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STUDIES ON THE RELATIONSHIP BETWEEN ERYTHROCYTES AND LEUCOCYTES UNDER THE CHIMERISM OF HETEROSEXUAL TWINS IN CATTLE

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The chimera phenomena in a few kinds of cells as the result of vascular anastomosis between heterosexual twins in the fetal period have been reported on by the present authors (7, 12, 15) and several other investigators (2, 3, 6, 16, 19). However, it was Owen, the discoverer of erythrocyte chimera, who provided the proof for this phenomenon with an immunogenetic method. This subsequently prompted the brilliant technique for the early diagnosis of freemartin. In the field of cytogenetics, the progress in the technique of tissue culture and the development of the study of the chromosome led to the report on the chimera of sex-chromosome by Ohno et al. Subsequently the present authors (7) and Hershler et al. suggested the possibility of freemartin diagnosis.

From such a viewpoint it seems to be of considerable significance in the developmental factor and diagnosis of freemartin to clarify the relationship between blood groups and leucocytes under the phenomenon of chimera. Comparative analysis was therefore made in the antigen analysis of erythrocytes and sex-chromosomes of cultured leucocytes.

Materials and Methods

The 22 cases of heterosexual twins of Holstein breed used in the present study consisted of 12 freemartins, 8 male co-twins with freemartin and 2 non-freemartin normal cows with calving. In the 8 co-twin pairs, both twins were examined.

The blood type analysis was based on the standard method of Neimann-Sørensen which was partly modified from that of Kosaka, as shown shematically in

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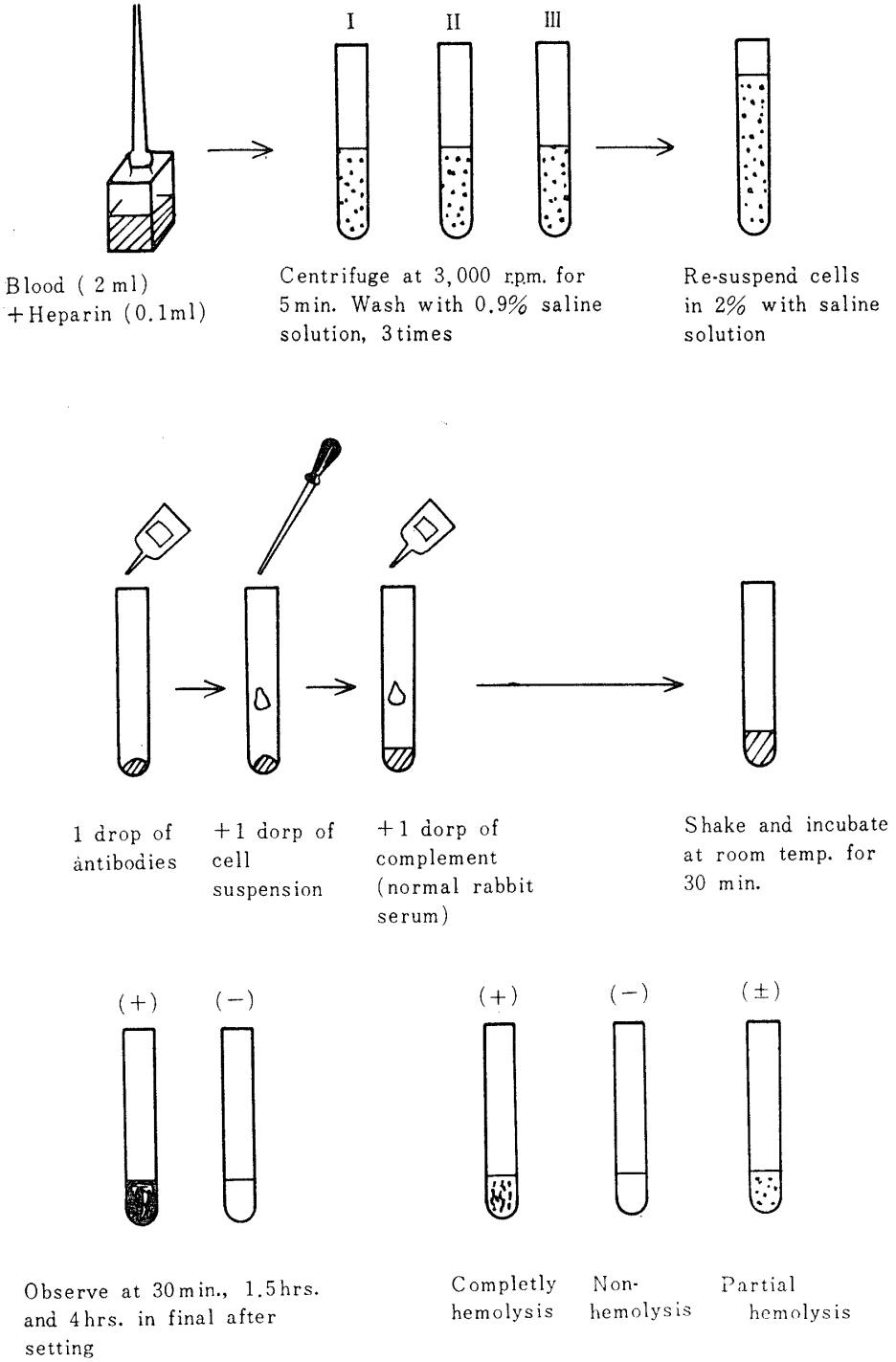

