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SURFACE AREA AND VOLUME CHANGES DURING MATURATION OF RETICULOCYTES IN THE CIRCULATION OF THE BABOON

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ABSTRACT

Changes in the surface area and volume of reticulocytes were measured during late stage maturation *in vivo*. Baboons were treated with erythropoietin to produce mild reticulocytosis. Reticulocyte-rich cohorts of cells were obtained from whole blood by density gradient centrifugation. The cohorts were labeled with biotin, reinfused into the animal, and recovered from whole blood samples by panning on avidin supports. Changes in the surface area, volume and membrane deformability were measured using micropipettes during the two to six weeks subsequent to reinfusion. For the entire cohort, membrane area decreased by 10-15% and cell volume decreased by ~8.5%, mostly within 24 hours after reinfusion. Estimates of the cellular dimensions of the reticulocyte subpopulation within this cohort indicate larger reductions in the mean cell area (12 to 30%) and mean cell volume (~15%) of the reticulocytes themselves. Two weeks after reinfusion the distribution of cell size for the cohort was indistinguishable from whole blood. There was slight evidence of elevated membrane shear rigidity in some reticulocytes prior to reinfusion, but this slight increase disappeared within 24 hours after reinfusion. These are the first direct measurements of changes in the membrane physical properties of an identifiable cohort of reticulocytes as they mature *in vivo*.

Running title: Surface loss during reticulocyte maturation in vivo

Keywords: Erythroid maturation, Cell deformability, primates, erythrocytes

List of Abbreviations:

CPD	Citrate phosphate dextrose
EPO	Erythropoietin
PBS	Phosphate-buffered saline
RBC('s)	Red blood cell(s)

List of symbols:

A	Cell area
a_{ex}	Excess cell area
d	Distribution equalling the weighted sum of two gaussian distributions
F_1,F_2	Weighting coefficients of distributions
L_p	Length of cell projection in pipette
P	Aspiration pressure
R_c	Radius of cell portion outside of the pipette
R_f	Reticulocyte fraction
R_p	Radius of the pipette
S	Sphericity of the cell (dimensionless V/A)
V	Cell volume
x	independent variable of distribution
x_{c1},x_{c2}	Means of distributions
μ	Membrane elastic modulus
σ_1,σ_2	Standard deviation of distribution

Introduction

The surface to volume ratio of mammalian red blood cells is an important parameter in determining the cell's ability to circulate. The absence of intracellular structures in mature cells and the small resistance of the membrane to extensional (surface shear) deformations contribute to the high degree of deformability that these cells exhibit[1,2]. Nevertheless, red cell deformations take place under strict constraints of fixed surface area and fixed volume. The resistance of the membrane to changes in area is large, and the area can expand by no more than a few percent without fracture and loss of cell contents[3]. The volume is constrained by the impermeability of the membrane to cations, and is regulated by a balance between the slow leak of cations and the Na^+K^+-ATPase of the membrane[4]. Thus, establishment and regulation of an appropriate ratio of membrane area to cell volume appears to be essential for cell viability and function.

In late stage maturation of reticulocytes, transferrin receptor and other "unwanted" proteins are endocytosed and subsequently released from the cell[5]. This membrane remodeling involves the preferential loss of membrane lipid, and by implication, a reduction in the surface area of the cell[6]. This phenomenon was first documented and is most evident in "stressed" reticulocytes induced to form by phlebotomy or treatment with phenyl hydrazine[6-8]. Subsequently, evidence has been obtained that similar events occur in reticulocytes in normal animals or mildly stressed individuals[9], although in these studies the amount of surface area lost was not measured. In the present work, we report the first direct measurements of surface area changes in an identifiable reticulocyte-rich cohort of cells as the reticulocytes mature *in vivo* in a primate model.

Methods

Preparation of the animals. Baboons (*Papio papio*) were phlebotomized on two occasions in the amount of 10 ml/kg (~500-600 ml) 2-3 months prior to the study. This blood was stored frozen[10] and was reinfused along with the biotinylated reticulocytes on the day of the study. On three separate days during a period three to ten days prior to the study, the baboons were injected intramuscularly with 500 IU/kg with EPO (IV) and 50 mg of Imferon. Two different animals were used in the study. One was tested three times and one was tested twice. Five to eight months were allowed to elapse between the end of one series of experiments and the beginning of the next to allow the animals to recover fully from the procedures.

Experimental manipulations involving RBC's in this study were performed under sterile conditions. The various solutions used in this study were sterilized by filtering through a 0.22 uM/L filter (Corning). Penicillin G (2,500U/ml) was added to the PBS (0.9% NaCl plus 5.0 mM PO_4 and 10 mM glucose) used for washing RBC's before reinfusion. The sterility of the reticulocyte-rich fraction which was reinfused was determined by microbiologic testing using blood agar plates to detect aerobic organisms, and thioglycollate broth tubes for detecting both aerobic and anaerobic organisms.

