ORIGINAL REPORT

Proposed criterion for distinguishing ABO mosaics from ABO chimeras using flow cytometric analysis

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Differentiation of ABO mosaics from chimeras is performed using flow cytometry (FCM) analysis. Although mosaics and chimeras have been distinguished by presence or absence of clear resolution using FCM analysis, the lack of quantitative metrics and definitive criteria for this differentiation has made some cases difficult to differentiate. In this study, therefore, we attempted to establish a definitive and quantitative criterion for this differentiation. When FCM histogram gates for group "A" or "B" antigen-negative and -positive red blood cells (RBCs) were set such that group O RBCs were classified as 99 percent negative and group A or B RBCs as 99 percent positive, the percentages of RBCs in the middle region of six chimeras and 23 mosaics (12 A mosaics and 11 B mosaics) were 0.1–0.6 percent and 7.0–19.0 percent, respectively. This result suggested that ABO mosaics and chimeras can be unambiguously differentiated when the cutoff point of the intermediate region is set to 1 percent. ***Immunohematology* 2015;31:24–28.**

Key Words: ABO, mosaic, chimera, flow cytometry

The differentiation of ABO subgroups is usually determined serologically on the basis of the following: (1) agglutination tests with human polyclonal anti-A, anti-B, and anti-A_1 lectin; (2) identification of anti-A or anti-B in serum; and (3) ABO transferase activity. ABO mosaics and chimeras are usually differentiated from other subgroups using these tests.[1–5] Consequently, mosaics and chimeras are differentiated by coil planet centrifugation (CPC)[6] or flow cytometry (FCM) methods.[7–9] Genotyping is useful as a supplementary tool to confirm the final differentiation of these ABO subgroups.[1,2] Although ABO mosaics and chimera can be easily differentiated from other subgroups, it is difficult to differentiate them from each other using standard serologic methods,[3,4] and only CPC and FCM methods are available for this purpose. The coil planet centrifuge is no longer manufactured and is thus difficult to obtain. Furthermore, distinguishing ABO mosaics from chimeras using the FCM method is based simply on the presence or absence of clear resolution of positive and negative peaks. This lack of quantitative metrics has made some cases difficult to differentiate because there are no definitive criteria for this determination. In addition, a quantitative FCM method appears promising for diagnosing early relapse or rejection after hematopoietic progenitor cell transplantation using ABO-incompatible cells.[10] Therefore, in the present study, we developed a quantitative criterion for FCM-based differentiation of ABO mosaics from chimeras by setting a gate between the "A" or "B" antigen-negative and -positive red blood cell (RBC) populations in the FCM histogram and then determining the proportion of cells in the middle gate. Quantitative tests such as this FCM system should be applied to other qualitative serologic tests.

Materials and Methods

Blood Samples

Twelve A mosaic samples, 11 B mosaic samples, and 6 ABO chimera samples were obtained from volunteer blood donors in the Kinki area of Japan. These samples were previously identified as mosaics or chimeras based on the following: (1) agglutination tests with monoclonal anti-A, anti-B, anti-A_1 lectin, and anti-H lectin; (2) identification of anti-A or anti-B in serum; and (3) ABO transferase activity in plasma. Consequently, the possibility of other subgroups including A_1, A_2, A_3, and A_x were ruled out.[1,2] Similarly, B, B_3, and B_x were ruled out. In addition, their phenotypes as mosaics or chimeras were confirmed by the CPC method, which can detect ABO chimerism as low as 0.1 percent.[6] A flow chart outlining the classification of ABO mosaics, chimeras, and other subgroups is presented in Figure 1. We did not perform a family study to determine if any of the donors were related. It is likely that most of the donors were unrelated, however, because they all had different family names and lived in widely scattered regions.

ABO Genotyping

Genomic DNA was prepared from 200 μL ethylene-diaminetetraacetic acid (EDTA) whole blood using a collection system (Quick whole blood kit, KURABO Industries, Osaka, Japan). The polymerase chain reaction–reverse sequence specific oligonucleotide (PCR-rSSO) method was performed

