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00Allo-anti-M

Allo-anti-M: detection peaks around 2 years of age, but may be attenuated by red blood cell transfusion

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Abstract

Background

Anti-M is frequently observed as a naturally occurring antibody of little clinical significance. Naturally occurring anti-M is often found in children, although the specific triggers of production, persistence, and evanescence of anti-M have yet to be elucidated.

Methods

In a retrospective, multicenter, nationwide cohort survey conducted from 2001 to 2015, alloantibody screening was performed before and after transfusion in 18,944 recipients younger than 20 years. Recipients were categorized into 6 cohorts based on their age at transfusion; within and among these cohorts, allo-anti-M was analyzed in regard to its production, persistence, and evanescence.

Results

In 44 patients, anti-M detected before and/or after transfusion was an age-related phenomenon, with a median age of 2 years and an interquartile range of 1-3 years; anti-M was most frequently detected in a cohort of children 1 to <5 years (0.77%, 31 of 4,035). At least 5 patients were presumed to have concurrent infections. Among 1,575 adolescents/young adults (15 to <20 years), no anti-M was detected. Of 29 patients with anti-M prior to transfusion, the antibody fell to undetectable levels in 17 recipients (89.5%, of whom at least 13 received only M-negative red cells) after anywhere from 5 days to 5.8 years; anti-M persisted in 2, and was not tested in 10. Only 15 recipients (0.08%) produced new anti-M after transfusion.

Conclusion

Naturally-occurring anti-M is a phenomenon of younger ages, predominantly between 1 to 3 years. After transfusion, it often falls to undetectable levels.

Introduction

Anti-M, frequently observed as a naturally occurring antibody, is usually not clinically significant when reactive at room temperature but not at 37 ℃[1]. Anti-M ranks fifth in frequency (10.3%) among red cell-associated alloantibodies in Dutch patients [2], and is the fourth most common antibody among Japanese patients, accounting for 7.2% (890) of 12,285 detected alloantibodies [3]. Anti-M is more commonly found in children than adults, although the specific mechanisms or triggers of anti-M formation are yet to be elucidated [4]. For ABO and other naturally occurring IgM antibodies, the gut microflora, pathogens, and food-associated proteins are thought to be immunogenic stimuli for antibody formation mediated by the innate immune system [5].

"Transfusion-Related Alloimmunization to Red Blood Cell Antigens in Japanese Pediatric Recipients" was a large, retrospective, multi-institutional cohort study [6] showing that alloimmunization did not occur from RBCs transfused within the first month of life (0%) and rarely occurred (0.46% - 0.80%) after transfusion within the first decade of life, whereas alloimmunization occurred in 1.15% - 1.88% of young pubescents and adolescents/young adults.

As part of this cohort study, naturally occurring or transfusion-related anti-M, in samples collected between 2001 and 2015, were investigated to assess the extent to which allo-anti-M arises or disappears from natural causes or by transfusion within different age brackets. This study has provided some insight into anti-M formation, persistence, and evanescence among transfusion recipients under 20 years of age.

Patients and Methods

Study design

As part of the retrospective, multicenter, nationwide cohort survey, "Transfusion-Related Alloimmunization to Red Blood Cell Antigens in Japanese Pediatric Recipients" [6], this study investigated alloreactive anti-M found before and/or after RBC transfusion. Pregnancy history was also solicited in the survey. This study was approved by the independent ethics committees of the Japan Society of Blood Transfusion and Cell Therapy, Hirosaki University Post-Graduate School of Medicine (#2016-224 and #2019-084), and the ethics committees of other participating institutions, as necessary. These ethics committees are guided by local policy, national law, and the World Medical Association Declaration of Helsinki.

Institutions and patients included

Criteria for inclusion of medical institutions and patients were described elsewhere [6]. Briefly, these were medical facilities in Japan that fulfilled criteria of having certified and continuously trained active staff, and had 30 or more pediatric patients every year who received RBC transfusions.

No exclusions were made for congenital or autoimmune diseases, transplantation, or the need for intravenous globulin. Eligible patients, including fetuses who received intrauterine transfusion, were included in the cohort, provided that informed consent was obtained and documented. Patients not transfused were excluded. Patient data from medical/transfusion service or chart records were anonymized and sent to Hirosaki University. Details about adverse reactions, including hemolysis or incorrect transfusions were not collected.

