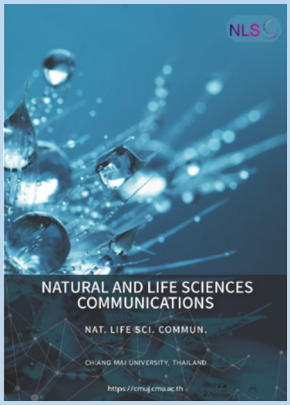
Research article



Editor:
Veerasak Punyapornwithaya,
Chiang Mai University, Thailand

Article history:
Received: March 1, 2023;
Revised: May 12, 2023;
Accepted: May 16, 2023;
https://doi.org/10.12982/NLSC.2023.045

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Mouse Monoclonal Antibody Specific To Human Ena Blood Group Antigen

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ABSTRACT

The aim of this study was to produce mouse monoclonal antibodies (mAbs) specific to human blood group antigens. Two percent solution of normal human red blood cells (RBCs) O, RhD+ in normal saline was freshly prepared, and 100 uL was immunized weekly into BALB/mice, intraperitoneal route for 3 weeks. Prior to each immunization, 100 uL of mouse blood was collected and serum was separated. The rising of anti-RBCs antibody titer was performed by standard hemagglutination compared to pre-immunized serum. Mouse was then sacrificed, and the spleen was collected. The fusion between mouse spleen cells and mouse myeloma (X63Ag8.653) cell lines was performed according to the standard hybridoma technique. Hybrid clones were grown in DMEM high glucose supplemented with 20% FCS at 3 °C, 5% CO_2 incubator with 5% humidity. The anti-RBCs in hybrid cell culture supernatant were tested by standard hemagglutination and a monoclonal antibody-producing single clone of the hybrid cell was generated by limiting dilution technique. Antibody screening and identification were performed using screening cells, panel cells, and rare blood type RBCs, followed by enzyme treatment technique. The Isotype of mAb was also identified. The results showed that amongst those obtained, mAb clone 1E10-2B2, was confirmed to be anti-En^a characterized as ficin resistant (anti-En^{a}FR) and isotype IgG2b, kappa. The anti-En^a mAb is very helpful and can be applied as specific mAb for the phenotyping of En^a which is the high-frequency blood group antigens in all populations.

Keywords: Glyphorin, MNS blood group, Ena, alloantibody, HDFN, HTR

Funding: The authors are deeply grateful for the research funding from the Faculty of Associated Medical Sciences, the Fundamental Fund (FF2022) from NRCT, and Chiang Mai University.

Citation: Wutti-in, Y. and Vongchan, P. 2023. Mouse monoclonal antibody specific to human en^a blood group antigen. Natural and Life Sciences Communications. 22(3): e2023045.

INTRODUCTION

Hemolytic transfusion reaction (HTR) is an episode from increased destruction of transfused donor red blood cells (RBCs) in patients with alloantibodies and/or autoantibodies. These antibodies have been shown to bind complement and have been involved in acute intravascular hemolysis and fatal/life-threatening complications (Poole and Daniels 2007). The antibodies can be cold or warm reactive. One or more antibodies specific to RBCs in an individual is approximately 1% per transfused unit and may reach 50% in transfusion-dependent patients (Quirino et al. 2019). Phenotyping of donor and patient's RBCs prior to transfusion of selected RBCs, thus, is necessary and helps to provide a significant quality of blood transfusion safety. Accordingly, pretransfusion testing is focused on screening and identification of these antibodies in a patient blood sample. This strategy eliminates the need for repeat patient workups when transfusion is more required based on using of compatible for extended blood group antigens and avoids the least incompatible units (Petz 2003; Ziman et al. 2017; Delaney et al. 2020). Importantly, donor units with ABO blood group antigen matched and lack of antigen-specific to identified antibodies is the must. To date, more than 190 blood group antigens are reported according to the International Society of Blood Transfusion (ISBT). High-incidence blood group antigens are defined as the antigens expressed on more than 90% of the population but most of them are more than 99% (Quirino et al. 2019).

