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A Procedure for Granulokinetic Investigation in the Developing Bovine Using Chromium-51[1] (38457)

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The study of intravascular kinetics of the granulocyte has provided an understanding of the normal kinetic flow of granulocytes and a basis for the alteration of the leukogram in disease states. Most of the kinetic studies have been conducted in man. There are, however, several published accounts of granulocyte kinetic studies in the canine (1–4) and other animal species (5, 6). In general, the basic kinetic patterns of the granulocytes in the various mammalian species are similar.

However, there are no studies during the critical neonatal period. The potential risks involved with the use of radioisotopic labels and technical difficulties related to body size have restricted such studies in human neonates. Most of the experimental animals used as human models are so small at birth as to severely limit their usefulness in this type of investigation. The large size of the bovine neonate thus provides a major advantage in this type of investigation. However, largely for technical reasons, definitive data on the bovine species have not been available. It was for this reason that the following procedure was developed for the study of intravascular granulocyte kinetics in the bovine.

Methods. Eight clinically normal Holstein Fresian calves were used in this series of investigations. The calves ranged in age from 8 to 380 days. A total of 15 granulocyte kinetic trials were conducted on these calves.

Granulocyte isolates were prepared for radioactive labeling utilizing a modification of a previously described procedure (7). Two hundred and twenty milliliters of blood were drawn into a specially formulated ACD solution (ACD solution at 50% greater concentration than standard Formula A) at a ratio of 1 ml of anticoagulant to 10 ml of blood. Aliquots of 60 ml were transferred to four sterile 240-ml siliconized tubes and centrifuged at 1000*g* for 15 min. An aliquot of 50–60 ml of plasma was harvested, cleared of platelets by centrifugation at 2500*g* for 10 min, and saved for later use. The remaining plasma, buffy coat, and upper erythrocyte layers in each tube were removed by sterile suction and discarded.

The packed erythrocyte fractions in each tube were mixed with 20 ml of sterile 0.8% phosphate buffered saline (PBS) to which special formula ACD at a ratio of 1:100 was added. In all the subsequent steps ACD was added to the PBS at this ratio, designated PBS·ACD. This step effectively prevents granulocyte clumping and disperses the erythrocytes to facilitate their lysis. One hundred and twenty milliliters of sterile distilled water were added to each tube and mixed gently for 30 sec. Isotonicity was restored with the addition of 60 ml of sterile 2.7% PBS·ACD. After gentle mixing, each tube was centrifuged at 200*g* for 8 min. The hemolyzed supernate was then removed by sterile suction, leaving a white cell button consisting primarily of granulocytes. The white cell button from each of the tubes was then dispersed in 5 ml of sterile 0.8% PBS·ACD and pooled. The cells remaining in the tubes were collected by the addition of 5 ml of sterile cleared plasma to each tube and pooled. The resultant pooled granulocyte suspension consisted of an equal mixture of 0.8% PBS·ACD and plasma with a total volume of approximately 40 ml.

The granulocyte suspension was incubated in

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a 38° water bath for 45 min with 300–600 μCi of ^{51}Cr[3] with frequent gentle mixing. After the incubation period, 30 mg of ascorbic acid[4] was added, mixed, and allowed to stand for at least 5 min. After careful mixing to disperse the cells, 40 ml of the suspension was drawn into a sterile 60-ml plastic syringe and the volume noted. The labeled granulocyte suspension was then ready for infusion into the calf. A 1-ml aliquot of these labeled cells was submitted for total and differential leukocyte counts and viability staining. Total leukocyte counts were performed on a Coulter Model (F) electronic cell counter and were based on a minimum of two dilutions counted three times individually. Smears for differential leukocyte counts were stained with a modified Wright Leishman stain (8). Differential counts were made on two smears counting at least 500 cells. Cell viability was estimated utilizing the Trypan blue dye exclusion technique (9), and based on cell counts of 500–1000 cells.

A 2- to 4-ml aliquot of the labeled suspension for determination of injected radioactivity was washed twice in 40 ml of 0.8% PBS·ACD by centrifugation at 200*g*. The final leukocyte button was dispersed in 2.7 ml of 0.8% PBS·ACD. Two milliliters of the cell suspension were placed in a 4-ml glass counting vial and 1 ml of 1 *N* NaOH was added to dissolve the cells. The dissolved leukocyte suspension with a volume of 3 ml was counted for radioactivity. The remaining cell suspension was submitted for total and differential leukocyte counts. These determinations were performed in the same manner as with the initial leukocyte suspension except that after counting, the cell suspensions were diluted 1:1 with plasma for smear preparation. The entire procedure to this point, including the isolation and the incubation steps was completed in about 2 hr.