Fig. 1. Blood typing technique.

Figure 1. The antisera used were made for the most part by iso-immunization, and only anti J, V and A from isonormal hemolysin and anti F from normal goat serum. The 45 bovine Blood-typing reagent and their sources are shown in Table 1.

For leucoytes culture, approximately 10 ml of blood was obtained from the

Table 1. Blood-Typing reagents used as their sources

Reagent by systems and their sources*		Reagent by systems and their sources*	
A system		F-V system	
A_1	1	F_1	1,3,4
A_2	1,2	F_2**	1,5
Z′	1,2	V	1,2
B system		J system	
B	1	J_0	2
G**	1,3	J_1	2,3
I_2	1	L system	
K**	1	L	1
O_1	1	S-U system	
O_3**	1	S_1	1
Q	1	S_2	1
T	1	U_1	1
Y_1	1	U_2	1
Y_2**	1,3	U′	1
D′	1,2	H′	3
E_1′	1	Z system	
E_2′**	1	Z**	1,3
E_3′	1,3	R′-S′ system	
F′	1	R′**	1
G′	1		
I′	1		
J′	1		
K′	1		
O_1′	1		
O_2′	1		
hi	1		
C system			
C_1	1		
C_2	1,2		
W_1	1		
W_2	1		
R	1		
X_1	1		
X_2	1		
L′	1		

* 1 (Cattle isoimmune); 2 (Normal cattle serum); 3 (Rabbit anticattle); 4 (Normal goat serum); 5 (Sheep anticattle) ** Homo-Reagent

jugular vein by means of a syringe and inoculated into a heparinized tube. Cultures were set up by a mixture of the heparinized blood and the culture medium in proportion of 1:3 with the additon of phytohemagglutin-M, and cultivated in T D-15 flasks. After incubation of 3 to 4 days, mitotic cells were arrested by colchicine treatment ($50 \sim 10^{-8}$M) for about 1.5 hours.

Following the water pretreatment, chromosomal slides were made according to the routine air drying method and stained by the Giemsa method. The procedure was shown also shematically in Figure 2.

