A POSSIBLE SECOND CASE OF SOMATIC CELL MATING IN TWIN CATTLE WITH ERYTHROCYTE CHIMERISM¹

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THE majority of dizygotic cattle twins show erythrocyte chimerism (OWEN 1945; STONE, STORMONT and IRWIN 1952). Each member of a pair possesses its own antigenically distinct cells as well as the antigenic type of its twin. Ordinarily, only two cell types are present in chimeric twins. However, we found one chimeric twin at three years of age whose blood contained two antigenic types: 10% representing his own genotype and 90% his co-twin's (STONE, FRIEDMAN and FREGIN 1964). When the blood of this chimera was retested at eight years of age and thereafter, three blood types were found: the two original types, each representing 2%, and a third type representing 96% of the cell population. It was postulated that the third type was a hybrid cell type resulting from mating between the two hematopoietic tissues in the chimeric mixture. The possibility that a second example of this phenomenon (Somatic Cell Mating) may have been observed is described in this paper.

MATERIALS AND METHODS

Providing that each of the two cell types in a chimeric mixture has at least one unique antigenic factor, the proportions of the two red cell populations in a twin can be measured accurately by a differential hemolysis test first described by OWEN (1945) and OWEN, DAVIS and MORGAN (1946). The principle of the test is simple—one of the two cell types (call it Type I) is hemolyzed (lysed) with appropriate blood typing reagent and the cells remaining (not lysed) are counted. This count, subtracted from the count on the untreated controls gives the percent cells of Type I present in the mixture. The percent of cell Type II is determined in the same way using another reagent specific for that type. Theoretically, the sum of the two counts equals 100 percent.

All of the differential hemolysis data reported here were obtained using a Coulter Counter (NIECE 1964, 1968). However, a Spectrophotometer (MANGE and STONE 1959) was often run in parallel and gave comparable results. Both methods assay the percent cells remaining after lysis of one or the other cell type by an appropriately chosen blood typing reagent. A direct count of cells remaining after lysis is made by the Coulter Counter, whereas the spectrophotometer measures the cells remaining after lysis by recording transmittance of hemoglobin. Both techniques provide accurate and reproducible results.

Briefly, the technic used was as follows: A model B Coulter Counter with Automatic Size Distribution Plotter and 100 μ aperture tube was used. The settings were chosen to allow intact

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cells to be distinguished from stroma after lysis (NIECE 1968). Blood samples were prepared for testing by washing in saline and standardizing so that the suspension contained between 3×10^7 and 7×10^7 cells/ml. The ratio of cell suspension: reagent: undiluted complement in the experimental tube was 1:2:1. The control tube contained saline in place of reagent. The total volume was 0.4 ml. The test was incubated $4\frac{1}{2}$ –5 hours at about 30°C with frequent shaking. After incubation, the samples were diluted for counting with saline which had been filtered through a Sela porcelain filter candle (porosity 01). Dilutions were made by transferring a 50 μl aliquot of sample to 25.0 ml of saline. The diluted samples were thoroughly mixed, plotted, and counted twice. Each count was corrected for coincidence using the values provided with the counter. The percent cells lysed was obtained by taking the average number of cells in the experimental tube divided by the average number of cells in its control tube subtracted from 100.

RESULTS AND DISCUSSION

At the time of purchase in September 1963, female Holstein-Friesian twin calves 143 and 144 were approximately one year old. They were then blood typed along with their dam and sire. The twins showed antigenic chimerism at the B locus only. The alleles (phenogroups) at this locus for these four animals are as shown in Table 1. Clearly the factors G and D' should be on opposite cell types in these twins since they are members of alternative alleles in the sire. Additional progeny from the sire confirmed that the factors G and D' were segregating as alleles.

Table 2 shows the results of periodic differential hemolysis tests on the blood of both twins. During the first two years after purchase, the blood of twin 143 contained about 75% type D' cells and 25% of type G, whereas in twin 144, D' cells represented about 35% of the mixture and G cells about 65%. Total lysis in each twin averaged about 100% (actually 103.3% in ten determinations). Generally, the sum of the two percentages determined by hemolyzing reciprocal types in a mixture equals 100 with a standard deviation of 2% for the spectrophotometer method and 4% for the Coulter Counter method (NIECE 1968).

