

Special Article

Innovation in monoclonal anti-Mi^a production

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Introduction

Miltenberger subsystem (Mi subsystem) or MNS blood group variants refer to a group of low-frequency antigens in the MNS blood group system. The MNS blood group system is the second most complex blood group system after the Rh system and it consists of 50 antigens¹ carried on glycophorin A (GPA), glycophorin B (GPB), or hybrids of these glycophorins. These variant alleles give rise to antigens known as the Miltenberger subsystem (Mi) subsystem, or Mi^a.^{2,3}

Levine and colleagues⁴ reported the discovery of the Mi^a antigen in 1951. Hart and colleagues reported the discovery of the Verweyst antigen (Vw) in 1954, although it was later found to be the same as the Graydon antigen (Gr) reported in 1946. Furthermore, Cleghorn⁵ classified the Mi subsystem into five classes (Mi.I to Mi.V) based on the reactions of red blood cell antigens with anti-Mi^a, anti-Vw, anti-Hut, anti-Mur, and anti-Hil. In 1992, the additional discoveries expanded the Mi subsystem to 11 classes (Mi.I to Mi.XI), that were referred to as GP. These include GP. Vw (Mi.I), GP. Hut (Mi.II), GP. Mur (Mi.III), GP. Hop (Mi.IV), GP. Hil (Mi.V), GP. Bun (Mi.VI), GP. Nob (Mi.VII), GP. Joh (Mi.VIII), GP. Dane (Mi.IX), GP. HF (Mi.X), and GP. JL (Mi.XI).^{5,6} (Table 1) In a recent study of Kallaya Kirdkaewngam found that GP.Mur (Mi. III) was the most common MNS hybrid GPs in Thai blood donors. GP.Vw (Mi.I), GP.Hut (Mi.II), and GP.Bun (Mi.VI) were less common and reported a novel phenotype (GP.Nuk).⁷

GP.Vw or Mi.I is part of the GP(A-B-A) hybrids subgroup: GP (A-B-A). In this subgroup, there are five types of antigens: GP.Vw, GP.Hut, GP.Nob, GP. Joh, and GP. Dane that were controlled by the GYP(A-B-A) hybrid gene.⁸ The GP.Vw phenotypes are often found to be associated with Ns alleles or are occasionally found

with NS alleles, but less frequently with Ms alleles. In individuals of Swiss descent, GP.Vw phenotypes are found in approximately 1.43%.^{9,10}

Anti-Mi^a is clinically significant as it can cause hemolytic transfusion reactions (HTRs) and hemolytic disease of the fetus and newborn (HDFN).^{3,11,12} Similarly, anti-Vw, which is the IgG type, has been reported as a cause of HTRs and HDFN.^{13,14} Anti-Mi^a was first discovered in the serum of Mrs. Miltenberger as a polyspecific antibody, which consists of antibodies against various antigens of the Mi subsystem.^{5,6} Anti-Mi^a was found in patients or blood donors that it can be stimulated during pregnancy and blood transfusion. It often has specificity as anti-Mur, anti-MUT, anti-Hil, anti-Hop, and anti-Hut when tested against Mi subclasses. Since anti-Mi^a is clinically significant and can cause HTRs and HDFN, patients receiving blood transfusion must be screened for anti-Mi^a and tested with Mi(a-) antigens to avoid the issue. It is necessary to use an anti-Mi^a reagent that is specific for GP. Vw (Mi.I), GP. Hut (Mi. II), GP. Mur (Mi.III), GP. Hop (Mi.IV), GP. Bun (Mi.VI), and GP. HF (Mi.X). The anti-Mi^a reagent of the National Blood Centre, Thai Red Cross Society (NBC, TRCS) is produced by two methods: polyclonal antibody and monoclonal antibody which have differences in process and specificity.

Production of polyclonal anti-Mi^a

Previously, the NBC, TRCS produced polyclonal antibodies against anti-Mi^a from the plasma of donors who had anti-Mi^a for use in the operation of the NBC, TRCS and Regional Blood Centres, which were subsequently distributed to hospital blood banks. The plasma is then transformed into serum by adding 24.7% calcium chloride and incubating at 37°C for at least 30 minutes

Table 1 Terminology and antigens presented in Miltenberger subsystem⁵

Terminology		Reaction of red cells with anti-										
Mi.	GP.	Mi ^a	Vw	Hut	Mur	MUT	Hil	TSEN	MINY	Hop	Nob	DANE
Mi.I	GP.Vw	+	+	0	0	0	0	0	0	0	0	0
Mi.II	GP.Hut	+	0	+	0	+	0	0	0	0	0	0
Mi.III	GP.Mur	+	0	0	+	+	+	0	+	0	0	0
Mi.IV	GP.Hop	+	0	0	+	+	0	+	+	+	0	0
Mi.V	GP.Hil	0	0	0	0	0	+	0	+	0	0	0
Mi.VI	GP.Bun	+	0	0	+	+	+	0	+	+	0	0
Mi.VII	GP.Nob	0	0	0	0	0	0	0	0	0	+	0
Mi.VIII	GP.Joh	0	0	0	0	0	0	0	0	+	+	0
Mi.IX	GP.Dane	0	0	0	+	0	0	0	0	0	0	+
Mi.X	GP.HF	+	0	0	0	+	+	0	+	0	0	0
Mi.XI	GP.JL	0	0	0	0	0	0	+	+	0	0	0

