A Differential Hemolysis Technique Using a Coulter Counter to Assay Erythrocyte Chimerism* (32936)

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Embryonic vascular anastomosis in dizygotic cattle twins usually leads to erythrocyte chimerism (1; see 2 for a recent review). Each chimeric twin possesses a mixture of two genetically distinct antigenic types of erythrocytes, his own type and the type transplanted from his co-twin. Erythrocyte samples from chimeric twins treated with appropriate blood typing sera (reagents) and complement do not give complete hemolysis, because the sample contains two populations of cells, one with and one without the antigenic factor for which the reagent is specific. One procedure for determining the proportions of the two cell types in a mixture is to measure the hemoglobin concentration of cells remaining after hemolysis. A colorimeter (Bausch and Lomb, Spectronic 20) has been used successfully for this differential hemolysis technic (3). Direct counting of residual cells has been employed using a hemocytometer (4) or hematocrit tubes (5). But these differential hemolysis techniques are tedious and require large amounts of costly reagents.

The best features of these techniques have been incorporated into a new procudure (6–8) utilizing a Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.). The Coulter counter has been used extensively in a variety of cell-counting procedures (for example, see 9). The technique reported in this paper represents another application in which large numbers of red cells remaining after hemolysis are individually counted in a few seconds. The technique has the advantage of requiring small quantities of reagent and less labor.

Materials and Methods. A model B Coulter counter with automatic size distribution plotter and 100 μ aperture tube was used. Operation of the Coulter counter is described by Brecher *et al.* (10). The proficiency of each operator was such that he could dilute and count 10 aliquots of a red blood cell suspension with a coefficient of variability of 5% or less. Different combinations of aperture current, amplification, gain, and scale factor settings may be used on any particular instrument and vary from one instrument to another. The settings were chosen to allow intact cells to be distinguished from stroma after hemolysis.

Blood samples were prepared as described by Mange and Stone (3) with the exception that cells were standardized by visual observation so that the control tubes contained between 3×10^7 and 7×10^7 cells/ml. This was a lighter suspension than used by Mange and Stone (3) and nearer that concentration for which the reagents were prepared. The ratio of cell suspension:reagent:undiluted complement in the experimental tube was always 1:2:1. The control tube consisted of cell suspension:saline:complement (1:2:1). Thus, saline was substituted for reagent and the control tube was effectively a complement control. After it was determined that total volume did not affect results, the volumes were reduced from 2.0 ml (3) to 0.40 ml (0.10 ml cell suspension, 0.20 ml reagent, 0.10 ml complement). With these smaller volumes, shell vials (Kimax model 60935-L, Owens-Illinois, Toledo, Ohio) were used in place of test tubes.

An incubation period of 4.5–5 hours at about 30°C was found necessary for hemolysis. Stromal material and intact cells were not sufficiently different in size when shorter incubation periods or lower temperatures were used. Shaking at intervals throughout the incubation period was important. Thirty- to 45-min intervals were convenient. After incubation, the samples were diluted for counting with saline (0.9%) which had been filtered through a Selas porcelain filter candle (poros-

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ity 01). Merthiolate (Thimerosal, Sigma Chemical Co., St. Louis, Mo.) 1/10,000 (w/v) was used as a preservative. With an automatic pipetter (Kimax model 37077 F, Owens-Illinois, Toledo, Ohio), 25.0 ml filtered saline was delivered to a 30-ml beaker. The experimental and control samples were thoroughly agitated and a 50-μl aliquot was transferred to the beaker of saline with a Drummond "Microcap" (Kensington Scientific Corporation, Berkeley, Calif.). The diluted samples were then mixed by careful swirling, plotted, and counted twice. The complement control was plotted first; the threshold for counting was determined from the plot. This threshold was taken as the upper limit of that window below the intact cells that gave a minimum number of counts (window number $\times$ 4 equals counting threshold). This procedure eliminates background counts so that only intact cells are similarly plotted and counted.

The results of Coulter counter determinations were calculated as follows: The average number of cells in the experimental tube was divided by the average number of cells in its control tube and expressed as percent. Each count was corrected for coincidence using the values provided with the counter before being used in calculating percentage. No correction was made for coincident counts of stroma. However, this error is negligible. For Spectronic 20 determinations, the proportions remaining after hemolysis were calculated by the method used by Mange and Stone (3).

Artificial mixtures of red cells of different antigenic types were prepared by mixing appropriate quantities of two thoroughly washed erythrocyte samples to give suspensions ranging form 0 to 100% by 10% intervals of each antigenic type. Also natural mixtures were obtained from chimeric twins. With both natural and artificial mixtures, a variety of blood typing reagents was used.

Results and Discussion. Preliminary trials with artificial mixtures gave a standard deviation of 4% based on 93 determinations performed on the Coulter counter. No difference was observed between conventional volumes and reduced volumes (t-paired comparison test, $p>0.3$). No effect of various reagents different from that seen in standard blood typing tests was observed. That is, reagents that gave incomplete lysis in standard tests also gave incomplete lysis in differential hemolysis tests. The corresponding Spectronic 20 standard deviation was about 2% (66 determinations). When determinations using natural mixtures were done simultaneously on the Coulter counter and Spectronic 20, no significant differences were observed ($0.10>p>0.08$).

Standard deviations varied little over the range of mixtures. For the Coulter counter technique, standard deviations ranged from 2 to 5%, and for the Spectronic 20 technique, standard deviations ranged from 1 to 4%. Ordinarily the sum of the two types in a chimera (or mixture) should equal 100%. The standard deviation for the departure of the sum from 100% was 2% for the Spectronic 20 and 4% for the Coulter counter. There was little difference among the standard deviations for the different percentage mixtures (0–100, 10–90, etc.).

Over a period of 30 months, 1149 differential hemolysis tests using the Coulter counter have been performed in this laboratory by seven different operators. In more than 82% of these routine tests the sum of the two cell types fell between 95% and 105% (approximately 1 standard deviation). Only 7% were not between 92% and 108% (2 standard deviations).

The most useful estimation of variability for long term twin studies (2) would be to compare results of tests on twins tested over periods of months or years. Table I summarizes results of tests on 2 pairs of twins that have shown no change in their red blood cell proportions. Twin pair A was tested over a period of 15 months and pair B over 20 months. The unequal number of tests for both cell types results from more reagents being available for one cell type than for the other. It is clear from these results that the Coulter counter technique provides an efficient and precise method for determining the cell proportions in chimeric cattle twins.

Summary. An improved technique for measuring erythrocyte chimerism in cattle is described. The method utilizes a Coulter counter to count directly the proportion of cells

TABLE I. Summary of Differential Hemolysis Test Using the Coulter Counter on Two Sets of Chimeric Twins.

Twin pair	Animal no.	Cell type	No. of differential hemolysis tests	Av cells remaining[a] (%)	SD (%)	SD of sum of both cell types	Confidence interval[b]
A	151	I	10	15	2	3	96–107
		II	9	87	4		
	152	I	10	22	2	2	96–106
		II	9	80	3		
B	169	I	24	99	3	3	97–108
		II	14	3	2		
	170	I	24	100	4	4	95–109
		II	14	2	2		

[a] After treatment with reagents reactive with opposite cell type.
[b] 95% level.

remaining after differential hemolysis by appropriate reagents. The technique is as precise as previous methods and has a standard deviation of less than 5%. The method requires less time and reagent than previous methods.

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