor Reza Parwaresch at the Institute for Hematopathology of the University of Kiel, Germany. Umbilical cord blood samples that were used for flow cytometric tests were provided by the Paracelsius Clinic birth unit in Langenhagen, Germany. Samples were collected after informed consents were obtained from blood and tissue donors.

Cell lines K-562 and KMOE-2 were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH).

Development of monoclonal antibodies: Antigen preparation and immunizaton

The isolation of fetal erythroid cell membranes used for immunization was performed by Genelab (Gelsenkirchen, Germany). Erythroid cord blood cells from term pregnancies were isolated based on differential expression of known surface antigens using multi-parameter FACS. Isolated cells were CD71+ and antigen i+ and negative for CD3, CD14, CD19, and CD45. Those cells were lysed and the cell membranes separated from the nuclei by centrifugation. Five mice were immunized with cell membranes

Table 1 – Overview of number and provenance of embryonic, fetal, and adult tissue samples.

Tissue	Total number of samples	Gestation week	Analyses performed
Yolk sac	4	6 and 8	Microscope
Fetal liver	8	6, 8, 20 and 30–38	Microscope
Fetal bone marrow	4	10, 20 and 30–38	Microscope
Fetal blood	14	6, 8, 20 and 30–38	Microscope
Umbilical cord blood	35	38–40	FACS
Fetal lymph node	14	6, 8, 20 and 30–38	Microscope
Adult bone marrow	32	N/A	Microscope
Adult liver	8	N/A	Microscope
Adult peripheral blood	5	N/A	FACS
Adult lymph node	8	N/A	Microscope

of 10^6 erythroblasts per immunization. The immunizations were done according to a standard protocol [21]. Next, spleen cells from the immunized mice were fused with the *mouse myeloma* cell line NSO [22]. The five fusions were divided into 2–3 portions each and stored in liquid nitrogen for further investigation.

Hybridoma cloning and antibody purification

Selection and cloning of antibody producing hybridomas were performed by Labsoft AG (Halle/Saale, Germany) using standard methods. The cell culture supernatants from positive clones were then sent to AdnaGen AG (Langenhagen, Germany) for screening and selection of fetal erythroid cell-specific reactivity using a specifically established multi-parameter FACS approach described below. Hybridomas producing antibody of desired specificity were then extensively sub-cloned using two sequential 12-steps limiting dilution processes. Finally, larger scale hybridoma cell cultures were grown from single cell clones and purified antibody was produced using a standard protein-G affinity column chromatography. Where indicated, purified antibodies were used unlabeled or after labeling with biotin and/or other detection moieties.

Isolation and processing of blood mononuclear cell fraction

Mononuclear cell (MNC) fractions from umbilical cord blood and bone marrow were isolated by density gradient centrifugation on Ficoll-Paque 1.077 g/mL according to the manufacturer's instructions (Amersham Biosciences). For preservation, 10^7 cells were suspended in 1 mL fetal calf serum (FCS; Gibco) and 500 μL freezing medium was added. The freezing medium consisted of 10% DMSO (dimethylsulfoxide) and 40% FCS. After 24 h incubation at −80 °C, the cells were then placed in liquid nitrogen for long-term storage.

Prior to use, the cells were thawed at 37 °C and resuspended in about 50 mL Medium A (Iscove's Modified Dulbecco's Medium with 10% FCS and 20 U/mL DNAse I) that was added drop-wise (10 mL). After centrifugation the cell pellet was resuspended in 10 mL medium B (Iscove's Modified Dulbecco's Medium with 10% FCS) and centrifuged again. Supernatant was removed and cells resuspended in the remaining medium.

Antibody screening by multi-parameter flow cytometry

To screen and select for antibodies that specifically bind surface antigens of fetal erythroid cells, a six-parameter fluorescence-activated cell sorting (FACS) method was developed. This multi-marker and high throughput approach facilitated reproducible screening of large numbers of culture supernatants of antibody-producing hybridomas. The components of the system included four fluorescence channels combined with determinations of forward (FSC) and side (SSC) scatters. Allophycocyanin (APC)-labeled anti-CD45 (CD45-APC), phycoerythrin (PE)-labeled anti-CD71 (CD71-PE), and the DNA dye LDS 751 were used to simultaneously identify leucocytes, erythroid, and nucleated cells, while the sixth channel set for detection of fluorescein isothiocyanate (FITC) was used for detection of the new antibodies under investigation.