The culture medium contains 14 different kinds of amino acid and 8 kinds of

Table 2-1 Blood type analysis

No,	AGE	SEX	BLOOD TYPE				
			A	B	C	FV	J
	Days						
1	1	♂	A_1/	$C_3J'K'O'_2$/−	CW_1X_2	F/F	−/−
2	1	♀	A_1/	O_1/−		F/F	−/−
3	3	♂	−/−	$GY_2E'_2D'QO'_2$	C/W_1	F/V	−/−
4	3	♀	−/−		C/−	F/V	−/−
5	3	♂	−/−	O_1/−	C/W_1R	F/V	−/−
6	3	♀	−/−	$O_3J'K'$/−	W_1R/−	F/V	−/−
7	6	♂	A_1/−	$BGKI'O'_2/I_2O'_2$	W_1X_1	F/V	−/−
8	6	♀	A_1/−	$GY_2E'_2/GY_2$		F/V	−/−
9	7	♂	−/−	$GY_2E'_2I_2O'_2$	CW_1R	F/F	−/−
10	7	♀	−/−			F/F	−/−
11	12	♂	A_1/−	$GY_2E'_2$/−	C/−	F/F	−/−
12	12	♀	−/−	O'_2−	−/−	F/F	−/−
13 *		♂	A_1/−	$D'Y_2O_2/O_3$	CR/−	F/F	−/−
14	25	♀	A_1/−	$BGKY_2I'O'_2/D'$	C/W_1	F/F	−/−
15 *		♂	A_1/−	$BGY_2O_3D'J'QO'_2Y'$	−/−	F/F	−/−
16	33	♀			C/C	F/F	−/−

Notes *: Not examined, estimated from systematic analysis.

Table 2-2 Blood type analysis

No.	AGE	SEX	BLOOD TYPE				
			A	B	C	FV	J
	Days						
17 *		♂	−/−	$O'_2E'_2/D'$	W_1−	F/V	−/−
18	90	♀	−/−	O_1E_2/D'	C/−	F/F	−/−
19 *		♂	−/−	I_2Q/	W_1/−	F/V	−/−
20	111	♀	−/−	$D'O'_2$/−	C/W_1	F/F	⊥/−
21	120	♂	A_2/−	$GY_2E'_2/O_3J'K'$	CW_1	F/F	−/−
22	120	♀	A_1/−	$O_3J'K'/O_3J'K'$		F/V	−/−
23	Unknown	♂	A_1/	$O_3J'K'$/−	C/−	F/V	J^{cs}/−
24	Unknown	♀	A_1	$D'E'_2$/−	−/−	F/V	−/−
25	9 yrs	♀	A_1/	$BGY_2G'O'_2$	OW_1X_2	F/F	−/−
26	12	♀	A_1/	GO_3	C/	F/F	⊥/−

Notes *: Not examined, estimated from systematic analysis.

and chromosomal findings.

SYSTEM				CULTURED LEUCOCYTES			
L	SU	Z	R′S′	No. of cells	2A-XX	2A-XY	DIAGNOSIS
−/−	H′/	−/−	S′/S′	32	16	16	
+	+	+	+				Freemartin
−/−	H′/	−/−	S′/S′	37	22	15	
−/−		−/−	S′/S′	29	8	21	
+	S_1H'	+	+				Freemartin
−/−		−/−	S′/S′	60	17	43	
−/−	H′/′	−/−	S′/S′	50	21	29	
+	+	+	+				Freemartin
−/−	−/−	−/−	S′/S′	23	9	14	
L/−	U_2U'/H′	−/−	S′/S′	31	19	12	
+	+	+	+				Freemartin
−/−	H′/−	−/−	S′/S′	26	16	10	
L/	H′/	−/−	S′/S′	29	6	23	
+	+	+	+				Freemartin
L/	H′/	−/−	S′/S′	28	6	22	
−/−	S_1H'/−	−/−	S′/S′	49	25	24	
+	+	+	+				Freemartin
−/−	S_1H'/H′	−/−	S′/S′	39	20	19	
L/−	$U_2U'H'$/−	−/−	S′/S′				
+	+	+	+	239	7	232	Freemartin
−/−	S_1H'/−	−/−	S′/S′				
L/−	H′/−	−/−	S′/S′				
+	+	+	+	28	6	22	Freemartin
−/−	S_1H'/−	−/−	S′/S′				

): Both of the twins were examined as chimerism.

and chromosomal findings.