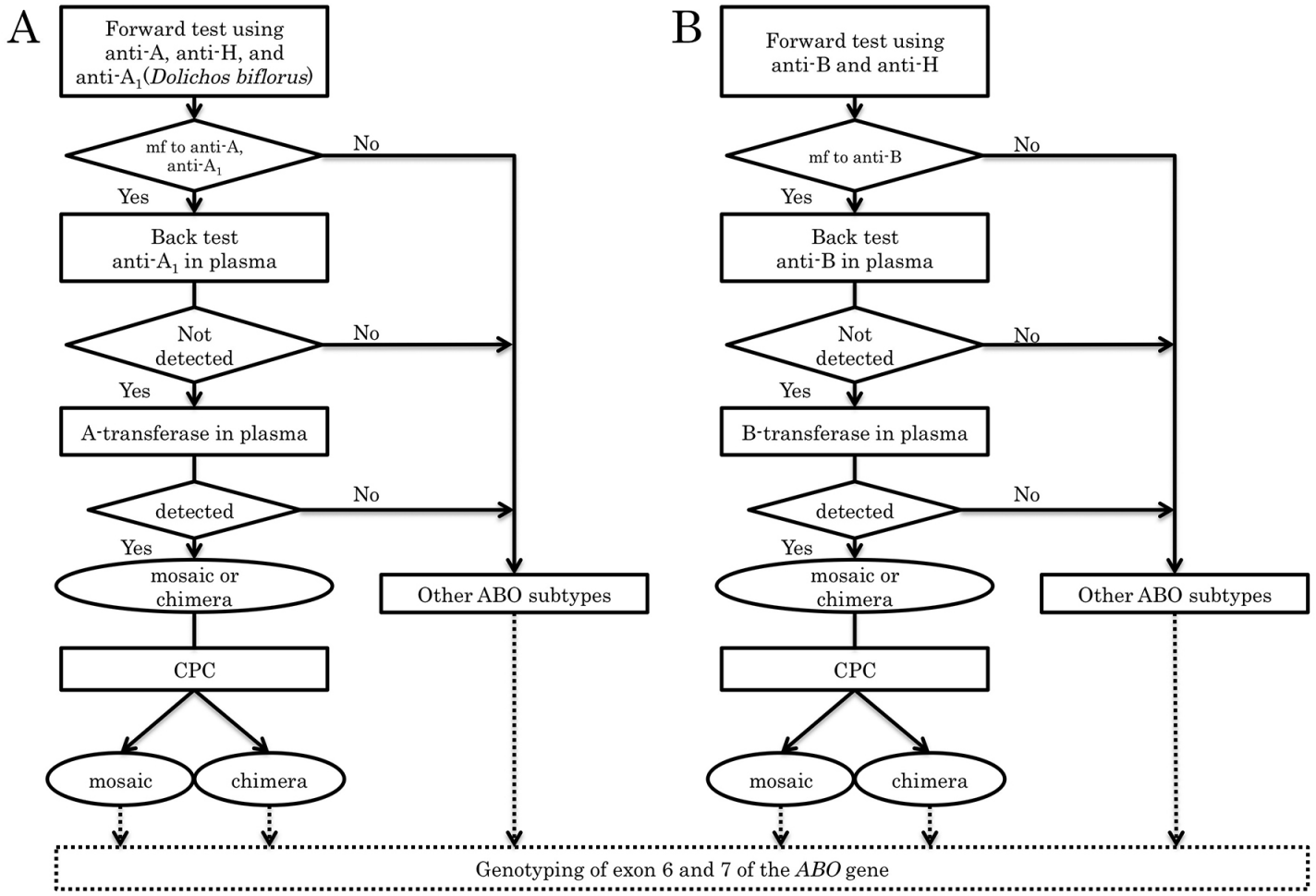

Fig. 1. Flow chart outlining the classification of ABO mosaics, chimeras, and other subgroups. Broken arrows and broken squares indicate supplementary tools used to confirm the final differentiation of these ABO subgroups.

to detect single nucleotide polymorphisms (SNPs) on ABO alleles. Amplicons labeled with fluorescence were separately amplified by PCR from ABO exons 6 and 7 using an automated system (GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA,) and the group-specific reagent (Genosearch ABO reagent, Medical & Biological Laboratories, Nagano, Japan). We detected SNPs on *ABO* alleles using a florescent system (Luminex System 200, Hitachi Solutions, Tokyo, Japan). In addition, we confirmed the DNA sequence of exon 7 on both A and B alleles by direct PCR sequencing. We first amplified exon 7 on both alleles using the primers GA22 and GA23 (Table 1). Each PCR contained 2.5 µL genomic DNA, 1 µL GA22, 1 µL GA23, 35.25 µL sterile water (distilled deionized sterile water, Nippon Gene Co. Ltd., Toyama, Japan), 5 µL of a dNTP mix at a concentration of 2 mmol/L of each dNTP (GeneAmp Applied Biosystems), 5 µL of a 10× buffer (PCR buffer, Applied Biosystems), and 0.25 µL of a Taq amplifier in a concentration of 250 U at 5 U/µL (AmpliTaq Gold, Applied Biosystems). PCR amplification was performed with initial denaturation at 96°C for 2 minutes, followed by 35 cycles at 96°C for 1 minute, 62°C for 1 minute, and 72°C for 4 minutes. For DNA sequencing analysis, the PCR fragments, which were purified, (QIAquick PCR purification kit,QIAGEN, Hilden, Germany), were fluorescently labeled with primer GA62 or GA03 (Table 1) using a cycle sequencing kit (BigDye Terminator v1.1, Applied Biosystems). The DNA sequences