Hybridoma supernatants were screened on cryopreserved or freshly isolated MNCs using direct or indirect immunofluorescence staining. Cell count and viability (not described) were always checked prior to staining and isotype controls of all antibodies were determined. Briefly, 2×10^5 cells in 15 μL PBS (phosphate-buffered saline), pH 7.2, containing 0.5% bovine serum albumin (BSA) was placed in 5 mL-tubes and mixed with FC receptor (FcR) blocking reagent (Miltenyi Biotech). Cell culture supernatant (35 μL) from antibody-producing hybridomas was then added to the cell suspension, mixed, and incubated for 10 min at room temperature (RT). The cells were washed with 3 mL PBS buffer and centrifuged for 5 min at $400 \times g$ and 4 °C. The supernatant was vacuumed off to about 50 μL and the cells were resuspended in the remaining buffer. For indirect staining, the primary antibody-labeled cell suspension was next incubated with a secondary anti-species antibody labeled with FITC. In a third step, the labeled cells were incubated with fluorescence labeled antibodies against CD71 (anti-CD71-PE), and CD45 (anti-CD45-APC), and with the nucleus dye LDS 751. All incubation steps with fluorescent dyes have been performed for 10 min in the dark. After the last wash, the cells were fixed by re-suspension in 300 μL 1 × Cellfix solution (BD Biosciences) and stored at 4 °C in the dark until analysis with a FACSCalibur (BD Biosciences). The data were collected and evaluated with the CellQuest Pro software (BD Biosciences).

Immunostaining, immunocytochemical, and immunofluoresence microscopy

Immunostaining of various cells and tissue sections was performed using the commercially available alkaline phosphatase anti-alkaline phosphatase (APAAP) kit (Universal APAAP kit, Dako Cytomation). The preparations were incubated for 30 min at RT with the undiluted antibody under evaluation. After washing in tris-buffered saline (TBS, pH 7.4), the preparations were incubated with a secondary rabbit anti-mouse IgM antibody diluted 1:25 in TBS containing 10% human serum for 30 min at RT. This was then followed by washing and incubation with the APAAP-complex diluted 1:50 in TBS containing 10% human AB-serum (blood group antigens AB) for 30 min. The washing and incubation with the secondary antibody and the APAAP complex was repeated twice as above. The preparations were finally developed with Neufuchsin substrate solution for 10–20 min at RT, washed with tap water, and counterstained with Mayers Hemalaun. The preparations were sealed with Kaiser Glycerol gelatin (Merck) and analyzed with a universal microscope (Axioplan 2, Carl Zeiss).

Immunostaining was also performed using the UltraVision-HRP method (UltraVision™, LabVision Corporation). Briefly, endogenous peroxidases were blocked for 10 min at RT with peroxidase blocking reagent (Dako Cytomation), the sections washed twice with TBS containing 0.05% Tween-20, and incubated for 10 min with Ultra V Block. The sections were then incubated with the undiluted antibody to be tested for 30 min at RT. After washing as above, the biotinylated goat secondary antibody detection reagent was added. The reaction was developed using the streptavidin-horseradish peroxidase (HRPO) conjugate and the chromogenic ACE+ substrate system (Dako Cytomation). Finally, the reaction was stopped by incubation with aqua dest. Counter staining was performed with Mayers Hemalaun for 2 min and the “blueing” was done under flowing tap water.

For double immunofluorescence staining with anti-GPA and new mAbs under investigation, fixed and dried cytospins or

frozen tissue sections were first incubated for 60 min at RT with mouse anti-GPA Ab appropriately diluted in PBS containing 1% BSA. After washing with PBS, the preparations incubated as above with goat anti-mouse IgG Alexa Fluor 488 conjugate (Molecular Probes). After a second washing, the GPA tagged preparations were sequentially incubated as above with undiluted new mAb for 60 min (mouse IgM isotype) to be tested, and then similarly with goat anti-mouse IgM-Alexa 594 conjugate (Molecular Probes). This was followed by 3 min staining of cell nuclei with DAPI (Molecular Probes) appropriately diluted in PBS. Preparations were viewed with a universal microscope (Axioplan 2) equipped with appropriate filters and documented with the digital camera system (MetaFluor Imaging System; Visitron Systems GmbH).