During the following 37 week period (November 1, 1965 to July 18, 1966) 16 independent differential hemolysis tests on the cells of twin 144 with respective reagents for G and D' gave a total lysis averaging 114.3%, representing about 14% overlysis. We are confident that the overlysis noted with twin 144

TABLE 1

B alleles of chimeric twins 143–144 and their parents

	Apparent blood type*	Genetic blood type†
Sire 14559	$\text{GY}_2\text{E}'_1/\text{D}'\text{E}'_3\text{G}'\text{O}'$	$\text{GY}_2\text{E}'_1/\text{D}'\text{E}'_3\text{G}'\text{O}'$
Dam 14079	BI'/E'_3	BI'/E'_3
Twin 143	$\begin{smallmatrix} \pm \pm \pm \pm \\ \text{GY}_2\text{E}'_1/\text{D}'\text{E}'_3\text{G}'\text{O}'/\text{BI}' \end{smallmatrix}$	$\text{D}'\text{E}'_3\text{G}'\text{O}'/\text{BI}'$
Twin 144	$\begin{smallmatrix} \pm \pm \pm \pm \\ \text{GY}_2\text{E}'_1/\text{D}'\text{E}'_3\text{G}'\text{O}'/\text{BI}' \end{smallmatrix}$	$\text{GY}_2\text{E}'_1/\text{BI}'$

* $\pm$ indicates incomplete reaction typical of erythrocyte chimerism.

† Three normal calves were obtained from each twin, and additional offspring from sire were tested to determine the correct alleles.

TABLE 2

Results of differential hemolysis tests on chimeric twins 143 and 144

Dates	Percent cells lysed			
	Twin 143 Reagent		Twin 144 Reagent	
	G	D'	G	D'
1-18-64	32.8	75.0	69.8	45.5
2-15-65	31.5	75.0	70.0	43.5
3-15-65	31.7	78.2	59.2	28.8
4-12-65	27.0	79.6	65.0	42.3
5-10-65	27.8	74.2	60.6	35.8
7-12-65	23.8	73.4	67.9	31.0
8- 9-65	27.5	75.2	70.4	35.0
8-23-65	15.0	76.4	67.6	35.0
9- 6-65	18.0	80.0	67.6	37.4
10- 4-65	22.0	83.0	69.9	36.3
11- 1-65	15.0	83.3	66.9	41.8
12-15-65	20.7	81.2	68.7	44.6
12-15-65	21.3	82.8	76.8	39.6
12-17-65	19.3	80.8	67.9	40.8
1-10-66	17.5	82.9	71.0	37.7
1-12-66	12.8	83.4	71.5	43.6
1-21-66	19.0	72.4	69.0	28.8
1-27-66	20.1	82.1	69.6	38.5
2- 7-66	15.4	78.4	66.0	39.6
2-21-66	15.8	84.5	69.4	44.5
3- 7-66	17.1	82.4	69.9	48.9
3-10-66	15.0	85.2	68.1	49.7
5-16-66	13.2	84.3	70.0	57.5
5-20-66	17.6	85.7	69.6	66.3
6-20-66	15.2	86.4	72.2	41.3
7-18-66	13.7	82.5	69.2	46.0
8- 8-66	17.0	85.5	64.3	34.0
9- 5-66	12.5	86.2	63.2	36.4
9-26-66	14.1	86.4	69.1	26.8
10-24-66	14.0	88.0	68.8	38.9
11-14-66	10.4	85.4	69.0	35.5
12- 5-66	13.1	87.7	77.2	25.4
1- 9-67	17.3	84.8	71.4	29.8
1-30-67	15.6	87.9	68.5	34.6
3- 6-67	16.5	88.0	74.8	40.4
5- 1-67	15.1	89.5	68.2	41.7
Statistic	Twin 143		Twin 144	
$\bar{G}$	18.7		68.8	
$\bar{D'}$	82.2		39.5	
Var. (G)	34.10		12.05	
Var. (D')	21.88		66.60	
Cov. (G,D')	-19.89		+2.53	
<i>n</i>	36		36	

was significant because the counts of the cells not lysed by each reagent were done spectrophotometrically (MANGE and STONE 1959) and on the Coulter Counter (NIECE 1964) with excellent agreement between these independent tests. About 70% lysis was obtained with the G reagent and about 44% lysis was obtained with the D' reagent. Treatment with Y₂ and E'₁ reagents detecting factors of the phenogroup GY₂E'₁ gave results which agreed closely with those obtained with the G reagent. Unfortunately, it was not possible to measure the percent lysis for factors G' and O' of the phenogroup D'E'₃G'O', and to compare it to D' because strong and specific reagents for G' and O' were not available.