Table 1. Primers used for amplification and direct sequencing of ABO gene fragments

Primer designation	Primer sequence (5′–3′)	Direction	Primer location	Amplified region
GA22	CTAAAACCAAGGGCGGGAGG	reverse	3′-UTR	
GA23	GAAGGATGTCCTCGTGGTGA	forward	exon 6	exon 6–7
GA62	TCAGGACAGGGCAGGAGAACG	forward	intron 6	exon7
GA03	TGCTGGAGGTGCGCGCCTAC	forward	exon 7	exon 7

were measured using a genetic analyzer (ABI PRISM 3130×L, Applied Biosystems). The loci c.261 and c.297 in exon 6 and c.467, c.526, c.547, c.646, c.657, c.681, c.703, c.771, c.771, c.784, c.796, c.802, c.803, c.829, c.871, c.930, c.1006, c.1054, and c.1060 in exon 7 were analyzed, and the results indicated that all of the samples that we could analyze were either *A102* or *B101* (Table 2).

Preparation of Cell Samples for FCM Analysis

A 10-μL RBC suspension (whole blood collected in K_2EDTA) was washed three times in phosphate-buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO, USA) and fixed by mixing with 50 μL of 0.25% glutaraldehyde (Nacalai Tesque, Inc., Kyoto, Japan) for 15 minutes at 5°C. The RBC suspension was subsequently washed four times using PBS containing 0.2% fetal bovine serum (FBS) (EIDIA Co. Ltd., Tokyo, Japan) and adjusted to a 0.25% cell suspension. Thereafter, 25 μL of this suspension was mixed with 50 μL monoclonal anti-A or anti-B (Bioclone Anti-A and Bioclone Anti-B, Ortho-Clinical Diagnostics, Tokyo, Japan) for 30 minutes at 5°C.[11,12] After washing twice with PBS containing 0.2% FBS (PBS/FBS), a tagged antibody (goat anti-mouse IgG-FITC, BD Biosciences, San Jose, CA, USA) was added for 30 minutes at 5°C. After a final wash with PBS, the cells' florescence was measured by FCM (FACSCalibur, BD Biosciences).

Table 2. Summarized results of nucleotide substitutions in *ABO* exons 6 and 7

	Position									
	Exon 6		Exon 7							
Sample*	261 G	297 A	467 C	526 C	657 C	703 G	796 C	803 G	930 G	Allele *A101*
A mosaic 1										NT
A mosaic 2	—	—	T	—	—	—	—	—	—	*A102*
A mosaic 3										NT
A mosaic 4	—	—	T	—	—	—	—	—	—	*A102*
A mosaic 5	—	—	T	—	—	—	—	—	—	*A102*
A mosaic 6										NT
A mosaic 7	—	—	T	—	—	—	—	—	—	*A102*
A mosaic 8	—	—	T	—	—	—	—	—	—	*A102*
A mosaic 9	—	—	T	—	—	—	—	—	—	*A102*
A mosaic 10										NT
A mosaic 11	—	—	T	—	—	—	—	—	—	*A102*
A mosaic 12	—	—	T	—	—	—	—	—	—	*A102*
B mosaic 1	—	G	—	G	T	A	A	C	A	*B101*
B mosaic 2	—	G	—	G	T	A	A	C	A	*B101*
B mosaic 3	—	G	—	G	T	A	A	C	A	*B101*
B mosaic 4	—	G	—	G	T	A	A	C	A	*B101*
B mosaic 5	—	G	—	G	T	A	A	C	A	*B101*
B mosaic 6										NT
B mosaic 7										NT
B mosaic 8	—	G	—	G	T	A	A	C	A	*B101*
B mosaic 9	—	G	—	G	T	A	A	C	A	*B101*
B mosaic 10	—	G	—	G	T	A	A	C	A	*B101*
B mosaic 11	—	G	—	G	T	A	A	C	A	*B101*
Chimera 1										ND
Chimera 2										ND
Chimera 3										ND
Chimera 4										ND
Chimera 5										ND
Chimera 6										ND

*GenBank accession no. AF134412. NT = not tested; ND = not determined.