All 18,944 eligible patients, i.e., those who received any volume of allogeneic RBCs, were assigned to Cohorts A-F according to their age at transfusion: A, neonates <1 month old; B, infants 1 to <12 months; C, children 1 to <5 years; D, prepubescents 5 to <10 years; E, young pubescents 10 to <15 years; and F, adolescents/young adults 15 to <20 years. Sex was assigned as male or female according to external phenotype.

Antibody tests and RBC transfusion

Details about antibody testing and RBC transfusion were described elsewhere [6]. Briefly, transfused RBC units were matched or compatible with patients' ABO and RhD. Neonates with any maternally derived alloantibodies received RBCs compatible with those antibodies. RBC antibody screening (except in emergencies) on pretransfusion samples, follow-up testing, or maternal samples for pre-transfusion neonatal care, proceeded according to institutional protocols and included indirect antiglobulin tests. RBCs for transfusion were always matched for clinically significant antibodies and also matched, whenever possible, for antibodies of lesser significance (e.g., Lewis, P1, Xg) when reactive at 37 ℃.

Allo-anti-M was rigorously confirmed by excluding auto-anti-M; after RBC M/N phenotyping, allo-anti-M was imputed only in patients with NN phenotype. During the study period, most facilities (~80%) transfused M-negative RBCs to patients with anti-M, even when the antibody

was not reactive at 37 ℃.Information of allo-anti-M characteristics (titer, cold-reactive only or 37 ℃ reactive) were not collected for this study.

Statistical analysis

Quantitative variables are shown as medians with interquartile ranges defined by 25% and 75% boundaries. Comparisons to group were made using the chi-square test, Fisher exact test, Bonferroni's multiple comparison test, and 95% confidence intervals (CI) for categorical variables, using StatMate IV for Microsoft Windows, version 4.01 (ATM, Niigata, Japan), and SAS Enterprise Guide 7.1 (SAS Institute, Cary, NC). Results were deemed to be statistically significant if the 95% CI did not contain its reference value, which is equivalent to a *P* value of <0.05.

Results

Detection of allo-anti-M according to patients' age groups (Table 1)

Among 5,253 neonates (Cohort A), maternally derived passive anti-M was detected in 4 (0.08%), born to 3 mothers. Out of 4,628 infants (Cohort B), anti-M was detected in 7 (0.15%, 95% CI: 0.04-0.26%). Cohort C patients accounted for 65% (31/48) of anti-M observed and predominated over all other age groups: 31 (0.77%, 95% CI: 0.52-1.09%) of 4,035 children aged 1 to <5 years. In older children, anti-M was identified in 5 of 1,708 pre-pubescents (Cohort D, 0.29%, 95% CI: 0.04-0.55%), and 1 of 1,575 young pubescents (Cohort E, 0.06%, 95% CI: 0-0.35%). Of note, there were no cases of anti-M among 1,745 adolescents/young adults (Cohort F, 0%, 95% CI: 0-0.21%). When compared by age cohort, the incidence of anti-M among Cohort C was significantly higher than Cohorts A ($p < 0.00001$), B ($p < 0.00001$), E ($p < 0.0005$), and F ($p < 0.00001$), but did not differ statistically from Cohort D.

Characteristics of patients with allo-anti-M

Anti-M was identified in 44 patients, 29 before and 15 after transfusion, aged between 6 months and 12 years. Four neonates with maternally-derived antibody (Cohort A: 2 males, 2 females) were excluded from further analysis. Anti-M was identified in 22 males and 22 females with a median age of 2 years (interquartile range 1-3 years); this was consistent with Cohort C patients (age 1 to <5 years, 56% male, 44% female). In all 44 of these patients, only anti-M was identified.

Table 2 summarizes the characteristics of patients found to have anti-M on pre- and/or post-transfusion testing. Anti-M was identified primarily in patients undergoing surgery/trauma (50%) or having malignancies including leukemia (25%). Of 8 patients with an "other" diagnosis, it is noteworthy that 5 (63%) were presumed to have had severe viral/bacterial infections. None of these 44 patients with anti-M had a known history of pregnancy. Overall, the distribution of underlying diseases among patients with anti-M did not differ statistically from Cohort C children: surgery/trauma (44%), malignancy (39%) and others (18%).