In the case of antibodies specific to high-incidence blood group antigens, the chance of availability of antigen-negative RBCs is a big problem for the blood banking (Seltsam et al. 2003). Detection and identification of antibodies to high-incidence blood group antigens is of utmost importance. RBCs with positive and/or negative RBCs blood group antigens are required to support the antibody identification. Most laboratories are unable to get success dues to a lack of resources especially specific antibodies used to phenotype blood group antigens. Thus, a reference laboratory has to be sought and this takes time to get results for further on preparation of compatible donor RBCs for patients.

Individual with negative expression of high incidence blood group antigens, therefore, is classified as having rare blood phenotype. In this situation, rare blood group inventory and/or family donors have to be concerned. Detection of RBCs antigen can be performed in various techniques. Several molecular methods have been developed and shown to be effective and advantageous in relation to some cases of the phenotyping (Gorakshakar et al. 2017; Telen 2000). However, the blood group system is characterized by the presence or absence of antigens on RBCs. Therefore, tube hemagglutination is still a classical standard method. The limitation of the hemagglutination technique is a very small number of specific antibodies for rare blood group antigens. Moreover, as recommended in quality assessment for blood banking, the antibody must not possess only specificity but also shows high sensitivity and avidity to react with RBCs expressing very few numbers of antigen as well (Cohn C.S. 2020).

Glycophorin A (GPA) and glycophorin B (GPB) are the major glycoproteins on the RBCs membrane. Blood group antigens reside on these two molecules including antigen in MNS blood group system (ISBT 002) (Crookston 1986; Heathcote et al. 2011; Siebert and Fukuda 1987). Individuals with both *GPA* and *GPB* genes deletion and may lack GPA and GPB on their RBCs are classified as M^kM^k (En^a(-)) (Metaxas and Metaxas-Buehler 1964). Such individuals may develop anti-En^a to determinants located on GPA.

Anti-En^a targets to external regions of GPA can activate the complement system and is reported to associate with severe hemolytic transfusion reaction (HTR) and hemolytic disease of the fetus and newborn (HDFN) (Al-Jada 2017; Okubo et al. 1988; Tokunaga et al. 1979; Walker et al. 1987). Anti-En^a can be developed in En(a-) individuals as non-red blood cell stimulating circumstances or patients with a history of blood transfusion and/or pregnancy (Al-Jada 2017).

According to the patterns of antigen response to hydrolysis of proteolytic enzymes, specific antibodies to En^a blood group antigen are classified into 3 groups. They are anti-En^{a}TS, anti-En^{a}FS, and anti-En^{a}FR where TS is trypsin sensitive, FS is ficin sensitive, and FR is ficin resistant, respectively (Tanner and Anstee 1976).

Since En(a-) is a very rare phenotype, patients with anti-En^a, thus, need En(a-) RBCs which is difficult to find out. In some situations, an autologous blood transfusion may require. However, autologous blood is a process that is based on criteria such as the patient must have hemoglobin more than 11.5 gm/dL (Cohn C.S. 2020). According to this limitation, En(a-) donor is a need.

Phenotyping En^a on donor RBCs needs a reagent anti-En^a antibody with highly sensitive and avidity. This report presents a new clone of monoclonal anti-En^a which has been proven to be anti-En^a with ficin resistant properties (anti-En^{a}FR). This obtained product will certainly be applied as a standard reagent antibody in routine blood banking use. The product is of reasonable price since it was produced in our country. In addition, this helps in reducing the import of an expensive required reagent antibody for laboratory in transfusion sciences.

MATERIALS AND METHODS

Animal, cell lines, and materials

BALB/cAJcl-Female-4wk mice were purchased from Nomura Siam International Limited Company, Thailand, and were taken care of under standard animal care protocol at the Laboratory Animal Center, Chiang Mai University.