The day of the study the baboon was anesthetized and approximately 500 ml of blood was drawn into CPD to be used for reticulocyte isolation. On two occasions reticulocytes were isolated on two consecutive days from 500 ml of whole blood on each day. After separation of a reticulocyte-rich fraction and labeling of this fraction with biotin the cells were reinfused into the animal. Residual RBC's following fractionation were discarded. The reinfused reticulocyte fraction was supplemented with a unit of stored blood to maintain the animal's blood volume.

Reticulocyte isolation. Reticulocytes were isolated using Percoll-Hypaque density gradients[11]. Forty milliliters of packed washed baboon RBCs were mixed thoroughly with a solution of Percoll and Hypaque (240 ml Percoll, 192 ml of distilled water, 128 ml Hypaque). Only enough of this red cell suspension was prepared at one time that could be spun immediately, necessitating several preparations throughout the procedure. The suspension was then spun for 20 minutes at 15,000 rpm in 50 ml polycarbonate test tubes in a refrigerated centrifuge (Sorvall SS-34 rotor; tubes cat# 03146; Dupont Sorvall, Wilmington, DE). After centrifugation the uppermost layers (~0.5 ml) were carefully isolated from each tube and were washed three times in PBS prior to biotinylation. Prior to reinfusion of the reticulocyte-rich cohorts, reticulocyte fractions were determined using new methylene blue staining. A total of 1,000 cells were counted for each determination, 500 by each of two technicians working in parallel. Accuracy of the determination was tested using Retic-chex standards (Streck Laboratories, Inc., Omaha, NE). The variability in our determinations was well within the established 95% confidence interval for the method. For example, for a reticulocyte fraction of 1.0 %, the established 95% confidence interval of the method is 0.4-1.6%.

Reticulocyte biotinylation and post-infusion recovery. Biotinylated reticulocytes were prepared by a procedure adopted from Dale and Norenberg[12] and described by McKenney and coworkers[13]. On the day following infusion and periodically during the following two to six weeks the surviving biotinylated RBC's were recovered using an avidin support system previously described[13]. RBC volumes in the samples just prior to reinfusion were determined using ^{125}I albumin[14]. Cell preparation, biotinylation and cell recovery were performed at the University of Massachusetts Medical Center in Worcester, MA. Treated RBC's were then reinfused into baboons at the Naval Blood Research

Laboratory in Boston, MA. Cell samples were shipped overnight to the laboratory of Dr. R. Waugh for mechanical testing and measurement of cell surface area and volume.

Micropipette Aspiration Tests. These procedures essentially followed the protocols described previously[15]. Micropipettes were prepared by fracturing off the tip of a glass capillary pulled to a needle point. The pipettes had an inside diameter between 1.5 and 2.3 μm. Cells were suspended at low hematocrit in phosphate buffered saline (160 mM NaCl, 25.0 mM Na_2HPO_4, 6.2 mM KH_2PO_4) adjusted to an osmolarity of approximately 292 mOsm, pH 7.35. (The osmolarity for the different series of experiments varied from 289 to 298 mOsm, but within a given series, the osmolarity was kept constant, ± 1.0 mOsm.) Cells were placed in a chamber on the stage of an inverted microscope and the micropipette was introduced from the open side of the chamber. The pressure in the pipette was controlled by adjusting the height of a water-filled reservoir connected to the back of the pipette by water-filled tubing. The image from the microscope was sent to a television camera and recorded on video tape for subsequent analysis. To measure membrane shear elasticity, a single cell was aspirated in the biconcavity of the disk and positioned in the field of view. A series of aspiration pressures was applied in the range of 15 to 60 Pa (1.5 to 6.0 mm H_2O pressure, approximately) and the length of the cell projection in the pipette was measured as a function of the applied pressure. An effective elastic shear modulus was calculated according to the theory originally set forth by Evans[2]. The modulus is approximately related to the slope of the length, pressure data pairs by:

$$\mu = \frac{R_p^{\,2}}{2.45} \cdot \frac{dP}{dL_p} \qquad (1)$$

where L_p is the length of the membrane projection into the pipette, P is the pressure difference between the pipette and the suspending fluid, R_p is the pipette radius and μ is the modulus.