The possibility that these results were due to non-specific reactions of the reagents seems to be eliminated because the average total lysis of the sixteen determinations done on co-twin 143 during the same 37 week period was only 101.3%. Also, in ten tests performed between July 18, 1966 and May 1, 1967, twins 143 and 144 averaged 100.4% and 100.8% lysis, respectively. The same technic and reagents were used throughout the experiments.

Is it reasonable to ascribe the overlysis noted with twin 144 to chance? Our null hypothesis is that the percentage lysis brought about by the two reagents, G and D', sums to 100. If G and D' are used to represent the values obtained in each individual test, then

$$\text{Var. (G+D')} = \text{Var. (G)} + \text{Var. (D')} + 2 \text{Cov. (G,D')}$$

and

$$\text{Var. } (\overline{\text{G+D'}}) = 1/n \text{Var. (G+D')}.$$

The significance of the departure of $(\overline{\text{G+D'}})$ from 100 during the entire period of the experiment may be found using tables for the Standard Normal Deviate (S.N.D.).

$$\text{S.N.D.} = \frac{d}{\sigma}$$

where d equals the departure of (G+D') from 100 and σ is estimated by the standard error of $(\overline{\text{G+D'}})$, i.e. the square root of var. $(\overline{\text{G+D'}})$. From the data in Table 2— $\text{S.N.D.} = \frac{8.3}{1.6} = 5.2$, for which $P \ll 0.0001$, which is a highly significant departure from expectation.

In actual practice the lysis figures (see Table 2) for the contrasting reagents do not quite sum to 100 percent, because a few cells are left unreacted. Of 1494 determinations carried out on 34 animals (17 pairs of chimeric twins, not including 143 and 144) the average sum was 98.8%. Thus, the significance test used above is in fact a somewhat conservative test. There can be little doubt that a significant degree of overlysis has occurred with twin 144.

Further statistical analysis of the data strengthens the evidence for more than two types of cells in the blood of twin 144. If there are two and only two populations of cells in the blood being tested, one marked by the blood factor G, the other by D', we expect that any change in the percent of the G population will be matched by an equal but opposite change in the percent of the D' population. Leaving aside the effect of different reagents we predict accordingly (1) that Var. (G) equals Var. (D') , and (2) if there are real changes in the proportions

of the two types of cells, the percent of lysis by G will be negatively correlated with the proportion of lysis by D'.

For twin 143, the co-twin of 144, we find that Var. (G) is not significantly different from Var.(D'). Thus, $F = 1.56$ (where F is the variance ratio) and $P > 0.05$. We note also that there is a negative correlation coefficient between the percent lysis by the two reagents (Correlation Coefficient = 0.530, $P \sim 0.001$). That is, the results for 143 are in agreement with two and only two populations of cells. Moreover, they suggest that differences in reagent (between G and D') do not significantly affect the variances of the estimates of the percent lysis. For 144, the results are quite the contrary. Var. (D') is highly significantly greater than Var.(G), $F = 5.53$, $P \ll 0.001$. There is no significant correlation between the percentage of lysis by the two reagents (Correlation Coefficient = 0.008, $P > 0.05$). Such results suggest that the assumption of two and only two cell populations is not valid for twin 144.

One explanation for the significant overlysis in the differential hemolysis tests on the blood of twin 144 is the presence of a third (hybrid) cell type which possesses *both* antigens. Such a cell type might be present if there had been a third animal *in utero* which did not survive the early period of gestation—in other words, if there had been chimeric triplets *in utero*. However, since the factors G and D' are members of alternative alleles in the sire, no offspring of this mating could normally possess both. The possibility of superfecundation is even more remote because sire 14559 was the only bull kept on the farm where the twins were born. However, it is not possible to completely rule out double fertilization of the type reported by GARTLER, WAXMAN and GIBLETT (1962).

