American Journal of Medical Genetics 61:264–268 (1996)

Blood Group Chimerism in Human Multiple Births Is Not Rare

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Twin blood group chimerism seems to be very rare in humans. The 30–40 previously reported cases usually were found by mere coincidence during routine blood grouping in hospitals or blood banks. Usually in these cases frank blood group mixtures of, for example, 50/50%, 25/75%, or 5/95% at most were seen. Smaller percentages are very difficult to notice during routine work-up. Using a sensitive fluorescence technique (sensitivity >0.01%) we detected blood group chimerism in 32/415 (8%) twin pairs and 12/57 (21%) triplet pairs, respectively, which is a higher incidence than reported previously.
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KEY WORDS: chimerism, twins, triplets, blood groups, erythrocytes

INTRODUCTION

Chimera referred to the monster of Greek mythology with the head of a lion, the body of a goat, and the tail of a serpent. In biology the word *chimera* is used when an organism has cells from two or more zygotes [Race and Sanger 1975]. When chimerism is confined to blood groups only, one can classify it as artificial chimerism, dispermic chimerism, and twin chimerism.

Artificial chimerism can arise from transfused blood stem cells, either by intrauterine transfusion—though nowadays this type of transfused blood usually is irradiated to prevent proliferation of donor stem cells in the host and consequently graft-versus-host disease—or by allogeneic bone marrow transplantation.

Dispermic chimerism can occur in cats, mice, goats, mink, horses, and as a rarity in humans [Tippett, 1983]. It is induced by the fertilization of two maternal eggs with two paternal spermatozoa and the fusion of these products into one body. This tetragametic chimerism is not limited to blood, but may also result in hermaphroditism, mixture of skin colours or two-cell lineage of fibroblasts. In humans 32 cases have been summarized [Tippett, 1983] and other cases have been published since.

Twin chimerism is a well-known phenomenon in dizygotic twin cattle, less common in other animals (e.g., marmosets), but an exception in humans. Some 30–40 cases have been reported in humans [Tippett, 1983], though not all cases are published. In cattle, chimerism is due to blood vessel anastomoses in the placenta, through which one twin can transfuse blood cells, including stem cells, and also primordial germ cells to the other and vice-versa. Some invading stem cells survive in the host by nidation and proliferation in the bone marrow. Since the fetal immune system is incompetent to recognise foreign antigens, this results in the coproduction of blood cells from host origin as well as from donor origin for the rest of the individual's existence. This way, two non-compatible blood groups can coexist in one individual.

Although hemopoietic twin chimerism is not confined to red cells only (leukocytes, for example, also show the phenomenon) [Race and Sanger 1975], it is usually found by coincidence during routine ABO/Rhesus blood grouping in hospitals and blood banks. Mostly it is noted on the basis of 2 populations of red cells, in blood group serology known as "partial agglutination" or "mixed field." Mixtures of 50/50% or 25/75% are detected easily; however, a 5/95% minor cell population can already be missed in routine blood grouping. Another way of detecting chimeras is on the basis of lack of the expected naturally occurring anti-A or anti-B isohemagglutinin. For example, a blood group O chimeric person with 5% minor cell population A-cells will only show anti-B in his or her serum and lack anti-A because of immune tolerance during fetal life against invading A red cells from his or her twin. On the other hand, a blood group A person with 5% O-cell minor population will not be recognized as being a chimera.

Twin chimerism seems to be very rare in humans [Tippett, 1983]. The 30–40 reported cases were found by coincidence. There are seven reports of systematic search for chimerism in twins [Race and Sanger 1975]. The combined searches included approximately 300

Received for publication June 3, 1994; revision received February 22, 1995.

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dizygotic twins. Only one case in a 4-month-old twin-half was was found; 2 months later it was undetectable.

Our laboratory has developed a fluorescence technique that can detect very subtle blood group mixtures [De Man et al., 1988]. We have used this technique in a prospective study to establish the frequency of blood group chimerism in human dizygotic twins and di- or trizygotic triplets, and to test the hypothesis that blood group chimerism in human multiple births is less rare than generally believed.