Results

Identification of fetal cell-specific antibody-producing hybridoma clones 4B8, 4B9 AND 5D10

Establishment of the multi-parameter FACS of differentially labeled MNCs from umbilical cord blood allowed reproducible screening of large numbers of hybridomas by enabling simultaneous identification of erythroid cells, differentiation of leucocytes from enucleated erythrocytes, and determination of binding specificity of the new Abs in a single step process. Scattered light characteristics of cells with different size (FSC) and granularity (SSC) was used to gate for the target cells (Fig. 1A; region R1). Erythroid target cells were expected to express the marker CD71 (Fig. 1B; region R2). Leukocytes were excluded with CD45 (Fig. 1C; region R3) and nucleated cells were detected with the DNA-dye LDS 751. The target cells (NRBCs) were identified by logically linking R1+R2−R3. A total of 690 cell culture supernatants from four immunizations were screened for antibodies that specifically bound NRBCs from umbilical cord blood. The screening resulted in the identification of three antibody-producing hybridoma clones 4B8, 4B9, and 5D10 (Fig. 1D–F). These novel Abs bound nucleated cells that were CD71-positive and CD45-negative (upper right quadrant). Antibodies 4B8 and 4B9 bound ~72–82% of the CD71-postive cells while Ab 5D10 detected ~35%.

MABs 4B8 AND 4B9 show no reactivity to adult blood group antigens and specific cell lines

The new mAbs 4B8, 4B9, and 5D10 are of the IgM isotype. By competitive binding analysis, mAbs 4B8 and 4B9 were found to bind the same or highly overlapping epitopes of a surface antigen on fetal NRBCs, while mAb 5D10 detected a different determinant and also showed some reactivity with monocytes (data not shown).

The new mAbs did not bind adult erythrocytes or blood group antigens as pre-incubation of the antibodies with increasing numbers of AB adult erythrocytes did not reduce their reactivity against umbilical cord erythroblasts. As negative and positive control the binding of anti-CD71 and anti-GPA to fetal NRBCs was tested after pre-incubation with adult erythrocytes, respectively. In accordance to the known surface expression a pre-incubation of anti-GPA with adult erythrocytes completely removed its binding capacity to cord blood cells, while similar to mAbs 4B8, 4B9, and 5D10, pre-incubation of the anti-CD71 did not block its anti-cord blood erythroblast reactivity (data not shown).