SYSTEM				CULTURED LEUCOCYTES			
L	SU	Z	R′S′	No. of cells	2A-XX	2A-XY	DIAGNOSIS.
−/−		−/−	S′/S′				
+		+	+				
−/−	S_1H'	Z/−	S′/S′	17	6	11	Freemartin
−/−			S′/S′				
+			+				
−/−	$S_1H'U'$	Z/	S′/S′	49	23	26	Freemartin
−/−	S_1/−	−/−	S′/S′	53	14	39	
+	+	+	+				
−/−	S_1/−	−/−	S′/S′	42	13	29	Freemartin
−/−	S_1H'/H′	Z/−	R′/S′	46	33	13	
+	+	+	+				
−/−	−/−	−/−	S′/S′	11	8	3	Freemartin
−/−	−/−	−/−	S′/S′	50	50	0	Non-freemartin
L/−	$S_1H'U_2$	−/−	R′/S′	50	50	0	Non-freemartin

): Both of the twins were examined as chimerism.

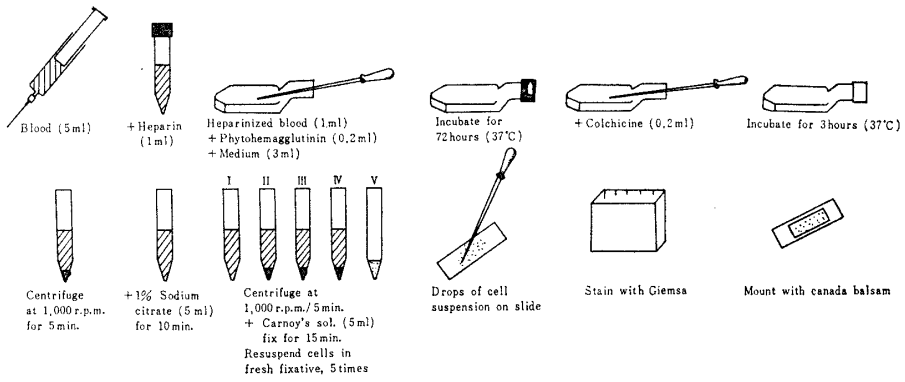

Fig. 2. Blood culture technique.

vitamin dissolved in EARL's balanced salt solution. The chemical components of the medium have been reported by Kanagawa et al. (7) previously.

Results and Discussion

Blood type analysis and chromosomal findings are shown in Tables 2–1 and 2–2. The results of the analysis of the blood groups in 13 freemartin and 8 male co-twins with freemartin show without exception the states of erythrocyte chimerism. On the other hand, the reaction of the 2 normal cows from the non-freemartin heterosexual twins revealed no chimerism.

In the chromosomal analysis, all the freemartins and the male co-twin showed 2A-XX/2A-XY chimerism, and findings of normal cows (Nos. 25 and 26) revealed a normal female complement with an XX-mechanism in cultured leucocytes. The chromosomal results is in good agreement with the blood type analysis (Fig. 5). Morphologically and histologically the rudimentary gonads of many freemartins showed an apparent likeness to those of the immature testis of a normal bull, associated with seminal vesicle-like structures and degenerated uterus horns like peanut (Fig. 6).

Since Lillie reported that in about 87 per cent of heterosexual bovine twins, the female was generally sterile, and only the remaining female twin was fertile, many investigators and practioners (1, 5, 15, 20) have paid attention to the clinical signs from the viewpoint of diagnosis of freemartinism. In such cases a decisive diagnosis cannot be made until 6 months or more, when a rectal examination of the internal sex organs can be allowed or the first esterus can be checked. This may cause the owner considerable economic loss.

Recently, Owen, Ohno et al. and several researchers in Japan reported erythrocytes chimerism and sex chromsome chimerism in heterosexual twins respectively, and also that the means of blood type analysis and leucocyte culture technique based on sex chromosome chimerism are very important to the diagnosis for freemartinism. Comparative analysis in the present reports was made in the antigen of erythrocytes and sex chromosome of cultured leucocytes, because

previous reports were limited to only erythrocytes or leucocyte.