To measure cell surface area and volume, cells were aspirated at a pressure of approximately 1,000 Pa (10 cm H_2O) to ensure that the membrane was fully extended into the pipette. Care was taken that there were no folds or creases in the membrane projection. The cell area A and the cell volume V were calculated from measurements of the outer cell radius, R_c, and the length of the projection in the pipette, L_p:

$$A = 2\pi R_c\left(R_c + \sqrt{R_c^2 - R_p^2}\right) + 2\pi R_p L_p \tag{2}$$

$$V = \frac{2\pi}{3}\left[R_c^3 + \left(R_c^2 + \frac{R_p^2}{2}\right)\sqrt{R_c^2 - R_p^2} + R_p^3\right] + \pi R_p^2\left(L_p - R_p\right) \tag{3}$$

The sphericity of the cell S is a dimensionless quantity proportional to the ratio of the 2/3 power of the volume to the cell area:

$$S = \frac{4\pi}{(4/3\,\pi)^{2/3}} \cdot \frac{V^{2/3}}{A} \tag{4}$$

The coefficient makes the maximum value of the sphericity 1.0 (a perfect sphere). The smaller the value of the sphericity, the greater is the "excess" surface area of the cell, that is, the area in excess of the area required to enclose the spherical volume of the cell. The percentage of excess area can be calculated as:

$$a_{ex} = (1/S - 1.0) \times 100\% \tag{5}$$

Results

Hematological Parameters

Five series of experiments were performed in which the cohorts were monitored for two weeks or more. Reticulocyte counts for the reticulocyte-rich fractions prior to infusion are tabulated in Table 1. The fractions of cells remaining in the blood after infusion are also shown in the table for the first day after reinfusion and approximately two weeks later. Note that the day 0 fractions are estimated based on the blood volume of the

animal and the volume of cells that was reinfused. In two cases (experiments 2 and 3) a second reticulocyte-rich cohort was prepared and infused into the animal 24 hours after the first re-infusion. The data indicate that, with the possible exception of experiment 5, greater than 80% of the cells reinfused into the animal continue to circulate 24 hours after reinfusion. We note that (with the possible exception of experiment 4) the outcome of the study was not influenced by an animal having been subjected to prior study.

Surface Area and Volume

Although there was some quantitative variability from experiment to experiment, all of the different series of measurements exhibited the same general trends. The changes in cell dimensions as assessed by micropipette aspiration during each of the five series of measurements are summarized in Table 2. The difference between the mean cell area of the cohort sample and the mean cell area of its matched control is given as a percentage of the control mean value. The changes in cell volume and sphericity are expressed in the same way. In the last grouping of the table the mean percentages of excess area for both cohort and control samples are given. For the day 0 samples, the cohort consisted of the biotin-labeled reticulocyte-rich fraction prior to infusion, and the control consisted of an untreated whole blood sample from the same animal. For subsequent days, the cohort consisted of cells that were adherent to an avidin support, and the control consisted of the non-adhering cells.

Pre-infusion, Day 0. Micropipette measurements revealed that prior to infusion the reticulocyte-rich fractions had larger mean surface area and larger mean cell volume than whole blood. The mean sphericity was slightly smaller than normal, reflecting a larger than normal excess area. (See Table 2.) New methylene blue staining indicated clearly that the labeled cohort consisted of two populations of cells, reticulocytes plus the lightest of the mature cell population. This is reflected in the histograms of the areas of

the reticulocyte-rich cohorts prior to infusion. (See Figs. 1A and 1D.) There appear to be two populations of cells in the labeled cohort, one having a distribution of surface areas similar to mature cells, and one having a distribution of areas larger than mature cells. The latter population, presumably, consists of the reticulocytes. These data indicate that the mean area for the entire cohort underestimates the mean area of reticulocytes because of the significant percentage of mature cells in the labeled cohort. It is possible to estimate the characteristics of the population of true reticulocytes by fitting the frequency distribution of cell areas for the labeled cohort with two gaussian curves, one having the mean and standard deviation of the control population plus a second population with a larger mean area. The frequency histogram for the cell areas of the cohort were fit to a distribution (*d*) consisting of the sum of two gaussian curves, one having mean x_{c1} and standard deviation σ_1, and one having mean x_{c2} and standard deviation σ_2:

$$d = \frac{F_1}{2\sigma_1}\sqrt{\frac{2}{\pi}} \cdot e^{-(x-x_{c1})^2/2\sigma_1^2} + \frac{F_2}{2\sigma_2}\sqrt{\frac{2}{\pi}} \cdot e^{-(x-x_{c2})^2/2\sigma_2^2} \qquad (6)$$