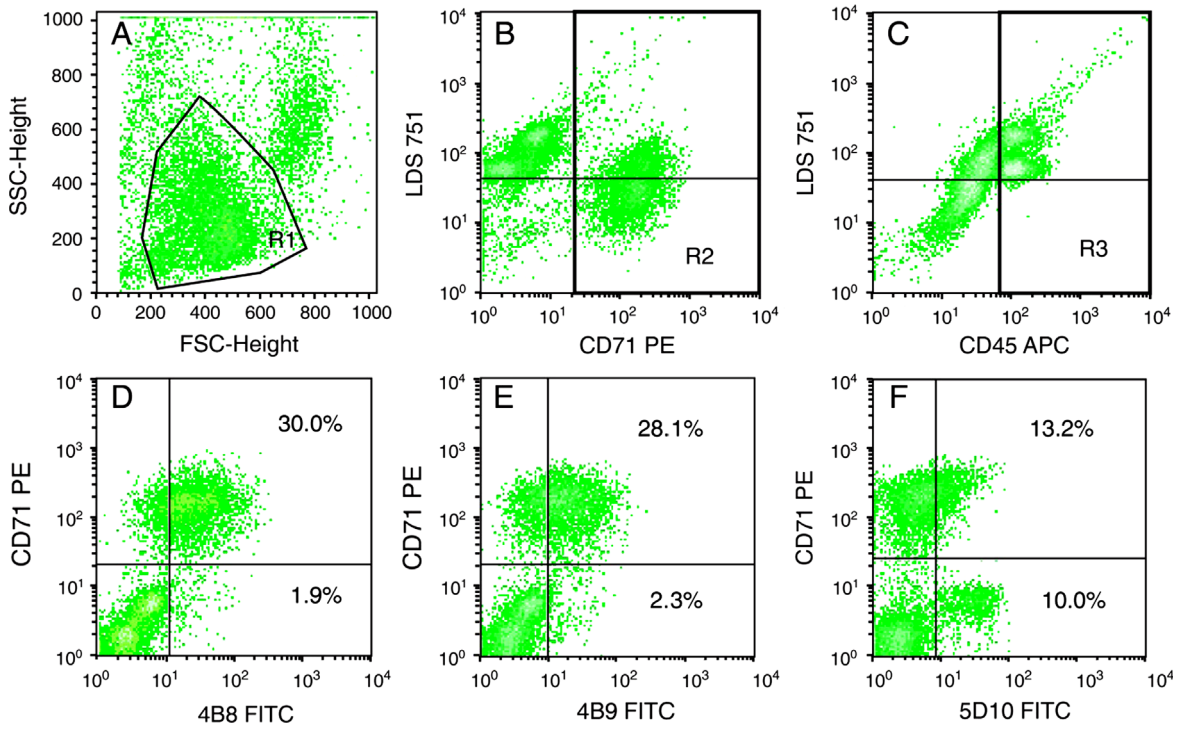

Fig. 1 – FACS-based pre-selection of erythroid cells for antibody screening and detection of hybridoma clones. (1A) Scattered light characteristics based on FSC and SSC. R1 gates lymphocyte and erythroid precursor populations. (1B) R2 gates CD71-positive nucleated cells. (1C) CD45-positive cells were gated in R3 and excluded. (1D–1F) Detection of antibody clones 4B8, 4B9, and 5D10.

Additional studies showed that mAbs 4B8, 4B9, and 5D10 do not bind to the leukemia cell line K-562 and the erythroid cell line KOME-2 which exhibit distinct developmental stages of erythroid cells and thus, a specific biomarker expression pattern [23,24]. This was revealed by comparative FACS analysis of the new mAbs versus binding of commercial Abs to known surface markers such as CD71, CD36, GPA, galactose, and CD45. MNCs from umbilical cord blood served as positive control (data not shown). Further specificity studies have primarily been done with mAb 4B9.

Analyses of embryonic, fetal, and adult tissues further demonstrate specificity of 4B9

An extensive summary of histological and FACS comparative analyses of mAb 4B9 for its reactivity against various fetal and adult tissues is depicted in Tables 2 and 3, and Figs. 2–4.

Ab 4B9 tested positive against all examined embryonic and fetal tissues as well as fetal blood samples (Table 2). Immunohistochemical staining of frozen tissues from yolk sac showed binding of mAb 4B9 to the embryonic primitive erythroblasts found in yolk sac blood islands, but no reaction occurred with embryonic stem cells (hemocytoblasts) (data not shown).

Blood cells from fetal liver preparations of gestation weeks 6, 8, 20, and 30 were analyzed to study the early expression of 4B9-antigen in fetal development. Fig. 2A exemplarily shows the results of the stained frozen fetal liver sections of the 20th gestation week. The collections of GPA- and 4B9-positive cells are blood forming foci with erythroid precursors. The negative areas are formed by the liver cells. Fig. 2A shows 4B9-positive, nucleated cells (red fluorescence) that also expressed the erythroid marker GPA (green fluorescence). In addition, hepatocytes were detected that did not express either of these antigens (arrow head). Fetal erythroblasts were 4B9-positive in all samples of the different gestational weeks tested.

Fig. 2B and C represent results of fetal bone marrow analysis showing GPA-positive and 4B9-positive fetal NRBCs. Microscopic analysis of fetal blood samples stained with anti-4B9 and anti-GPA are shown in Fig. 3A and B.

To prove that the 4B9-antigen is exclusively expressed on fetal cells, adult tissues have been stained with anti-GPA and anti-4B9 for microscopic analysis (Table 2, Fig. 4). Adult liver cells, lymphatic cells, peripheral blood cells (data not shown) and bone marrow cells showed no reaction with mAb 4B9. However, there was a detectable reactivity with the 4B9 Ab in 1 of 32 (3%) adult bone marrow samples (Fig. 4C). In this particular sample about 5–10% of the erythroblasts, in fact euchromatic erythroblasts, were 4B9-positive.