MATERIALS AND METHODS

The Netherlands Twin Register (NTR) was started at the Free University in Amsterdam in 1987 and registers 40–50% of all newborn twins and triplets in The Netherlands [Boomsma et al., 1992]. Parents of 4-year-old twins or triplets born in 1987 and 1988 were asked to mail a blood sample of the twins/triplets (drawn by a general practitioner) to the Blood Transfusion Department of the University Hospital St. Radboud in Nijmegen. The request was put to the parents in the context of a research project on the inheritance of twinning. Parents were asked to let their offspring donate a blood sample to determine zygosity of the twins/triplets and were also asked permission to look for blood group chimerism in non-identical twins or triplets. Parents of twins of unlike sex were also asked to participate in the chimerism study. A total of 1,378 parents was approached and blood samples from 552 twin pairs and 24 triplets were received in time to be part of this study (Table I). After 18 months, a second blood sample was obtained from 14 twin and 6 triplet pairs in which at least one of the children showed a chimerism. All individuals from these triplet and twin pairs were retested. The study also included 48 16-year-old twin pairs who took part in an electroencephalogram study and from whom blood samples for zygosity determination were available.

After receipt the blood samples were centrifuged. The leukocytes and plasma were kept frozen separately for future research purposes. The red cells were typed with conventional, commercially available, test sera for the following red cell blood group antigens: ABO, CcDEe, MNSs, P_1, Kk, Kp^aKp^b, Fy^aFy^b, Jk^aJk^b, Lu^aLu^b. When in a pair of twins/triplets one or more differences were found (i.e., a blood group antigen present in one sib and absent in the other), the antigen involved was considered a marker antigen. The sib lacking the marker antigen was investigated for the presence of a minor subpopulation of erythrocytes possessing the antigen in his blood circulation by using a fluorescence assay (see below). This was possible for all blood group antigens except for P_1, Lu^a, and Lu^b for the following reasons. For P_1 no anti-IgG antibody (see below) is available, and Lu^a/Lu^b has not been validated for the fluorescence assay because it is a seldomly encountered blood group difference of low frequency. All determinations were performed once by an experienced group of 4 technicians.

TABLE I. Responding Multiple Pairs and Results of Zygosity Based on Blood Group Determination

	Parents asked	Blood sample received		Zygosity[a]
4-year-old twins	1,318	552	600	286 MZ 266 DZ
16-year-old twins	48	48		31 MZ 17 DZ
4-year-old triplets	60	24		2 MZ 8 DZ 14 TZ

[a]MZ = monozygotic; DZ = dizygotic; TZ = trizygotic.

The fluorescence assay that was used has been described before [De Man et al., 1988]. In short, red blood cells are first incubated with an anti-IgG antibody directed against the marker antigen under investigation. After washing the erythrocytes, fluorescent microspheres coated with anti-human IgG are added, followed by centrifugation. Marker positive erythrocytes which are intensively labelled with fluorescent microspheres can be visualized and counted under a fluorescence microscope. The sensitivity level of this assay is one positive cell per 10,000 negative cells, i.e., 0.01%. The mean percentage positive cells in 15 different blood group negative cell suspensions was 0.002 ± 0.004 (SD) with a range of 0–0.012%. The mean number of cells analyzed was 23,500 [De Man et al., 1988].

RESULTS

From the 600 twin pairs, 283 (47%) had one or more differences in the blood grouping profile and were consequently designated to be dizygotic (Table I). Because in 16 pairs P_1, Lu^a, or Lu^b were the only differing antigens, 267 dizygotic twin pairs could be investigated for blood group chimerism (see Materials and Methods). In 148 twin pairs both individuals had marker antigens, so that both could be investigated for mutual chimerism. In the remaining 119 pairs only one of both individuals could be tested.

From the 24 triplet pairs, 22 appeared to be di- or trizygotic. In 17 pairs all 3 had different blood groups that allowed for mutual chimerism investigation. In 2 pairs 2 individuals, and in 2 other pairs only one individual could be investigated.

In summary, 472 individuals were investigated for chimerism using 849 marker antigens (Table II). There was no significant difference in prevalence of any blood group marker antigen in either the non-chimeric or the chimeric group. In twins and triplets, respectively, 32/415 (8%) and 12/57 (21%) individuals appeared to have blood group chimerism of >0.01% (chi-square = 10.55, df = 1, P = 0.001). The individual results are shown in Tables III and IV. Only one of the evident cases (triplet 50-y) was detected with the unaided eye during the extended blood group typing because of "mixed field" reaction pattern. Thirteen twin pairs and 6 triplet pairs, 44 individuals in total, were retested after approximately 18 months; the results are shown in Table V. Twelve individuals still showed blood group chimerism, 8 individuals had become negative, and 6 individuals who initially were negative showed chimerism on the second occasion.