or until fibrin forms. The mixture is filtered to separate the fibrin from the serum. After that, 0.5 M disodium EDTA ($C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$) is added for decalcification, removing any remaining calcium and then sodium azide (NaN₃) is added at a concentration of 0.1% to inhibit bacterial growth. The serum is stored at 2-8 °C for at least 3 weeks to allow sedimentation and fat to settle, and is then roughly filtered using a 0.45-micrometer pore size filter. It undergoes a second filtration using a 0.2 micrometer pore size filter. The resulting serum is then tested for serum grouping, antibody screening and antibody identification.¹⁵ It is also tested against blood samples with Mi^a positive and negative antigens to ensure accuracy and in this case, it must be specified as anti-Mi^a. The strength of the antibody is tested through an antibody titration procedure.¹⁶ This process is complicated and time-consuming, prone to batch variability and limited in quantity which is not enough for customer needs.

Moreover, plasma from these blood donors is mostly specific as anti-Mur, anti-MUT, anti-Hil, anti-Hop, and anti-Hut but does not cover for the entirely Mi subclasses. At that time, the manufacturers were unable to check this anti-Mi^a with all Mi subclass cells. Due to these challenges, the initiative to produce the monoclonal antibody had begun to replace the aforementioned polyclonal antibody.

Production of monoclonal antibody

The NBC, TRCS has applied cell culture and hybridoma technology¹⁷ to the production of blood group reagents since 1987. Phikulsod S, et al^{18,19} promoted the study and research of blood group reagent production in the ABO system using cell culture and hybridoma technology. The antigen injection in laboratory mice to produce antibodies according to requirements (murine MoAb) succeeded in 1991. The first monoclonal antibody produced was MoAb anti-B. Subsequently, various blood group reagents including MoAb anti-A,²⁰⁻²¹ MoAb anti-AB,²² MoAb anti-D²⁰, MoAb anti-A1,²³ MoAb anti-M, MoAb anti-N,²⁴ and MoAb anti-H, are also currently in production.²⁵ NBC, TRCS has achieved success in producing blood group reagents (as murine MoAb) and has launched consecutive projects for the development of other blood group reagents from human B-lymphocytes (human MoAb). The new project presents a good opportunity for collaboration with the Japanese Red Cross Society, under the leadership of Dr. Soisaang Phikulsod, former deputy director of the NBC, TRCS and head of the antiserum and standard cell preparation department. As well, the NBC, TRCS initiated a training program on monoclonal hybridoma cell line production in collaboration with the Japanese Red Cross Society. This program aimed to study the production of hybridoma cell lines using monoclonal technology. As a result of the

collaboration, the NBC, TRCS gained precious expertise and successfully produced monoclonal antibodies, both murine and human monoclonal. The production of monoclonal antibody (human MoAb or murine MoAb), was reported by George Kohler and Caesar Milstein,²⁶ involves creating hybridoma cells through cell fusion, which can produce specific antibodies and be cultured for an extended period in laboratory tubes. This method has been proven effective by many scientists and then formed the basis for monoclonal antibody work, earning them the Nobel Prize in physiology or medicine in 1984. Hybridoma cells were subsequently modified and became a foundation for medical research, applied in the diagnosis and treatment of diseases²⁷ like infectious diseases and cancer.²⁸⁻³⁰

The United States Food and Drug Administration (US FDA) has certified over 79 monoclonal antibodies for market distribution.³¹ In addition to therapeutic use, monoclonal antibodies are used in the production of blood grouping reagents. The production of monoclonal antibodies for blood grouping began in 1980, when Voak D. and colleagues successfully produced monoclonal anti-A antibodies. In 1987 (B.E. 2530), Sacks S. and colleagues achieved the production of monoclonal anti-B antibodies. From then on, many monoclonal antibodies have been produced using this method for various blood group systems.

The NBC, TRCS has produced various types of murine monoclonal antibodies (murine MoAb) in the ABO system, including anti-A, anti-B, anti-AB, anti-A1, and anti-H. In the MNS system, it consists of anti-M and anti-N. Subsequently, they have developed the production of human monoclonal antibodies in different systems, such as anti-E, anti-P1, anti-Di^a, and anti-Mi^a. The latter is produced through a combination of anti-Vw (15D2 cell line), a murine monoclonal antibody, and anti-MUT(4E4) obtained from a human monoclonal antibody, similar to anti-P1.³²

The process of creating the 15D2 cell line using the murine monoclonal antibody method