Setting the Gate for FCM

Using control RBCs (O and A_1 or B groups), the G1 gate was set so that group O RBCs were 99 percent negative for A or B antigens, and the G3 gate was set so that group A_1 or B RBCs were 99 percent positive for A or B antigens. When these gates were applied, only 0.1–0.3 percent of group O control RBCs showed nonspecific binding to anti-A or anti-B and only 0.1–0.4 percent of group A_1 or B RBCs were false negative. The G2 gate was set as the region between G1 and G3 (Fig. 2). The proportion of cell population detected in the G2 gate was calculated as follows: [G2 / (G1 + G2 + G3) × 100].

Calculation of the Heterogeneity of Chimera Samples from a Standard Calibration Curve

The following artificial mixtures of A_1 or B and O RBCs were prepared [(A_1 or B) / (A_1 or B + O) × 100] and analyzed using FCM: 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 percent. A standard calibration curve of (G2 + G3) / (G1 + G2 + G3) was generated and used to estimate the ratios of cells in the chimera samples.

Results

The percentages of 23 ABO mosaics and 6 ABO chimeras detected in the G2 gate are presented in Tables 3 and 4. The mean percentages of mosaics and chimeras in the G2 gate were 13.1 percent (range: 7.0–19.0%) and 0.2 percent (range: 0.1–0.6%), respectively.

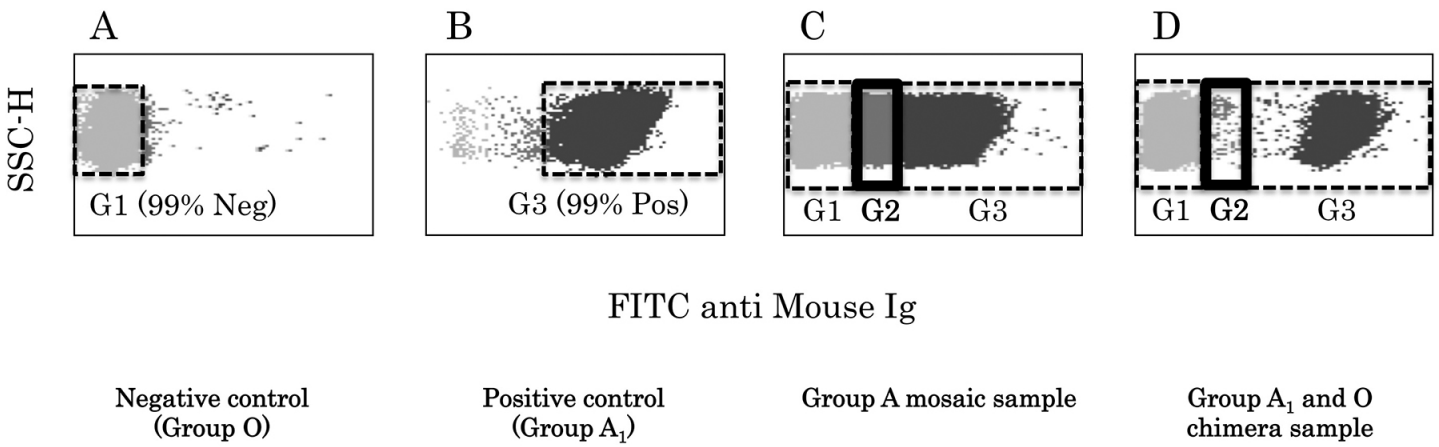

Fig. 2. Determination of gate. Dot plots of negative (A) and positive (B) control samples. Gating regions were determined as 99 percent negative (G1, dotted line) and 99 percent positive (G3, dotted line). The G2 (solid line) gate was defined as the region between G1 and G3 indicated in images C and D, which are example dot plots of an A mosaic and A_1 + O chimera sample, respectively. FITC = fluorescein isothiocyanate.

Although the number of samples was small, the G2 values of mosaics were significantly higher than the values of chimeras ($p < 0.001$: Mann-Whitney U test). When the cutoff point was set to 1 percent to differentiate mosaics from chimeras, all analyzed cases were differentiated accurately and unambiguously.