TABLE II. The 849 Marker Antigens Used in this Study (There Are More Markers Than Individuals (n = 472, See Results) Because Several Individuals Had >1 Marker Antigen*

	Non-chimeric cases		Chimeric cases	
Marker-antigen	(n)	(%)	(n)	(%)
A	62	8.4	8	7.1
B	25	3.4	2	1.8
C	70	9.5	8	7.1
c	41	5.6	10	8.9
D	41	5.6	5	4.5
E	56	7.6	8	7.1
e	9	1.2	1	0.9
M	52	7.1	9	8.0
N	52	7.1	5	4.5
S	64	8.7	8	7.1
s	23	3.1	5	4.5
K	26	3.5	2	1.8
k	—	—	—	—
Kp^a	4	0.5	—	—
Kp^b	—	—	—	—
Fy^a	60	8.1	13	11.6
Fy^b	43	5.8	9	8.0
Jk^a	58	7.9	9	8.0
Jk^b	51	6.9	10	8.9
	−+	−+	−+	−+
	737	100%	112	100%

*A, B: ABO system; C, c, D, E, e: Rhesus system; M, N, S, s: MNSs system; K, k, Kp^a, Kp^b: Kell system; Fy^a, Fy^b: Duffy system; Jk^a, Jk^b: Kidd system.

DISCUSSION

Our laboratory has developed a sensitive and reliable fluorescence technique for detecting very subtle minor red cell populations [De Man et al., 1988]. It is primarily used for the monitoring of patients after allogeneic bone marrow transplantation (BMT). Some 2–4 weeks after BMT the minor population of donor red cells that appears in the circulation of the recipient can be recognized with a sensitivity of 1:10,000 cells (0.01%). This artificially chimeric state increases in time, and in the first 4 months after BMT a change of blood groups can be seen. Although it seems logical that the blood groups of the BMT patient are completely changed into those of his donor (complete donor chimerism), a high number of mixed chimeras (i.e., patients with both autologous red cells and donor-type red cells) can be observed, especially in recipients of T-cell depleted grafts [Schattenberg et al., 1989]. Since 1986 we have had experience with this technique. Annually about 750 investigations for this purpose are performed. The technique is highly appreciated by hematologists, since it is very sensitive, simple, rapid, and inexpensive. The reliability was proven in a comparative study using genetic markers and restriction fragment length polymorphism (RFLP) studies [Schattenberg et al., 1989]. The technique is also used in our hospital as a non-invasive replacement for Cr^{51}-survival studies in cases of incompatible blood transfusion.

Since almost all reports concerning twin blood group chimerism concern mixture percentages of relatively gross character (50 individuals, >5%; 9 individuals, 1–5%; and only 5 individuals, <1%) [Tippett, 1983], we wondered if more subtle forms of chimerism could be detected using the fluorescence method. Indeed, we

TABLE III. Results of 32 Individuals With Twin Chimerism*

	Chimerism		Marker antigens with no detectable chimerism
Twin no.	Marker antigens	%	
1620-e	Fy^a-Jk^b	23.9–3.1	N
1622-e	N	15.3	s-E
1518-y	Fy^a	11.4	A
1181-e	Fy^b	8.4	Jk^a
1194-y	Fy^b-s	8.3–0.06	N
817-y	Jk^b	6.0	K
995-y	Fy^b	3.4	Jk^b
688-e	Fy^b	2.09	S
687-y	D-C	1.8–0.80	—
1721-e	Fy^b-Jk^a	1.6–0.08	—
1871-e	Fy^b	1.3	s
432-y	E	0.39	c-M
608-y	C	0.39	D
499-y	M	0.24	Jk^a
1473-y	Fy^a-c	0.24–0.08	—
1496-e	c	0.09	—
2415-y	E	0.07	—
260-e	S-Jk^b	0.06–0.04	M
1560-y	Jk^a	0.06	—
3069-y	B	0.06	E-Jk^a
68-e	E-D	0.05–0.03	S-Fy^a-Jk^a
589-y	A	0.04	C
526-y	C-A	0.03–0.02	—
972-e	c	0.03	—
1647-e	Jk^b	0.03	N-s
2093-y	M	0.03	c-S
71-y	Fy^b	0.02	N
653-y	C	0.02	D-E
714-y	c	0.02	—
761-y	c	0.02	Fy^a
893-e	c	0.02	Fy^a-M-S
1944-y	Jk^b	0.02	—

*Abbreviations as in Table I.