The occurrence, persistence, and evanescence of anti-M (Figure 1, and Table 3)

Out of 29 patients who had anti-M before transfusion, only 2 (7%, Subgroup I) were persistently positive at 28 days and 2.3 years after transfusion. On the contrary, testing revealed evanescent anti-

M in 17 patients (59%, Subgroup II), as early as 5 days to as late as 5.8 years after transfusion (median 53 days, interquartile range 11 days - 7 months). Out of these 17 patients, at least 13 (76%) received M antigen-negative RBCs exclusively. Five patients in Subgroup II lost anti-M within two weeks following transfusion of M-negative red cells. Ten (34%, Subgroup III) were not tested after transfusion.

In contrast, 15 patients developed de novo anti-M following transfusion. Among 5 patients with follow-up testing, anti-M was undetectable in 4 patients (Subgroup V) and persistently positive in one patient (Subgroup IV). In 10 patients (Subgroup VI), no further test results were available.

Discussion

This survey quantified the incidence of allo-anti-M before and/or after transfusion in pediatric patients aged between 1 month and 20 years. Anti-M was identified among infants, children, and prepubescents, i.e., patients 6 months to 12 years of age, with a median age of 2 years and interquartile range of 1-3 years. No one younger than 6 months or older than 13 years were found to have anti-M. These results concur with a previous report [7].

In infants and young children, anti-M is generally considered naturally occurring, often in the setting of infection. In this study, we found at least 5 instances of anti-M in patients with suspected viral/bacterial infection. Naturally occurring anti-M has been reported to be produced almost exclusively in young children after infections of *Hemophilus influenzae, Proteus mirabilis, Staphylococcus. aureus, Neisseria meningitis,* and others [8]. Studies of Hemophilus and Neisseria species have shown expression of sialylated glycans that could potentially stimulate cross-reactive antibodies to monosialylated type 1 O-glycan structures on the RBC membrane, including M/N antigen [9]. Based on shared epitopes using the BLASTp database, a potential connection between microbial infection and RBC alloimmunization has been investigated [10]. Curiously, anti-M was not detected among young adults, suggesting that these cross-reactive, naturally-occurred antibodies are not strongly specific to M-antigen and gradually evanesce below detection level. Still, we cannot explain why no young adults had cross-reactive anamnestic responses when RBCs were transfused.

In addition, some viruses (e.g., influenza) and bacteria secrete neuraminidase, which can modify glycans present on M/N antigen, thus altering their immunogenicity [5]. “Acquired B antigen” on RBCs in A_1 individuals is a well-known phenomenon observed with certain Gram-negative infections that secrete de-acetylase enzymes that convert A antigen to a B-like antigen [11]. Many pathogens, including rotaviruses, adenoviruses, influenza viruses, and some bacteria, exploit sialic acid structures as receptors for binding [12]. Given the abundance of sialic acids on glycophorin A ($1x10^6$/ RBC), on which MN antigens are present, some infected hosts may evoke so-called naturally occurring, or microbiota-cross-reacting anti-M during immune response to invading pathogens. An analogous example is paroxysmal cold hemoglobinuria, which classically follows infection and is associated with induction of a transient auto-anti-P [13]. Likewise, Epstein-Barr virus, the etiologic agent of infectious mononucleosis, induces a transient anti-i IgM and cold autoimmune hemolytic anemia [14].

Anti-M is reported to be detected in around 10% of pregnant women in the USA [15], 3.9% in Africa [16], and 10.8% in Japan [3] as follow-up to a positive antibody screen. Our findings are consistent with a recent article by Takeshita and colleagues [3]. Among 12,285 RBC alloantibodies, the detection of anti-M in men (7.0%) was similar to that of women (7.4%, p=0.40). Contrary to expectations, anti-M is less frequent in patients with a history of transfusion than in patients with no or unknown transfusion history (2.9% vs. 8.9%, p<0.001). However, the frequency of anti-M during pregnancy is more than that of women not pregnant (10.8% vs. 8.6%, p<0.029). The increase in anti-M during pregnancy may reflect restimulation by exposure to fetal M antigen. We hypothesize that childhood infection can provoke M-reactive antibodies that evanesce following infection and/or ageing. Among male military veterans (USA), anti-M reactive at 37 ℃persisted in 67%, similar to other allo-antibodies: 65% for anti-K, 64% for anti-E, and 70% for anti-c [17].