We then attempted to estimate the proportion of the two different types of RBCs in the chimera samples using a standard linear curve that defines the relationship between the mixing ratio of artificial chimera samples and their values in the G3 gate. We obtained a linear curve, with the known concentrations on the y-axis and the measured variable on the x-axis (data not shown). The estimated ratios were almost consistent with the G1 and G3 gate values (Table 4).

Discussion

In the present study, we successfully established a clear and simple criterion for FCM analysis to differentiate ABO mosaics from ABO chimeras by setting the FCM histogram gates (G1 and G3) for group "A" and/or "B" antigen-negative and -positive RBCs. We set these gates such that 99 percent negative RBCs and 99 percent positive RBCs were included in gates G1 and G3, respectively. The value of 99 percent set the cutoff point to 1 percent to differentiate mosaics from chimeras. This value allowed us to unambiguously differentiate both types of RBCs. Furthermore, we attempted to determine heterogeneity based on data from artificial chimera samples with a known number of RBCs of each blood group.

After having established the proposed criteria, we performed serologic ABO blood group testing on 777,617

Table 3. ABO mosaic samples

	% of gate (anti-A)		
Sample	**G1**	**G2**	**G3**
A mosaic 1	17.6	11.8	70.6
A mosaic 2	36.7	19	44.3
A mosaic 3	66.8	8.7	24.5
A mosaic 4	17.6	13.7	68.7
A mosaic 5	20.6	13.8	65.6
A mosaic 6	55.5	7	37.5
A mosaic 7	40.4	14.4	45.2
A mosaic 8	23.5	17.4	59.1
A mosaic 9	32.9	12.6	54.5
A mosaic 10	24.5	10.2	65.3
A mosaic 11	37.1	18.8	44.1
A mosaic 12	45	10.7	44.3
Mean	34.9	13.1	52
	% of gate (anti-B)		
Sample	**G1**	**G2**	**G3**
B mosaic 1	27.7	11.3	61
B mosaic 2	19.5	10.9	69.6
B mosaic 3	35.3	17.3	47.4
B mosaic 4	28.9	13.8	57.3
B mosaic 5	18.3	12	69.7
B mosaic 6	44.9	14.8	40.3
B mosaic 7	32.7	16.8	50.5
B mosaic 8	45.6	11.9	42.5
B mosaic 9	43.2	10.8	46
B mosaic 10	61.1	15.3	23.6
B mosaic 11	46.8	8.5	44.7
—	—	—	—
Mean	36.7	13.1	50.2

Table 4. ABO chimera samples

Sample	Constituent	Positive antigen	% of gate			Estimated chimera ratio	
			G1	G2	G3	Negative	Positive
Chimera 1	B + AB	A	82.9	0.1	17	76	24
Chimera 2	O + B	B	70.9	0.1	29	70	30
Chimera 3	A + AB	B	56.1	0.6	43.3	55	45
Chimera 4	A + AB	B	95	0.1	4.9	95	5
Chimera 5	O + B	B	91.7	0.1	8.2	88	12
Chimera 6	O + A	A	29.9	0.1	70	30	70
Mean	—	—	71.1	0.2	28.7	69	31

Constituent = red blood cell ABO group.

donors as a routine test from December 2013 to October 2014. Among the samples from all donors, nine samples were suspected to be mosaic or chimera based on serologic analyses; subsequent CPC analyses revealed that seven of these samples were mosaics and the remaining two samples were chimeras. When a validation test was performed using these nine samples, our proposed FCM criterion clearly discriminated between these mosaics and chimeras.

Although FCM analysis is widely used to distinguish ABO mosaics from ABO chimeras and hence our qualitative FCM system may not have a powerful impact on the differentiation of ABO mosaics and chimeras, we hope that it contributes to the introduction of more quantitative tests in the field of serologic blood typing.

Our FCM system has a drawback. Because it is necessary to set the G3 gate wide enough for a substantial number of mosaic RBCs to be counted in the G3 population, chimera RBCs in G2 should exhibit relatively high expression of A or B antigens. Therefore, A_2 chimeras (e.g., A_2 + O), the RBCs of which express only a limited amount of A or B antigens, cannot be distinguished from "A" mosaics. Although A_2 is a rare blood group in Asian populations and is not a major problem in Asia, the possibility of finding an A_2 blood type should be carefully considered when analyzing RBCs from people of European or African descent, because A_2 is found in 8–10 percent of these populations.[13]

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