TABLE IV. Results of 12 Individuals With Triplet Chimerism*

Triplet no.	Chimerism: Marker antigens	Chimerism: %	Marker antigens with no detectable chimerism
50-y	Jk^b	17.8	M
18-e	Jk^b	9.1	A-S
18-m	Fy^a	2.4	A
62-m	Jk^a	2.5	A
62-y	Jk^a-D-C	1.0–0.37–0.35	S
17-y	C	0.92	S-Fy^a
33-y	Fy^a	0.82	Jk^b
64-e	E	0.18	M
27-m	Fy^a	0.09	M
6-y	Jk^a	0.04	c
2-m	Jk^b	0.02	A
15-m	C	0.02	A-Fy^a-e

*Abbreviations as in Table I.

found 32/415 (8%) twin individuals and 12/57 (21%) triplet individuals to have blood group chimerism of >0.01%. Since 0.01% is the under limit of positivity of the test method, one might consider results of 0.02–0.1% as borderline cases. As can be seen from Tables III and IV, 15/415 (4%) twin individuals and 8/57 (14%) triplet individuals were very evident chimeric cases of ≥0.1%. This is a very exceptional finding and seems to put the concept that blood group chimerism in humans is a rarity, in a completely new context. It seems that with the usual techniques in blood group serology many cases of blood group chimerism in multiple births are not notified.

Perhaps the incidence of minor chimerism as we found it reflects in a correct way the true phenomenon of blood stem cell exchange from one fetus to the other. In cattle a twin pregnancy is almost always a result of the fertilization of an ovum from each ovary; development begins separately in each horn of the uterus [Lillie, 1916]. The rapidly elongating ova meet and fuse in the small body of the uterus at some time between the 10 mm and the 20 mm stage. The blood vessels from each side then anastomose in the connecting part of the chorion; a particularly wide arterial anastomosis develops, so that either fetus can be injected from the other. The arterial circulation of each also overlaps the venous territory of the other, so that a constant interchange of blood takes place. Blood vessel anastomoses occur, also in humans, sometimes between dizygotic twins in utero. In monochorionic placentas anastomoses between the two fetal circulations in some areas of the placenta seem to be found often [Nylander and Osunkoya, 1970].

The higher frequency in triplets than in twins is also remarkable. Possibly in the triplet group a common factor predisposes to placental blood vessel anastomosis. For instance, it is known that in twins, and even more in triplets, there is a close relation between the trophoblasts, especially in the first trimester of pregnancy. This could explain an easier exchange of blood stem cells between the triplet individuals. Since it is known that 40% of dichorionic twins have fused placentas, it would have been interesting to know whether the

TABLE V. First and Repeat Testing After 18 Months in 17 Twin Individuals (Upper Part of Table) and 9 Triplet Individuals (Lower Part); No. 656-y Was Tested Because on the First Occasion a Marginal Positive Result (0.01%) Was Seen*

Multiple no.	Markers	First determination	Second determination	Markers first negative, later positive
1194-y	Fy^b-s	8.3–0.06	0.11–0	—
817-y	Jk^b	6.0	1.2	—
995-y	Fy^b	3.4	4.2	—
687-y	D-C	1.8–0.80	0.02–0	—
432-y	E	0.39	0.39	M-c: 0.03–0.02
589-y	A	0.04	0.02	—
893-e	c	0.02	0.03	S: 0.02
688-e	Fy^b	2.09	0	—
499-y	M	0.24	0	—
260-e	S-Jk^b	0.06–0.04	0–0	—
68-e	E-D	0.05–0.03	0–0	—
71-y	Fy^b	0.02	0	—
499-e	B	0	0.2	—
589-e	K-E	0–0	0.1–0	—
260-y	Fy^a-s	0–0	0.04–0	—
656-y	c-Fy^b	0–0	0.02–0	—
995-e	M-Fy^a	0–0	0.02–0.02	—
50-y	Jk^b	17.8	0.15	—
18-m	Fy^a	2.4	0.06	—
6-y	Jk^a	0.04	0.02	c: 0.04
2-m	Jk^b	0.02	0.02	—
18-e	Jk^b	9.1	0	—
62-m	Jk^a	2.5	0	—
62-y	Jk^a-D-C	1.0–0.37–0.35	0–0–0	—
17-y	C	0.92	0	Fy^a: 0.08
6-m	Fy^b-E	0–0	8.3–0.33	—