In our study, 17 (90%) of 19 pediatric patients positive for anti-M before transfusion and tested after transfusion lost detectable anti-M as early as 5 days to as late as 5.8 years, with a median of 53 days, per post-transfusion testing. Thirteen (76%) of 17 patients who lost anti-M after transfusion received only M antigen-negative RBCs. The loss of anti-M after transfusion is a phenomenon we call “transfusion-related anti-M attenuation.” One mechanism may be transfusion-related clonal anergy following cross-match-compatible, M-positive red cells. Specifically, anti-M might go into clonal anergy after rapid transfusion that is massive in comparison to body size and blood volume, thus decreasing the production of antibody to undetectable levels. However, we should stop short of saying that anti-M clones are totally depleted, as in so-called clonal depletion or immunological accommodation in patients who lose anti-A (or B) following ABO-mismatched transplantations.

The mechanism would not, however, explain the loss of anti-M after transfusion of M-negative cells. In six children, anti-M disappeared within 2 weeks of M-negative RBC transfusion. This raises the question whether there was non-specific adsorption and clearance of anti-M by donor NN-red cells, which has not been described. Theoretically, some examples of anti-M might also possess an anti-Pr^{M} component [11]. Anti-Pr is a common, pH-sensitive autoantibody that recognizes the terminal sialylated O-glycans on both glycophorin A and B. Anti-Pr^{M} has M-specificity at warmer temperature that can be inhibited by glycophorin extracts from both M+ or N+ red cells [11].

Alternatively, the loss of anti-M may be coincidental to transfusion and simply reflect the removal of the immune stimulus.

For patients with allo-anti-M, M antigen-negative red cells were transfused even for cold-reactive anti-M at around 80% of Japanese facilities in our study, and allocated only for 37 ˚C reactivity at the remaining ~20% during our study period (2001-2015); Japanese policy has since been updated to specify that compatible RBCs should be transfused when the antibody is reactive at 37˚C, based on evidence from a nationwide survey [18].

That anti-M is usually of little clinical significance in transfusion is supported by two Japanese surveys [18,19]. No one developed hemolytic reactions after 41 M antigen-positive RBC transfusions into 14 patients who had cold reactive anti-M [18]. No adverse reactions were reported in 33 patients with anti-M following transfusion of M-positive RBCs (5 clinically relevant by IAT and 28 clinically not relevant by only saline/enzyme methods) [19]. Such clinical non-significance is presumptively attributed to the fact that almost all anti-M is mainly of IgM class. There are rare reports of anti-M, reactive both at 37 ˚C and at room temperature/ 4 ˚C, causing delayed hemolytic transfusion reactions [20,21,22]. When anti-M is detected, the vigilance in regard to any change of reactivity state is warranted, i.e., to assess a bi-phasic behavior at 37 ˚Cand lower temperatures [23].

In contrast, anti-M is one of the most clinically significant antibodies in cases of severe anemic disease of the fetus and newborn (ADFN) in Japanese and Chinese populations [24,25], even if maternal anti-M includes only miniscule amounts of IgG [26]. Although maternal anti-M IgG titers are often quite low, there is chronic transplacental transfer of maternal IgG into fetal circulation throughout the course of pregnancy, with ongoing cumulative adverse effects [27]. Glycophorin A is expressed on early erythroid precursors and it is believed that anti-M binding may lead to ineffective erythropoiesis and reticulocytopenia similar to that reported for anti-K [24,27], but with a distinct target molecule. This was demonstrated by Ishida et. al. who showed a decrease in erythroid colony formation by anti-M *in vitro* [26]. A subsequent case of a low titer, cold reactive anti-M IgG causing ADFN with reticulocytopenia was recently reported in the USA [28]. Similarly, ADFN with ineffective erythropoiesis is reported with anti-Ge3 antibodies against glycophorin C [29].