A preinfusion blood sample was taken in a 2-ml EDTA tube[5] and the total and differential leukocyte counts were performed as described. The ^{51}Cr-labeled granulocyte suspension was slowly infused into the jugular vein. Forty milliliters of blood were drawn into 4 ml of special formula ACD at various time intervals from 20 min to 12 hr after the infusion of the labeled granulocytes. Two milliliters of the blood were submitted for total and differential leukocyte counts. Granulocyte fractions were isolated from the blood as previously described (7), the only modification of this procedure being the addition of special formula ACD at a ratio of 1:100 to the 0.8% PBS and 2.7% PBS solutions to limit agglutination or clumping. The final leukocyte buttons from the granulocyte fractions were dispersed in 2.7 ml of 0.8% PBS·ACD. Two milliliters of these cell suspensions were placed in 4-ml glass counting vials, dissolved in 1 ml of 1 *N* NaOH, and then submitted for specific activity determination. Total and differential leukocyte counts based on 500 cells were performed as previously described on both leukocyte fractions and the whole blood from each of the serial samples.

The isolated NaOH dissolved leukocytes from the incubated granulocyte preparation and the serial granulocyte fractions were counted in a well-type scintillation counter (Tracerlab[6]). Each vial contained 3 ml of dissolved cells. The samples were counted for time sufficient to reduce counting error to less than 5%. Background counts were determined with each counting period and substracted from the total cpm to yield the net cpm.

Protein determinations on the dissolved leukocyte preparations were performed in duplicate on a Technicon dual channel autoanalyzer[7] using a modified Lowry Protein Technique. A standard curve was prepared with each series of samples, using serial dilutions of Albumin Stock Solution (Technicon No. T23-0204[7]). Protein concentrations were read directly from this curve and corrected for dilution. Since the volume, 3 ml, and the protein concentration of each sample were known, it was possible to calculate the total protein present in each sample vial.

Specific activity was expressed as net cpm/mg protein. Most of the sample vials contained approximately 10 mg of leukocyte protein. The actual activity of the early samples ranged from 200 to 2000 cpm. The granulocyte specific activity was plotted on a semilog scale, and a line of

[3] Abbott Laboratories, North Chicago, IL

[4] Cenolate, Abbott Laboratories, North Chicago, IL.

[5] Vacutainer, Beckton, Dickenson and Company, Columbus, Nebraska.

[6] Tracerlab, Waltham, Massachusetts.

[7] Technicon Corporation, Tarrytown, NY.

best fit was determined by the least-squares regression analysis of the log of the points. The line was extrapolated to zero time to yield the zero time specific activity used for pool size calculations, and the clearance half-time ($T_{\frac{1}{2}}$) was determined, as has been described for man (10).

Results. The data on the granulocyte isolate prepared for radioactive labeling are presented in Table I. The isolation procedure resulted in a marked enrichment in the granulocyte purity to greater than 89% from a blood granulocyte differential percentage that ranged from 29 to 63%. The cells were morphologically normal and the mean viability as assessed by the trypan blue dye exclusion technique was greater than 97%. After the washing procedure to prepare the labeled granulocytes for specific activity determination, there was a significant enrichment in the granulocyte percentage to nearly 95%. Washing resulted in the virtual elimination of all erythrocyte and thrombocyte fragments. The relative purity and viability of the isolated ^{51}Cr-labeled granulocytes was remarkably consistent in every trial and was essentially the same in the calves of various ages.

Granulocyte fractions were isolated from 7–8 serially drawn blood samples with each kinetic trial. The mean and SEM purity of these serially isolated granulocyte fractions is presented in Table II. The mean purity value was 93.9%. The estimated total recovery rate of granulocytes in these isolates as compared to the total present in the blood samples was 81.7%. The mean purity and recovery rate for the serially isolated samples were remarkably consistent. The correlation coefficients for the least-squares regression analysis of the log plot of the specific activity of the serial granulocyte isolates for each individual trial are presented in Table II. The mean correlation coefficient was 0.9940 ± 0.0012 SEM, indicating that the specific activity clearance curve was indeed an exponential function with a very close fit of points to the regression line. A typical granulocyte specific activity clearance curve is presented in Fig. 1. The granulocyte specific activity clearance curves were a single exponential function in all 15 trials on the normal calves.

Discussion. The development of the described procedure for the study of the intravascular granulocyte kinetics in the bovine was necessitated by several factors. Disopropylflurophosphate (DF^{32}P), the most commonly used label for granulokinetic studies in other species, was found to be unsatisfactory for this purpose in the bovine (11). Bovine granulocytes failed to preferentially take up significant amounts of this compound. When the concentration of DF^{32}P was increased above the levels suggested by Athens *et al.* (12), hemolysis of the erythrocytes became a major problem.