The most likely explanation of these results is that a hybrid cell type resulted from somatic cell mating of the two different hematopoietic stem cells in twin 144. If so, this would represent the second case of this phenomenon in chimeric cattle twins (STONE *et al.* 1964). Unfortunately, unlike the first case, it was not possible to isolate the hybrid cells, because there were no reagents that would hemolyze only the original types, leaving the hybrid cells for counting and typing. Therefore, it was impossible to determine the relative proportions of the various cell types in the mixture.

During the period of overlysis with G and D' reagents, the reactions of twin 144 with B and I' reagents remained complete, indicating that if there had been hybrid cells with *both* G and D' factors, they also possessed the BI' phenogroup (from the dam). This suggests that the hybrid cells may have been aneuploid (trisomic) or tetraploid. This contrasts with the previous case in which the hybrid cell was presumably diploid in that only two alleles of a given blood group system occurred. Short term cultures of peripheral blood lymphocytes from both twins (143 and 144) during the period of overlysis yielded normal karyotypes ($2n = 60$). Thus, there were no obvious quantitative changes in the chromosome number of the lymphocytes. The same results were obtained in the studies on the chimeric twin previously reported (STONE *et al.* 1964). Whether or not the progenitors of the non-nucleated terminal erythrocytes were aneuploid or tetraploid is unknown.

To prove the presence of a hybrid cell containing *both* G and D' antigens, we treated the washed cells of twin 144 with G reagent. Following lysis, we recovered the stromata by centrifugation and used them to absorb the D' reagent. After absorption, the reagent did not react with D'-positive cells, indicating that the stromata contained both D' and G antigenic factors. These tests were performed on an artificial three-way mixture of G-positive, D'-positive, and G + D'-positive cells gave the same results, whereas an artificial two-way mixture of G-positive and D'-positive cells (no cells possessing *both* G and D') weakened the reagent, but left the D' antibodies intact. We repeated the absorption experiments during the period when overlysis was not occurring—when presumably the hybrid cells were not present. As expected, the stromata recovered after lysis with G reagent did not absorb the D' antibodies. We cannot explain the transient nature of the hybrid cell type. If it is aneuploid, one might expect it to be at a selective disadvantage. On the other hand, this argument need not hold for tetraploid cells. As already indicated, the hybrid cell in the first case (STONE *et al.* 1964) must have had a selective advantage since it represented over 95% of the cell population and persisted indefinitely. During the entire four-year period of these experiments, twin 144 was in good health except for an infected leg treated successfully in February, 1966 with antibiotics.

It should be noted that both twins were being used in an experiment to determine the effects of irradiation on erythrocyte chimerism (STONE and CRAGLE 1964). Among 47 chimeric twins that had received varying doses of whole body gamma irradiation (Co^{60}) only twin 144 showed evidence of hybrid cells. Whether or not the irradiation caused somatic cell mating or favored the selection of the hybrid cells is unknown. Although the mechanism is obscure, the occurrence of the virus-mediated somatic cell fusion in tissue culture (HARRIS 1966; ROIZMAN 1962; YOSHIDA and EPHRUSSI 1967), the possibility of somatic mating and segregation in radiation chimeras (LENGEROVÁ 1967), and the somatic segregation observed within an ordinary monozygotic individual (OHNO 1966) emphasize the importance of this phenomenon in somatic cell genetics (LEDERBERG 1958).

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SUMMARY

The proportions of the two erythrocyte populations in chimeric twins can be determined accurately by measuring the percent cells remaining after hemolysis of one or the other cell type by appropriately chosen reagents. Ordinarily, the total lysis of the two cell types in a twin approximates 100 percent. However, in one twin, significant overlysis was observed with reagents presumably detecting factors unique to each cell type, suggesting the presence of a hybrid cell type possessing antigenic factors in common with the other two. This hybrid cell type

may have resulted from somatic cell mating in the hematopoietic tissues of the chimeric twin.

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