Mouse myeloma (P3-X63Ag8.653) was purchased from ANH Scientific Marketing Company, Thailand. Cells were grown in DMEM high glucose supplemented with 10% fetal calf serum (Gibco, Life Technologies, NY, USA). Other common reagents used in this study were purchased from local reputable companies including PCL Holdings (Thailand) and Pacific Sciences (Thailand). Three standard RBCs of each screening cells, panel cells, papain-treated screening cells, and papain-treated panel cells, and antiglobulin reagent (polyspecific) were purchased from Thai Red Cross Center, Bangkok, Thailand. Cord blood in EDTA with different ABO blood groups was collected from Labor room, Maharaj Hospital, Faculty of Medicine, Chiang Mai University.

Production of mAb anti-RBCs antibody

Screening cells (O1 and O2) were pooled and pelleted down by centrifugation at 1000 x g for 2 minutes. Packed RBCs were washed three times with normal saline solution (NSS, 0.85% NaCl solution) and prepared to 2% cell suspension in NSS. Antibody against RBCs was generated by immunization of 2% RBCs suspension to a female BALB/c mouse at 1-week intervals with 100 µL/dose *via* the intraperitoneal route. The rising titer of anti-RBCs antibodies in mouse serum was determined by standard tube hemagglutination. Splenocytes were collected and fused with P3-X63Ag8.653 myeloma cells by standard hybridoma fusion techniques using 50% polyethylene glycol and HAT medium selection. The monoclonal antibody-producing hybrid was then isolated by the limiting dilution technique and kept growing in 20% DMEM high glucose supplemented with 20% FCS in CO_2 incubator, 37°C, and 5% humidity. The isotype of the mAb was determined by an indirect ELISA technique developed in the laboratory compared to the standard immunoglobulin isotype.

Characterization of mAb specific antigen in hybrid cell culture supernatant

To obtain the antibody production clones in the cell culture supernatant, antigen-specific to mAbs was studied starting with screening, identification, and finally confirming by special techniques and/or rare blood type RBCs. Briefly, to

screen the mAbs, two drops of hybrid cell culture supernatant and one drop of various 5% RBCs suspension (screening cells, O1 and O2) were individually mixed and incubated at room temperature for 5 minutes before spinning at 1,000 x g for 15 seconds. The hemagglutination was observed and recorded. The reaction was then incubated for another 30 minutes in a water bath at 37°C. After completion, the reaction was spun down and hemagglutination was observed. According to indirect immunoglobulin techniques, the reaction was washed three times with NSS before adding one drop of antiglobulin reagent (AHG, polyspecific), and spun to observe hemagglutination. In case of a visible negative result, IgG-sensitized RBCs were finally added for one drop and checked for hemagglutination to confirm the activity of the antiglobulin reagent used. To identify and confirm the specificity of the selected hybrid cell culture supernatant was reacted with normal panel cells and papain-treated panel cells with the same technique. To confirm its specificity, diluted hybrid cell culture supernatant was individually tested with many rare blood type RBCs by standard tube agglutination test. All tests were performed in parallel with cell culture media (20% FCS, DMEM high glucose).

RESULTS

Screening of the antibodies specific to red blood cell by standard hemagglutination test

In the first step of screening for the antibody-producing clones, 5% RBCs of screening cells, O1, O2, and O3 were individually mixed with cell culture supernatant, and incubated at room temperature and 37°C followed with indirect immunoglobulin techniques to observe hemagglutination. Among those tested cell culture supernatant samples, 1E10 antibody was positive in three of the screening cells tested as shown in Table 1. This clone was further performed by limiting dilution. The antibody screening result of the clone name 1E10-2B2 was similar to the primary 1E10 (Table 2).

Table 1. Antibody screening of primary hybrid clone 1E10.