As shown in Table 2, both freemartins and their co-delivered males showed a similar finding in the mixing of chimera in leucocytes and also in erythrocytes. From these results, the cause of the development of chimera in both cells, including the stages of nidation and transition of the basic cells, therefore, appears due to the same factors. And the degree of histological deviation of gonad in heterosexual twins does not seen to depend upon the degree of chimerism. In order to obtain a decisive conclusion on this subject, however, much more work will be required.

Summary

Blood typing and sex-chromosomal analysis were carried out on 23 cases of heterosexual twins consisting of 13 freemartins, 8 male co-twins with freemartin and 2 normal cows from non-freemartin heterosexual twins.

Both erythrocyte and leucocyte chimerism were detected in all cases of the freemartins and its male co-twins. On the other hand, in 2 normal cows, no chimerism of erythrocytes or leucocytes were observed. The chimera phenomena in blood type and cultured leucocytes appear to be due to the same cause.

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Explanation of Plates

Plate 1.

Fig. 3. Special Blood-Typing Board used in the hemolytic reaction (containing 40×80. of test tube)

Fig. 4. Small grass test tubes for the blood-typing and ordinary tubes (:OT) size: 2.5×0.7 cm

Plate 2.

Fig. 5. Sex-chromosome chimerism in cultured leucocytes from the freemartin (No. 2)
Arrows show sex-chromosome (large: X, small: Y)

Fig. 6. Dorsal view of the sexual organ of the above freemartin
BG: Bilateral gonads
UT: Uterus
SV: Seminal vesicle-like structures
VA: Vagina
CL: Clitoris

Plate 1.

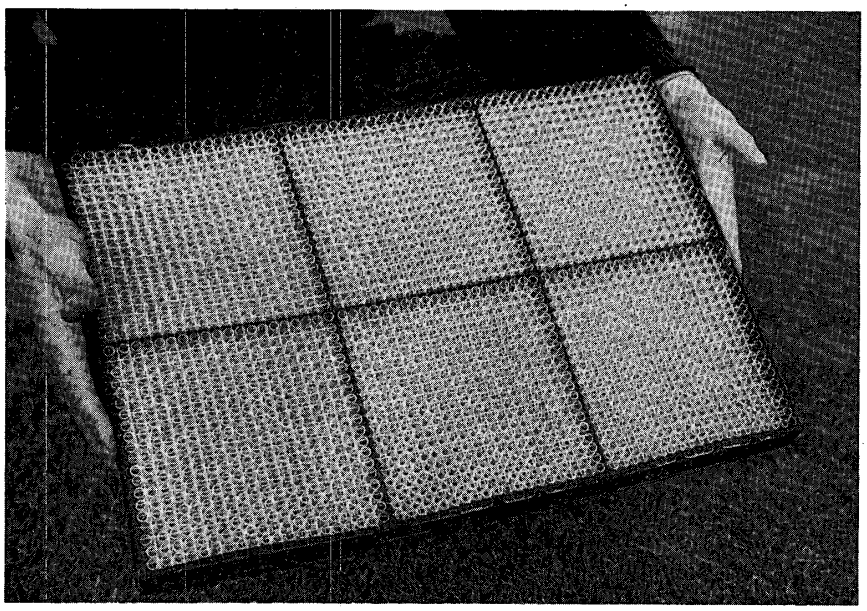

Fig. 3.