Through further FACS and microscopic analyses we compared the occurrence of the 4B9-antigen during ontogeny to that of GPA (Table 3). The 4B9-antigen is expressed during embryonic and fetal erythropoiesis as defined according to the published data concerning other well-known erythroid markers like CD36, CD71, and terminal α- or β-*N*-acetylgalactosamine, but – with one exception – not in adult erythropoiesis. GPA is expressed on all NRBCs, reticulocytes and erythrocytes formed in embryonic, fetal, and adult tissues.

Discussion

Despite significant progress in downstream chromosomal and genetic testing, unavailability of a highly specific anti-fetal erythroblast antibody has remained a major limiting factor to successful development of non-invasive prenatal diagnostic tests. To address these unmet analytical and clinical needs, we here described development and characterization of mAbs that specifically bind to surface epitopes distinctively expressed on plasma membranes of fetal erythroblasts. Development of a six-parameter FACS assay facilitated screening of large numbers of Ab-producing clones. Further test results showed that Abs from the suitable clones 4B8 and 4B9 appeared to detect a single or highly overlapping epitopes of a fetal-erythroblast specific surface antigen.

The cytological and histological data on the characterization of the 4B9-antigen expression indicate that this surface structure seems to be closely linked to the development of the body's blood forming system (Table 3). The results of the fetal tissue analyses (fetal liver, fetal bone marrow, and fetal blood) showed that the 4B9-antigen is present on normoblasts, euchromatic, polychromatic, and basophilic erythroblasts, on pro-erythroblast and weakly on most reticulocytes and erythrocytes (Table 2, Figs. 2 and 3). No reaction was detectable with fetal hemolymphatic cells or fetal myeloid cells. Accordingly, mAb 4B9 demonstrated binding to primitive erythroblasts found in

Table 2 – Summary of histologic and cytological analyses performed with anti-4B9 and anti-GPA.

Embryonic /fetal tissue	NRBCs[a]		Reticulocytes/erythrocytes		Macrophages, dendritic cells, T- and B-lymphocytes	
	4B9	GPA	4B9	GPA	4B9	GPA
Yolk sac	+	+++	–	+++	–	–
Liver	+	+++	(+)	+++	–	–
Bone marrow	+	+++	(+)	+++	–	–
Peripheral blood	+	+++	(+)	+++	–	–
Umbilical cord blood	+	+++	(+)	+++	–	–
Lymph node	–	–	–	–	–	–
Adult tissue	**4B9**	**GPA**	**4B9**	**GPA**	**4B9**	**GPA**
Bone marrow	[–]	+++	–	+++	–	–
Liver	–	++	-	++		
Peripheral blood	–	+++	–	+++	–	–
Lymph node	–	–	–	–	–	–

[a] Normoblasts, euchromatic/polychromatic/basophilic erythroblasts, proerythroblasts; NRBCs: Nucleated red blood cells; –No antigen expression;+/+++ Intensity of marker expression; (+) 4B9-expression was detectable in all samples but not on all cells of a sub-population; [–] In 1 of 32 adult bone marrow samples about 5–10% of the erythroblasts were 4B9-positive.

Table 3 – B9-antigen expression during ontogeny.

Marker	Embryonic erythropoiesis	Fetal erythropoiesis		Adult erythropoiesis
	Yolk sac primitive NRBCs	Fetal liver NRBCs	Fetal BM NRBCs	Adult BM NRBCs
CD71	(+)	++	++	++
CD36	–	++	++	++
GalNAc[a]	?	++	++	++
Glycophorin A	+++	+++	+++	+++
4B9	+	+	+	–

[a] GalNAc: Terminal α- or β-*N*-acetylgalactosamine (detected with SBA); yolk sac: expression of markers CD71, CD36, and GalNAc according to literature;? No data found in the literature; (+) marker expression low or undetectable; +/++/+++ increasing intensity of antigen expression.