1E10	Rh					MNS				P	Lewis		Mi^a	Kidd		Duffy		Kell		Diego		Xg^a	AGT	
	D	C	E	c	e	M	N	S	s	P1	Le^a	Le^b		Jk^a	Jk^b	Fy^a	Fy^b	K	k	Di^a	Dib		RT	AHG
R1R1	+	+	0	0	+	+	0	+	+	+	0	+	+	+	+	+	+	+	+	0	+	+	4+	4+
R1R2	+	+	+	+	+	0	+	0	+	+	+	0	0	+	+	+	0	0	+	+	+		4+	4+
R1R1	+	+	0	0	+	+	+	0	+	0	0	+	0	0	+	+	0	0	+	0	+		4+	4+

Table 2. Antibody screening of hybrid clone 1E10-2B2.

1E10-2B2 (undiluted)	Rh					MNS				P	Lewis		Mi^a	Kidd		Duffy		Kell		Diego		Xg^a	AGT	
	D	C	E	c	e	M	N	S	s	P1	Le^a	Le^b		Jk^a	Jk^b	Fy^a	Fy^b	K	k	Di^a	Dib		RT	AHG
R1R1	+	+	0	+	+	+	+	+	+	+	0	+	0	+	+	+	0	+	+	+	+		4+	4+
R1R2	+	+	+	+	+	+	+	0	+	0	+	0	+	+	+	+	+	0	+	0	+		4+	4+
R1R1	+	+	0	0	+	+	+	0	+	0	0	+	0	0	+	+	0	0	+	0	+		4+	4+

Identification of the antibodies specific to red blood cell by standard hemagglutination test

Monoclonal antibody (mAb) from hybrid clone 1E10-2B2 was investigated to identify their specificity by using 11 red cell panels along with cell culture media, 20% FSC, DMEM high glucose (Table 3).

Table 3. Antibody identification of hybrid clone 1E10-2B2.

1E10-2B2 (undiluted)		Rh					MNS				P	Lewis		Mi^a	Kidd		Duffy		Kell		Diego		Xg^a	AGT	
		D	C	E	c	e	M	N	S	s	P1	Le^a	Le^b		Jk^a	Jk^b	Fy^a	Fy^b	K	k	Di^a	Dib		RT	AHG
O1	R1R1	+	+	0	0	+	0	+	0	+	w	+	0	0	+	+	+	0	0	+	+	+		2+	3+
O2	R1R1	+	+	0	0	+	+	0	+	+	0	+	0	0	0	+	0	+	0	+	0	+		3+	4+
O3	R1R1	+	+	0	0	+	+	+	0	+	0	0	+	0	+	0	0	+	0	+	0	+		3+	4+
O4	R1r	+	+	0	+	+	+	0	+	+	+	0	+	0	+	+	+	+	0	+	0	+		3+	3+
O5	R1Rz	+	+	+	0	+	+	+	0	+	0	0	+	+	+	0	+	0	0	+	0	+		3+	4+
O6	R1R2	+	+	+	+	+	+	0	0	+	0	0	0	0	+	+	+	0	+	+	0	+		2+	3+
O7	R1R2	+	+	+	+	+	+	0	0	+	+	+	0	+	0	+	+	0	0	+	0	+		3+	4+
O8	R2R2	+	0	+	+	0	0	+	0	+	0	+	0	0	0	+	+	+	0	+	0	+		3+	3+
O9	rr	0	0	0	+	+	+	0	0	+	0	+	0	+	+	+	+	+	0	+	+	+		3+	4+
O10	r′r	0	+	0	+	+	+	+	0	+	0	+	0	0	+	+	+	0	0	+	0	+		2+	4+
O11	r′′r	0	0	+	+	+	+	0	+	+	+	0	+	0	+	0	+	0	0	+	+	+		3+	4+

Isotypic study of monoclonal antibody clone 1E10-2B2

Isotypic study of mAb 1E10-2B2 was then performed by sandwich ELISA compared to various isotypes of standard mouse immunoglobulins. Cell culture supernatant was diluted 1:50 and 1:100 before tested. It was confirmed that mAb 1E10-2B2 is IgG2b, kappa as shown in Table 4.

Table 4. Determination of mAb 1E10-2B2 isotype.