This study has some limitations. As a retrospective survey, it relied on post-transfusion data collection that was not necessarily part of routine care. This makes it likely that there were unobserved instances of attenuation and formation/reactivation of allo-anti-M. Moreover, pregnancy-

related anti-M sensitization should be elucidated more carefully. Although no case of anti-M was found in cohort F (15 to <20 years), this might be due to or relatively small sample size (n= 1,745); anti-M arising either from pregnancy or transfusion is plausible in this age cohort.

In conclusion, this retrospective survey revealed that naturally occurring anti-M was often found among children between 1 and 3 years of ages and was frequently attenuated, irrespective of whether transfused RBCs carried M antigen.

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Table 1. Allo-anti-M detected in pediatric recipients.

	Cohort A (neonates)	Cohort B (infants)	Cohort C (children)	Cohort D (pre-pubescents)	Cohort E (young pubescents)	Cohort F (adolescents/ young adults)
Age interval	0 - < 1 month	1 - < 12 months	1 - < 5 years	5 - < 10 years	10 - < 15 years	15 – < 20 years
No. of patients tested	5,253	4,628	4,035	1,708	1,575	1,745
Anti-M detected[a] (%, 95% CI) [d]	4[b] (0.08%) or 0[c] (0%, 0-0.07)	7 (0.15%, 0.04-0.26)	31 (0.77%, 0.52-1.09)	5 (0.29%, 0.04-0.55)	1 (0.06%, 0- 0.35)	0 (0%, 0 - 0.21)
Age distribution		6 m (1) 7 m (1) 8 m (3) 10 m (1) 11 m (1)	1 y (14) 2 y (11) 3 y (6)	5 y (1) 6 y (1) 8 y (1) 9 y (2)	12 y (1)	

a: including all patients who had anti-M before and/or after transfusion.

b: including 4 neonates (2 singletons, 2 twins: 3 mothers who had maternally-transferred antibody).

c: excluding 4 neonates who had maternally-derived anti-M

d: statistically significant difference between Cohort C vs Cohorts A ($p < 0.00001$), B ($p < 0.00001$), E ($p < 0.0005$), and F ($p < 0.00001$).

Table 2. Characteristics of the patients with allo-anti-M

Disease/condition for transfusion[a]		
Surgery/trauma	22	
Malignancy (including leukemia)	11	
Others	8	
Viral myocarditis[b, c]		1
Acute encephalopathy[b, d]		1
Viral associated hemophagocytic syndrome[b, e]		1
Respiratory distress[b, f]		1
Hemolytic uremic syndrome[b,g]		1
Pulmonary cyst		1
Pulmonary artery stenosis		1
Myelodysplastic syndrome		1
Anonymized	2	
Unknown	1	
Previous or present pregnancy	0	

a: excluding 4 neonates with maternally-derived anti-M

b: infection may be involved

c: not described, but frequently by adenovirus or enterovirus (including coxsackie virus)

d: not described, but often by herpes viruses, influenza viruses, rotavirus, or respiratory syncytial virus

e: not described, but often by Epstein-Barr virus

f: not described, but often by respiratory syncytial virus

g: no pathogen was identified

Figure 1. The occurrence, persistence and evanescence of allo-anti-M other than maternally-derived.