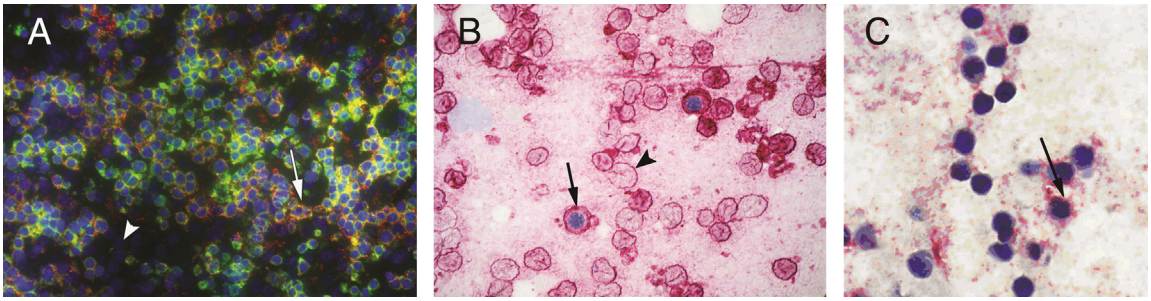

Fig. 2 – Immunofluorescence and immunohistochemical staining performed on fetal liver frozen sections and fetal bone marrow cells, gestation week 20. (2A) Fetal liver sections: 4B9-positive fetal NRBCs (arrow, red fluorescence) were also GPA-positive (green); nucleus staining (blue); hepatocytes (arrow head). Overlapping red and green fluorescence appears yellow. (2B) Fetal bone marrow cells: APAAP-staining with anti-GPA and (2C) with anti-4B9. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

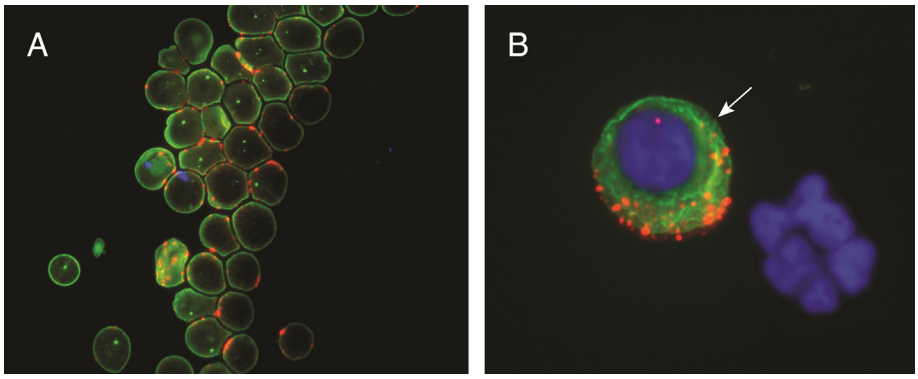

Fig. 3 – Immunofluorescence staining performed on fetal peripheral blood cells, gestation week 36. (3A) GPA-positive (green fluorescence) and 4B9-positive (red fluorescence) non-nucleated erythrocytes and reticulocytes, 400 ×. (3B) GPA- and 4B9-positive nucleated erythroid precursor cell (zoom). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

yolk sac blood islands, but no reactivity was found on embryonic stem cells (hemocytoblasts).

An expression of the 4B9-antigen was seen on all erythroblast stages of embryonic blood formation in the yolk sac. In umbilical cord blood, 4B9-antigen is expressed on all morphologically identifiable stages of fetal erythropoiesis: pro-erythroblasts, nucleated erythroid precursors, reticulocytes and erythrocytes. The 4B9-antigen was only found on erythroid precursors in one adult bone marrow sample but was not detectable in adult peripheral blood.

The results of adsorption studies with adult AB+erythrocytes demonstrated that 4B9 mAb does not bind surface structures expressed on the strongly glycosylated adult erythrocytes. The latter strongly supports the notion that the 4B9 Ab-targeted antigen is unlikely to be one of the main blood group antigens such as antigens i and I, GPA, and membrane-bound galactose chains detected by the binding of soybean agglutinin [25,26]. CD36, the thrombospondin receptor, was eliminated as a possible 4B9-antigen because it is expressed on both fetal as well as adult monocytes. Similarly, the possibility of 4B9 mAb recognizing the