Isotype control	Absorbance (OD)			
	1E10-2B2 (1:100)	1E10-2B2 (1:50)	IgM, kappa	IgG1, kappa
IgG1	0.044	0.041	0.015	0.748
IgG2a	0.050	0.046	0.015	0.039
IgG2b	0.687	0.854	0.122	0.308
IgG3	0.013	0.011	0.014	0.014
IgM	0.014	0.015	0.512	0.015
IgA	0.018	0.018	0.016	0.019
Kappa	0.671	0.676	0.371	0.700
Lambda	0.184	0.217	0.069	0.298

Reactivity of 1E10-2B2 with rare phenotype red blood cells

To identify the specificity of mAb 1E10-2B2, many rare red blood cell types were tested including normal cord blood samples of various ABO blood group antigens using diluted cell culture supernatant (1:100 in normal saline). The result confirmed that mAb 1E10-2B2 showed a negative reaction to En(a-) RBCs while strongly positive to all rare blood cell types tested (Table 5).

Table 5 Reactivity of 1E10-2B2 with Bombay, para-Bombay, adult I, cord blood cells, and other rare phenotype red blood cells.

Phenotype	N	1E10-2B2 mAb (1:100)
Para-Bombay	6	4^+
Bombay	1	4^+
Adult i	1	4^+
I	3	4^+
Cord blood	6	4^+
Rare phenotype (N=1)		
Ko		4^+
KEL: -14		4^+
Kp(b-)		4^+
McLeod (Kx-)		4^+
Ge: -2, -3, 4		4^+
p		4^+
En(a-)		negative
Jk(a-b-)		4^+
Rhnull		4^+
In(Lu)		4^+

Reactivity of 1E10-2B2 with enzyme or chemical-treated RBCs

The diluted cell culture supernatant of mAb 1E10-2B2 (1:100 in normal saline) was tested with various enzymes and chemical-treated red blood cells. Three types of anti-En^a mAbs were tested in parallel as a control. Table 6 demonstrated reactions of mAb 1E10-2B2 to various enzymes and chemical-treated RBCs/ However, the result indicated that mAb 1E10-2B2 is ficin resistant (anti-En^{a}FR).

Table 6. Reactivity of 1E10-2B2 with enzyme or chemical-treated RBCs.

RBCs treated with Enzymes or chemicals	1E10-2B2 (1:100)	Specificity of anti-En^a*		
		Anti-En^{a}TS	Anti-En^{a}FS	Anti-En^{a}FR
Ficin	4^+	neg	neg	4^+
Trypsin	4^+	neg	4^+	4^+
α-chymotrypsin	4^+	neg	4^+	4^+
Pronase	4^+	neg	4^+	4^+
AET	4^+	4^+	4^+	4^+
DTT (200 mM)	4^+	4^+	4^+	4^+
Acid	4^+	4^+	4^+	4^+
Untreated	4^+	4^+	4^+	4^+

Note: *Homemade mouse monoclonal antibodies used for control were generated in Department of Laboratory Japanese Red Cross, Japan.

DISCUSSION

Cell culture supernatant from each hybrid cells was screened for the antibodies specific to red blood cell with screening cells by standard hemagglutination test. One of all hybrids, 1E10 was very interesting in specific

reaction with screening cells in all three phases test (Table 1). The result demonstrated the specificity of antibodies to high incident blood group antigen(s) and/or sialoglycoproteins (GPA/GPB) which are strongly positive reaction molecules located on red blood cells.

The primary hybrid 1E10 was then performed to produce a monoclone by standard limiting dilution technique. One hybrid obtained, 1E10-2B2, presented a strong reaction to all screening cells tested in both room temperature and antiglobulin phase. Anti-Human Globulin Anti-IgG is prepared by immunizing rabbits with human IgG. The anti-IgG component contains antibody reactivity against light chain IgG and thus may also agglutinate IgA or IgM-sensitized red blood cells. Therefore, the positive results of mAb 1E10-2B2 were then observed in both phases. Moreover, the reaction confirmed its specificity to high incident blood group antigen or GPA/GPB (Table 2).