First of all, it begins with stimulating laboratory mice (BALB/c) with Verweyst antigen (GP.Vw) or Mi.I. to induce the production of Verweyst antibody (anti-Vw). Red blood cell pellets from blood donors with blood type O who are GP.Vw positive, are washed, and a 20% cell suspension is prepared. Mice are intraperitoneally injected three times with GP.Vw antigen; each injection is 200 microliters and repeated every two weeks. After the third injection, with a three-day interval, cell fusion is performed by sacrificing the mice. Spleen cells are separated and splenocytes are finely ground to isolate B lymphocytes, which are plasma cells incapable of prolonged cultivation due to their limited lifespan. Therefore, these cells are fused with myeloma cells (X63 Ag-8.653) using PEG as the cell fusion agent.³³ The cell fusion process involves the merging of memory cells before their nuclei pass into the cytoplasm of another cells. The fused cells with two or more nuclei form hybrid cells, or hybridoma cells capable of producing specific antibodies. As cancer cells continuously grow and are uncontrollable, a method is needed to selectively cultivate only the hybridoma cells. In 1964, he discovered a selection method using a selective medium called HAT medium, which includes aminopterin, hypoxanthine, and thymidine.

Aminopterin is a folic acid analogue that inhibits the synthesis of DNA by blocking the enzyme folic acid reductase, causing cancer cells to die. The B lymphocytes, which are normal cells, also die after approximately 7-10 days. Surviving cells in the HAT medium are hybridoma cells. Once antibody-producing clones with specificity were obtained, they were cultured, expanded, and then re-cloned to ensure specificity and lifetime stability.³³ In the case of the 15D2 clone, it has specificity as an anti-Vw antibody and can be accurately used to detect GP.Vw (Table 1).

The process of creating the 4E4 cell line using the human monoclonal antibody

First of all, the method involves obtaining B lymphocytes from individuals who produce the desired antibody.³⁴ The NBC, TRCS has isolated B-lymphocytes from whole blood or buffy coats from blood donors who produce anti-MUT. These B-lymphocytes are then transformed using Epstein-Barr virus (EBV) from the B-958 cell line. The transformed cells are tested, and cells that produce antibodies are selected using the hemagglutination method. Fusion is then performed with JMS-3 (Kanto Koshinetsu Block Blood Centre, Japanese Red Cross Society) cancer cells using 50% polyethylene glycol (PEG 1540) (Sigma, St. Louis, MO, USA). The resulting hybrid cells are cultured, and the culture supernatant is tested for antibodies. Selected wells that produce antibodies are cloned to obtain single cell, and then antibody screening is repeated to ensure that the antibodies are produced by individual cell. The selected cells, which are antibody-producing cells, are preserved in liquid nitrogen at -196°C to create a master cell bank for future use. The 4E4 clone obtained has specificity as an anti-MUT antibody, giving positive results for GP. Hut (Mi. II), GP. Mur (Mi.III), GP. Hop (Mi.IV), GP. Bun (Mi.VI), and GP. HF (Mi.X) cells (Table 1).

However, in the production of monoclonal antibody reagents for blood typing, it is necessary to use the supernatant of more than one combined clone, as each clone has different properties. The anti-Mi^a produced by the NBC, TRCS for example was prepared from the supernatant of the 4E4 clone, which is a human monoclonal antibody with specificity for anti-MUT, giving positive results for GP. Hut (Mi. II), GP. Mur (Mi.III), GP. Hop (Mi.IV), GP. Bun (Mi.VI), and GP. HF (Mi.X) cells. Still, it cannot be used to detect GP.Vw. Therefore, it needs to be combined with the 15D2 cell line, which has specificity as an anti-Vw (Mi.I) antibody. This allows the anti-Mi^a produced by the NBC to have specificity as anti-Mi^a covering GP. Vw (Mi.I), GP. Hut (Mi. II), GP. Mur (Mi.III), GP. Hop (Mi.IV), GP. Bun (Mi. VI), and GP. HF (Mi.X) which provides an advantage in covering antigens in the Miltenberger subsystem. In addition, the product was confirmed by Japanese Red Cross Kanto-Koshinetsu (Table 2).

In summary, the National Blood Centre, Thai Red Cross Society, can produce anti-Mi^a monoclonal antibodies with specificity covering antigens in the Mi subclasses. These products can be used as a replacement for polyclonal antibodies, reducing the complexity of the production process, offering unlimited production

Table 2 Comparison of anti-Mi^a human MoAb produced by the NBC, TRCS and JRC

Subclass	NBC, TRCS product			JRC product			
	Anti-Vw (15D2)	Anti-MUT (4E4)	Anti-Mi ^a (15D2+4E4)	Anti-Vw (CBC-430)	Anti-MUT (CBC-412)	Anti-Mur (HIRO-138)	Anti-Mi ^a (CBC-172)
Mi.I	4+	0	4+	4+	0	0	4+
Mi.II	0	4+	4+	0	4+	0	4+
Mi.III	0	4+	4+	0	4+	4+	4+
Mi.IV	0	4+	4+	0	4+	4+	4+
Mi.V	0	0	0	0	0	0	0
Mi.VI	0	4+	4+	0	4+	4+	4+
Mi.X	0	4+	4+	0	4+	0	4+
Mi(a-)	0	0	0	0	0	0	0

NBC = National Blood Centre, Thai Red Cross Society; JRC = Japanese Red Cross

quantities, and allowing precise control of antibody strength in each production batch. This achievement is particularly beneficial in blood service activities for providing suitable and safe blood for patients.

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