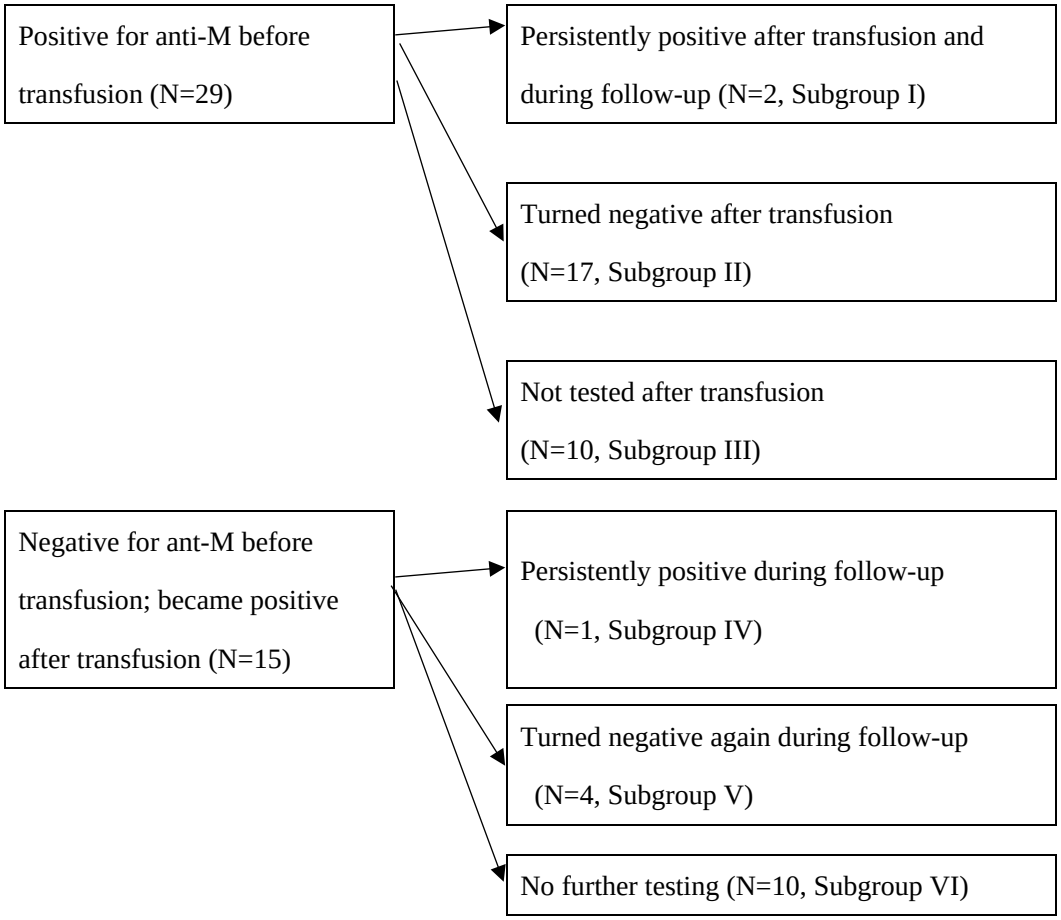

Table 3. Characteristics of patients with anti-M, and persistence/evanescence of anti-M following RBC transfusion

			Pre-Transfusion			Post-Transfusion	
Subgroup	**Disease/Condition**	**RBC transfusion**	**Patient Age**	**Anti-M Status**		**Time Interval**	**Anti-M Status**
Subgroup I							
01	Surgery/trauma	M-negative, 1 bag	2 years	Positive		28 days	Positive
02	Leukemia	M-negative, 25 bags	9 years	Positive		2.3 years	Positive
Subgroup II							
03	Cardiac surgery	M-negative, 4 bags	7 months,	Positive		3 years	Negative
04	Viral myocarditis	M-negative, 4 bags	8 months	Positive		11 days	Negative
05	Malignancy	M-negative, 3 bags	1 year	Positive		17 days	Negative
06	Malignancy	Random, 4 bags	1 year	Positive		20 days	Negative
07	Cardiac surgery	M-negative, 16 bags	1 year	Positive		10 days	Negative
08	Malignancy surgery	Random, 5 bags	1 year	Positive[a]		9 months	Negative
09	Malignancy	M-negative, 6 bags	2 years	Positive		7 months	Negative
10	Malignancy chemotherapy	M-negative, 4 bags	2 years	Positive		3 months	Negative
11	Cardiac surgery	M-negative, 1 bag	2 years	Positive		3 days	Negative