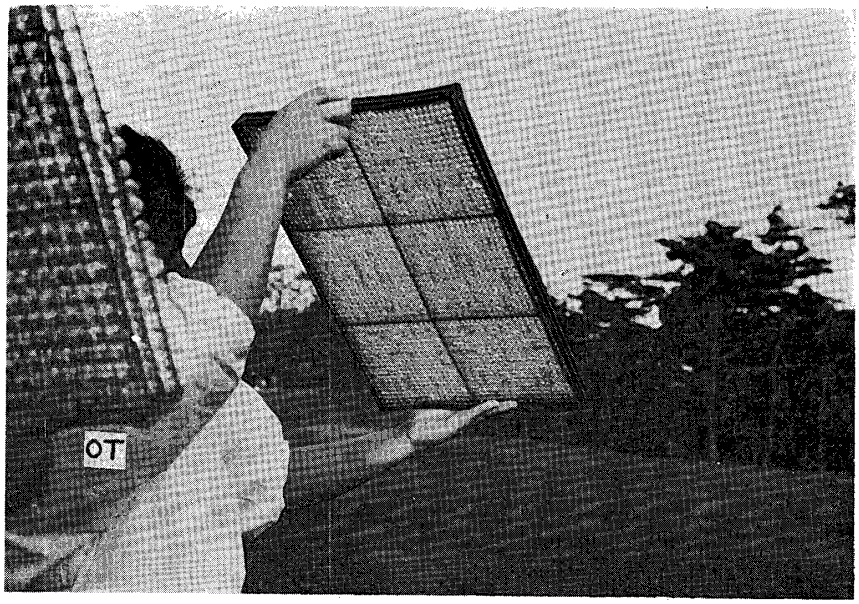

Fig. 4.

Plate 2.

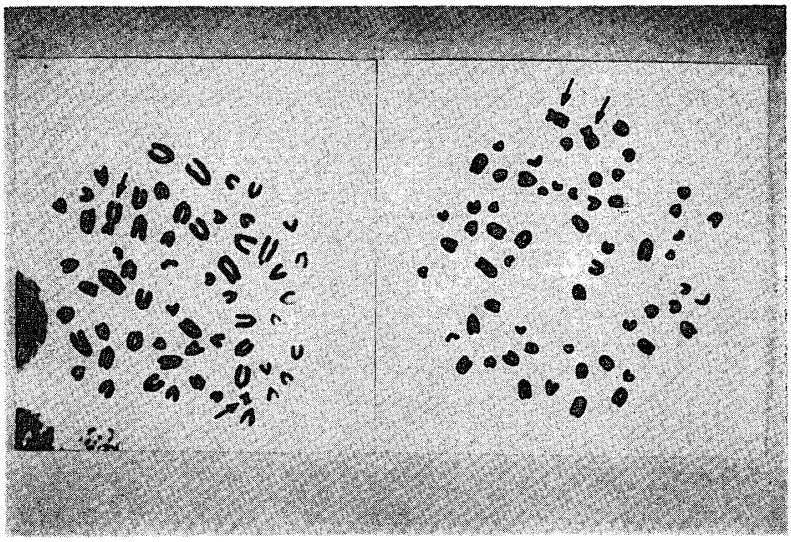

Fig. 5.

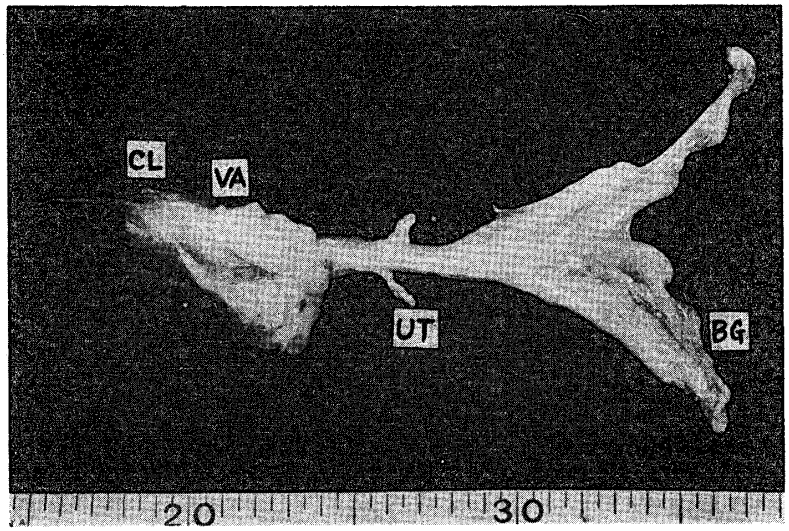

Fig. 6.