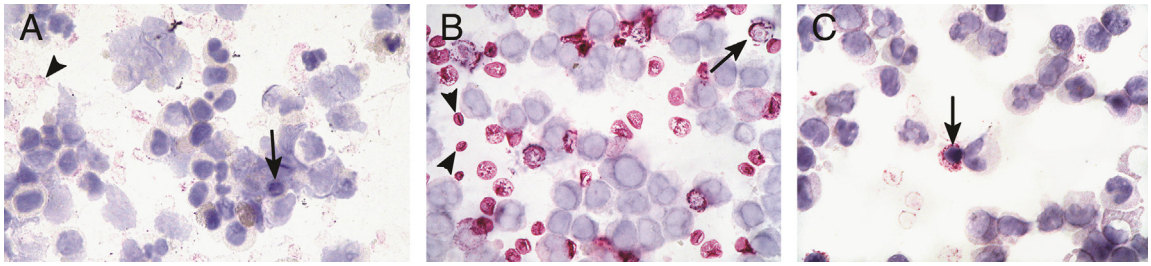

Fig. 4 – APAAP-staining performed on adult bone marrow cells. (4A) Anti-4B9 is negative for adult erythroid bone marrow cells and leukocytes; 400 ×. (4B) NRBCs, mature erythrocytes or reticulocytes are positive for marker GPA; 400 ×. (4C) One 4B9-positive adult bone marrow cell with cytoplasmic staining (arrow); 400 ×. Arrows head: non-nucleated erythrocytes/reticulocytes; arrow: erythroblasts.

erythropoietin receptor was excluded as the receptor is also expressed on both fetal and adult erythroid cells. In addition, the transferrin receptor, CD71, was excluded as the potential 4B9-antigen as antibodies specific to CD71 did not compete with 4B9 Ab for cell surface binding and as importantly, 4B9 Ab did not bind to cell lines such as the leukemia cell line K-562, which strongly expresses CD71 [27]. Furthermore, the earliest detectable 4B9-antigen was seen at the pro-erythroblast stage while CD71, according to literature, is already expressed on early CD34-positive BFU-E [28]. Accordingly, in our FACS analysis, a subpopulation of CD34 and CD71 double positive cells was found, but no CD34 and 4B9 double positive population was detected.

By immunofluorescence staining, the 4B9 antigen appeared to have a specific dot- or drop-like distribution on the surface of fetal erythroid cells (Fig. 3B), which is quite similar to the expression pattern CD71 demonstrated in our microscopic analyses (data not shown). Although CD71 can be eliminated as the targeted antigen, a comparison of the expression patterns might allow conclusions on the structure of the 4B9 antigen. Because CD71 is a highly glycosylated transmembrane receptor [29] with similar immunofluorescence expression patterns to that of 4B9, one might postulate that the 4B9 antigen is also a transmembrane molecule with possibly similar functions to CD71 in cell proliferation and differentiation.

Properties of erythroid cells are used for diagnosing hematologic diseases [30], but also for prenatal testing of non-hematologic disorders [20], namely early analyses of aneuploidies (e.g. trisomies 13, 18, and 21) or gene defects (e.g. cystic fibrosis, dystrophia). An ultimate goal is to develop reliable analytical methods that are safe for mother and fetus to replace invasive intervention such as the amniocentesis [2,9]. Fetal NRBCs are the most suitable cells that have been increasingly targeted for establishment of non-invasive prenatal diagnostic tests [31]. The molecular structure of the 4B9-antigen still has to be determined however, its potential to detect rare fetal cells in the blood of pregnant women has been demonstrated by its specificity for fetal erythroblast. The antigenic structure specifically targeted by 4B9 mAb is expressed on fetal primitive NRBCs as well as on fetal definitive erythroblasts, but not on adult peripheral blood cells. The specificity of this novel mAb to primitive fetal cells should enable future developments of simple fetal isolation strategies important to eventual development of reliable non-invasive prenatal diagnostic strategies. This remains to be shown in further studies.

In summary, the 4B9-antigen is an unknown membrane-bound structure that is almost exclusively formed on fetal erythroid cells and that is expressed during embryonic and fetal erythropoiesis. In adults, it was detectable only in one exceptional case in adult bone marrow but not in peripheral blood. Due to the specific expression of the 4B9-antigen this novel marker seems to be suited to specifically characterize and identify fetal erythroid cells and aid in their isolation from maternal blood.

Conflict of interest

KellBenx Inc., Great River, New York 11739 (info@kellbenx.com) has licensed the 4B9-antibody from AdnaGen AG and supported the author to publish the present data. The remaining authors have nothing to declare.

Acknowledgments

This work was supported by and performed at AdnaGen AG. We wish to thank Professor Reza Parwaresch for contributing fetal and adult tissue analysis. We also thank Genelab and Labsoft AG for the development of the antibody-producing hybridoma clones.

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