12	Hemolytic-uremic syndrome	M-negative, 5 bags	2 years	Positive[b]		9 days	Negative
13	Malignancy	M-negative, 4 bags[c]	3 years	Positive		62 days 82 days	Negative Positive
14	Leukemia	M-negative, 4 bags	3 years	Positive		21 days	Negative
15	Myelodysplastic syndrome	Random, 3 bags	3 years	Positive[d]		68 days	Negative
16	Leukemia	M-negative, 5 bags	5 years	Positive		53 days	Negative
17	Brain surgery	M-negative, 1 bag	6 years	Positive		91 days	Negative
18	Respiratory distress	M-negative, 1 bag	8 years	Positive		5.8 years	Negative
19	Malignancy	NIA[e], 1 bag	11 years	Positive		11 days	Negative
Subgroup III							
20	Acute encephalopathy	Random, 1 bag	8 months	Positive			Not tested
21	Cardiac surgery	M-negative, 1 bag	1year	Positive			Not tested
22	Surgery/trauma	M-negative, 1 bag	1 year	Positive			Not tested
23	Surgery/trauma	NIA[e], 3 bags	1 year	Positive			Not tested
24	Virus-associated hemophagocytic	NIA[e], 1 bag	1 year	Positive			Not tested

	syndrome						
25	Others	M-negative, 1 bag	1 year	Positive			Not tested
26	Anonymized	Random, 2 bags	2 years	Positive			Not tested
27	Others	Random, 2 bags	2 years	Positive			Not tested
28	Pulmonary-cystic disease	M-negative, 1 bag	2 years	Positive			Not tested
29	Cardiac surgery	M-negative, 3 bags	3 years	Positive			Not tested
Subgroup IV							
30	Cardiac surgery	Random, 3 bags[f]	<1 month	Negative		6 months 9.5 months	Positive Positive
Subgroup V							
31	Cardiac surgery	Random, 1 bag[g]	<1 month	Negative		17 months 3.3 years	Positive Negative
32	Surgery/trauma	Random, 3 bags[h]	<1 month	Negative		13 months 17 months	Positive[i] Negative
33	Pulmonary-artery occlusion	Random, 1 bag[j]	1 month	Negative		9 months 17 months	Positive Positive

						28 months	Negative
34	Surgery/trauma	Random, 2 bags[k]	8 months	Negative		24 months 32 months	Positive Negative
Subgroup VI							
35	Malignancy	Random, 1 bag	2 years	Negative		1 day	Positive[l]
		Random, 2 bags	2 years	Positive			Not tested
36	Cardiac surgery	Random, 12 bags	< 1 month	Negative		12 months	positive
37	Surgery/trauma	Random, 2 bags	<1 month	Negative		7 months	positive
38	Surgery/trauma	Random, 2 bags	5 months	Negative		15 months	positive
39	Retroperitoneal teratoma surgery	Random, 1 bag	6 months	Negative		24 months	negative
						31 months	positive
40	Asplenia surgery	Random, 3 bags	1 year	Negative		18 months	positive
41	Cardiac surgery	Random, 1 bag	<1 month	Negative		12 months	negative
		Random, 2 bags	1 year	Negative		11 months	positive
42	Patent-ductus arteriosus	Random, 1 bag	1 year	Negative		8 days	positive
43	Cardiac surgery	Random, 1 bag	2 years	Negative		18 months	positive
44	Leukemia, Graft-vs-	Random, 28 bags	4 years	Negative		26 months	positive

	host disease						

a. Case 08, positive by an enhanced IAT, but negative by saline-IAT at 37 ℃ for 60 minutes.

b. Case 12, weakly positive by PEG-IAT at 37 ℃, but negative by saline-IAT at 37 ℃ for 60 minutes.

c. Case 13 plus 2 bags of M-negative RBCs even after reverse-seroconversion to negative for anti-M.

d. Case 15, negative by saline-IAT at 37 ℃ for 60 minutes.

e. NIA, no information available.

f. Case 30, plus 2 bags of M-negative RBCs after seroconversion

g. Case 31, plus 2 bags of M-negative RBCs after seroconversion

h. Case 32, plus 2 bags of RBCs with unknown M/N antigens after seroconversion.

i. Case 32, anti-M positive by an enhanced IAT, but negative by saline-IAT at 37 ℃ for 60 minutes

j. Case 33, plus 7 bags of M-negative RBCs after seroconversion

k. Case 34, plus 3 bags of M-negative RBCs after seroconversion

l. Case 35, positive only at cold temperature