^{51}Cr has been utilized for kinetic studies of both lymphocytes and granulocytes in man. In these studies neither elution of label from the cells nor reutilization was found to be a major problem (13). ^{51}Cr is not a specific granulocyte label, and the cells must be isolated prior to incubation with the radioactive compound (13). For this purpose a recently described technique for the isolation of highly purified granulocyte fractions from bovine blood was employed (7). This procedure reproducibly yielded viable granulocyte fractions of high purity. Preliminary investigations indicated that incubation of the cells with ^{51}Cr for 45 min at 38° resulted in maximal granulocyte labeling. The addition of ascorbic acid after incubation reduces the unbound ^{51}Cr to the +3 state which is no longer capable of binding. This step effectively eliminates *in vivo* labeling of granulocytes in the circulation (14).

The use of hypotonic lysis of contaminating

TABLE I. Purity and Viability of Isolated Chromium-51 Labeled Granulocyte Fractions Prepared for Infusion.[a]

	Infused granulocyte preparation		Washed granulocyte preparations for specific activity determinations
	% Granulocyte	% Viable	% Granulocyte
Mean	89.2	97.4	95.0
SD	± 5.91	± 2.04	± 2.44
SEM	± 1.52	± 0.53	± 0.65

[a] Results of fifteen trials on normal calves.

TABLE II. Serial Granulocyte Isolate Purity, Recovery Rate and Specific Activity Correlation Coefficient.[a]

	Purity % granulocytes	Recovery rate (%)	Correlation coefficient (r)
Mean	93.9	81.7	− 0.9940
SD	± 2.95	± 5.42	± 0.0046
SEM	± 0.76	± 1.44	± 0.0012

[a] Correlation coefficient of log plot of granulocyte specific activity clearance curves.

erythrocytes for the preparation of cell fractions has previously been employed in lymphokinetic studies (15). This report, however, represents the first use of this technique for granulocyte kinetic studies. Labeled granulocytes used for kinetic studies must be viable, functional, and above all behave kinetically as normal granulocytes in the pool which they represent. The ^{51}Cr-labeled granulocytes appeared to be morphologically normal. The mean viability of these cells was 97.5% as assessed by the trypan blue dye exclusion technique. The high purity of the granulocyte fractions was remarkably consistent considering the wide range of the granulocyte differential percentage of the whole blood in the various calves. Significant lymphocyte contamination of granulocyte fractions can have a major impact on the granulocyte kinetic data evolved when ^{51}Cr is used as a cell label (13). The net effect of such contamination is to yield total blood granulocyte pool sizes that are inappropriately large and may be responsible for the biphasic clearance curve observed by some investigators. The ideal situation for granulocyte kinetic studies using ^{51}Cr is to have both the incubated labeled cells and the serial granulocyte isolates of 100% purity. In this series of investigations, the relative purity of the granulocyte isolates was 94%, thus approaching the ideal.

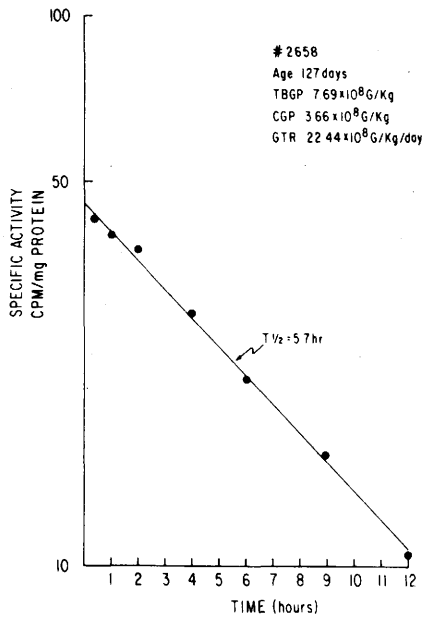

FIG. 1. Typical granulocyte specific activity clearance curve for calves.

The granulocyte specific activity clearance curve is clearly an exponential function in the bovine as in the human (10) and canine (1) species. The recovery rate, pool sizes, half-times, and turnover rates in the bovine are remarkably similar to those of the other species (11). The kinetic data are also in close agreement with those obtained by leukophoresis and tritiated thymidine labeling studies of granulocytes in calves (16–18). These data provide strong support to the validity of the methodology employed.

The described procedure offers the potential for investigation of granulocyte kinetic parameters in the neonate. The kinetic flow of the granulocytes during this critical period of life may have a major influence on the development of and resistance to various disease processes. The procedure should also be useful in the investigation of responses of the bovine to exogenous corticosteroids and induced inflammation. This is particularly true since the reliability of the granulocyte isolation procedure upon which the kinetic procedure is based is nearly independent of the granulocyte differential percentage in the peripheral blood.

Summary. A procedure for the study of intravascular granulocyte kinetics in the bovine utilizing chromium-51 as a cell label is described. The high degree of purity of labeled, serially isolated granulocyte fractions allows an accurate assessment of the various kinetic

parameters. The procedure is applicable to the developing bovine and may be of particular importance in the evaluation of granulokinetic parameters in the critical neonatal period.

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