*Abbreviations as in Table I.

chimerism was restricted to, or more frequent in, these pairs. Unfortunately, this kind of information is scarcely available and in many cases unreliable unless pathologic-anatomical investigation is performed. Another possibility is the transplacental transfer of stem cells from the mother to the child. In obstetrics this phenomenon usually is the other way around (fetomaternal transfusion of red cells, responsible in some cases for blood group immunization of the pregnant woman), but maternofetal transfusion of red cells is also reported [Cohen and Zuelzer, 1964]. Since we did not have blood samples of the mothers, we could not test this hypothesis. A control series to throw further light on the question of maternal versus co-twin transfusion is to perform the assay on a series of monozygotic twins who are the potential recipients of marker blood groups from their mother. This kind of study is currently underway. On the other hand, this explanation could not account for the higher incidence in triplets, which makes the hypothesis less probable. A second reason for this theory being less probable is the fact that blood group chimeras almost exclusively have been found in twins, not in single individuals.

In addition to the finding of higher frequency, we also found another, difficult to explain, phenomenon. When an individual had more than one blood group marker antigen, usually only one antigen reacted in the fluorescence microsphere method (Tables III and IV). In 9 out of 38 individuals with more than one marker antigen available, another marker antigen reacted along. Three of the 9 cases showed similar results when different marker antigens were compared (260-e; 68-e; 526-e). The other 6 cases showed quite different results, one marker giving much higher results than the other. No particular patterns of interference can be distinguished: the phenomenon affects all blood group antigens equally. These findings suggest differential expression of blood group antigens in one individual, or possibly the coexistence of several populations of bone marrow stem cells. Another possibility is a partial suppression of blood group antigen expression. Technically there seems to be no possibility to distinguish these hypotheses.

The 20 cases that could be retested on a second occasion 18 months later showed fluctuating results (Table V). Twelve individuals still had chimerism, though usually of low degree; 8 had no chimerism anymore; 6 new cases were detected whose twin individual had lost chimerism. The phenomenon was noticed in the 4-year-old twins and triplets only, not in the 16-year-old group. One could speculate that it is a developmental phenomenon, though the frequency of 20 cases out of totally 624 (3%) does not allow conclusions concerning the small group of 48 16-year-old cases. And even if it would seem a temporary chimerism of childhood, it is in contrast with the reported cases in the literature concerning adults predominantly [Tippett, 1983]. Changes with time in proportions of the two populations of cells in blood group chimeric twins have been described before [Tippett, 1983]. In our opinion, this phenomenon once again points to the direction of several populations of bone marrow stem cells.

All twin chimeras described in the literature show tolerance for their twin's genetic line; i.e., they do not recognise the twin "graft" as foreign. A blood group O propositus for example will lack the naturally occurring isohemagglutinin anti-A if he is chimeric for his A-blood group twin. However, our results show quite the opposite. The 13 cases with major ABO blood group incompatibility (i.e., blood group O with offending A or B cells) all had normal titres of isohemagglutinins anti-A or anti-B (data not shown). In 8/13 the chimerism was not demonstrated within the ABO-antigens itself, but in accompanying second available marker antigens. In the remaining 5 cases the percentages of offending ABO major incompatible cells were low (0.01–0.06%). Perhaps very low percentages are not able to induce immune tolerance. On the other hand, it is strange that these incompatible red cells are not destroyed by the anti-A or -B. This phenomenon needs more clarification. Possibly a crossmatch between the serum of the chimeric individual and the erythrocytes of the sib could give an answer whether there is some kind of tolerance: in that case one would expect a negative crossmatch. Unfortunately this experiment was not performed.

ACKNOWLEDGMENTS

We are very grateful for the willingness of all twin and triplet children and their parents to participate in this study. We also thank Dr. O. Bleker and Dr. A. Schattenberg for their critical comment on the manuscript. Last but not least the four technicians who performed the tests should be mentioned: Yvonne van Berkel, Ans Linders, Ben Steetzel, and Claudia Mouton.

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