The specificity of mAb 1E10-2B2 was also identified. Eleven standard panel cells were individually reacted with cell culture supernatant from mAb 1E10-2B2 by indirect antiglobulin test. It was found that all 11 panel cells tested provided a strong positive reaction with the grading of 2^+ to 4^+ in all phases according to different amounts of antigen expression on each panel RBCs (Table 3). More interestingly, the reaction was stronger in the antiglobulin phase. This result indicated that mAb 1E10-2B2 isotype was IgG since it was detected stronger after incubation at body temperature followed with antiglobulin reagents. Then the isotypic study of mAb 1E10-2B2 was performed by sandwich ELISA. It was confirmed that mAb 1E10-2B2 is IgG2b, kappa (Table 4).

Since the mAb 1E10-2B2 reacted to all cells tested, identification of its specificity was then tested with many rare blood type red blood cells including normal cord blood of various ABO blood group antigens. The results strongly suggested that mAb 1E10-2B2 is specific to Ena since it showed a positive reaction with all rare blood types tested but negative with En(a-) red blood cells (Table 5).

As reported, three broad categories of anti-En^a have been defined according to the effect of protease enzymes on the antigenic determinants, namely anti-En^aTS, anti-En^aFS, and anti-En^aFR (Howard et al. 2019). In this study, diluted cell culture supernatant of mAb 1E10-2B2 (1:100 in normal saline) was also tested with various enzyme and chemical-treated red blood cells. The results demonstrated that mAb 1E10-2B2 is anti-En^aFR since it could react with all enzymes and chemical-treated red blood cells (Table 6).

The monoclonal antibodies specific to red blood cells were raised in order to apply as a reagent antibody for laboratory typing of blood group antigens. Pooled screening cells O1 and O2 possessing various blood group antigens were used to immunize the mice and hybridoma technology was performed. One interesting clone, 1E10-2B2 was characterized since it strongly reacted with all screening cells and panel cells tested. The pattern of reaction demonstrated the antibody specific either to high incident blood antigens or red blood cell membrane molecules. The broad reaction indicated strong avidity, specificity, and sensitivity to red blood cells. In order to identify whether the specificity was to blood group antigen or membrane molecules, cell culture supernatant has to be diluted to the optimal dilution. Various rare blood phenotypes together with cord blood cells of various ABO blood group antigens were tested. The results confirmed anti-En^a with resistance to ficin treatment which could be categorized as anti-En^aFR compared to untreated red blood cells.

The obtained anti-En^aFR can be applied to routine laboratory work for red blood phenotyping. However, the titer of working concentration is recommended to optimize prior to any application. In transfusion science, the good antibody to be applied in phenotyping must be standardized and adjusted to a titer of >256 (Cohn C.S. 2020). Therefore, according to its strong reaction, mAb 1E10-2B2 will be considered as a good reagent antibody to characterize red blood with negative or weak expression of En^a antigen. However, since it was classified as IgG2b, the test is suggested to be a complete antiglobulin test.

CONCLUSION

In this study, a monoclonal antibody specific to En^a, the high incident blood group antigen was identified. The antibody is IgG2b, kappa. The product of this study is the new clone of monoclonal anti-En^{a}FR which can be beneficially applied as the specific reagent antibody for transfusion science research and routine blood banking.

ACKNOWLEDGMENTS

The author is indebted to Prof. Dr. Toyoda Chizu and Dr. Hatsue Tsuneyama, Department of Laboratory Japanese Red Cross, Japan, for their kind helps in confirmed identification of the monoclonal antibody.

ETHICAL APPROVALS

This study was approved by the Ethics Committee of the Faculty of Associate Medical Sciences, Chiang Mai University (AMSEC-63EM-006). The approval for the use of laboratory animals from Animal Care and Use Committee, Chiang Mai University was certified as MC008/2561[02-2561-08-15].

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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