In this formulation the relative numbers of cells in the two gaussian subpopulations is given by the ratio of F_1 and F_2. The independent variable x corresponds to cell area. A non-linear least squares algorithm (Marquardt-Levenberg) was used to fit the distribution to the frequency histogram (via the statistical package Origin, by MicroCal Software, Northampton, MA.) In practice, the mean x_{c1} and the standard deviation σ_1 were fixed at values corresponding to the distribution of the matched control cells, and F_1, F_2, x_{c2} and σ_2 were allowed to vary to fit the distribution to the frequency histogram of the cell areas of the reticulocyte-rich cohort. The fraction of "reticulocytes," that is, the relative number of cells in the population with larger surface area, was estimated from the ratio $F_2/(F_1 + F_2)$. These calculated values agreed well with values of the reticulocyte fraction obtained by new methylene blue staining. This is evident in Table 3, which contains the estimated reticulocyte fractions and the mean areas of the two fitted distributions. On

average the mean surface area of the larger cells exceeded that of the control population by approximately 20%, whereas the arithmetic mean area of the entire, mixed, labeled cohort exceeded the control population by an average of approximately 12%.

The distribution of cell volumes determined by micropipette aspiration for the reticulocyte-rich cohort prior to infusion did not separate clearly into two distinct populations of cells. Typically, the cohort population was skewed toward higher values for the volume, but in all but one case, the distribution of volumes for the cohort could be fit as well with a single gaussian as it could with a double gaussian distribution (Eq. 6). Nevertheless, the mean cell volume for the mixed sample was (in four out of five cases) substantially larger than for the matched control population. The mean cell volume of the reticulocyte sub-population V_{retic} was estimated from the reticulocyte fraction determined by new methylene blue staining R_f and the difference between the mean cell volume of the labeled, reticulocyte rich cohort V_{samp} and the mean cell volume of the whole blood control V_{con}:

$$V_{retic} = V_{con} + \frac{\left(V_{samp} - V_{con}\right)}{R_f}. \qquad (7)$$

Based on these estimates, the mean cell volume for reticulocytes was approximately 15% greater than mature cells, on average.

Post-infusion. After reinfusion there were significant changes in the distribution of surface areas in the labeled population. As is evident from the data shown in Table 2, there was some quantitative variability from experiment to experiment. Nevertheless the general behavior was similar for all experiments, and is reflected in the averaged values of the ratio of the mean cohort area (volume and sphericity) to the matched control area (volume and sphericity) depicted in Figure 2. After 24 hours in the circulation there was a

dramatic decrease in the areas of the labeled cells. In three of the five experiments, the labeled cell cohort 24 hours after reinfusion appeared to consist of a single population of cells with a gaussian distribution of cell areas. In these cases, the mean area of the labeled cohort on day 1 was not appreciably different from the control. (See Figs. 1A-1C). In two of the experiments, the labeled cell cohort on day 1 could be resolved into two populations of cells, one having a mean surface area equal to the control, and one having a larger mean cell surface area. However, for these cases, the proportion of cells in the population with larger cell areas on day 1 was smaller than had been on Day 0. In one case the proportion of cells in the larger group fell from 85% on Day 0 to 43% on Day 1, corresponding to a decrease in the mean surface area of the entire labeled cohort from 12% greater than control (on Day 0) to 7% greater than control (on Day 1). In the other case (depicted in Figs. 1D-1F), the proportion of cells in the larger subpopulation fell from 52% on Day 0 to 27% on Day 1, corresponding to a decrease in the mean area of the labeled cells from 10% greater than control (on Day 0) to 5.5% greater than control (on Day 1). In all experiments the mean areas of the fitted distributions for the cohort samples 14 days after reinfusion was within 2% of the mean of the distribution of the control population (Figs. 1C and 1F).

The difference in cell volume between labeled and control populations observed prior to infusion was not evident 24 hours or two weeks after infusion. On average, the difference in the mean cell volume for labeled and unlabeled cells 24 hours after reinfusion was essentially zero.

Membrane Rigidity

There was no clear evidence of increased shear rigidity of reticulocyte membranes in these studies. Average moduli calculated via Eq. 1 for reticulocytes were not different from the moduli determined for controls in any of the experiments. However, in two of

the five experiments, some individual cells in the reticulocyte population appeared to be more rigid than control cells in that the length of the projection in the pipette was shorter than it was for other cells in the same group and shorter than the matched control cells. Even in these cases, however, the change in the projection length with increasing pressure was not different from control, and so the modulus calculated according to Eq. 1 was not abnormally high. Such indications of increased rigidity were only ever observed in the reticulocyte cohort prior to reinfusion. In all cases examined, the rigidity of the biotinylated cells was indistinguishable from unlabeled cells within 24 hours after reinfusion into the circulation.

Discussion

Earlier studies have provided evidence that macroreticulocytes produced under conditions of severe hemorrhagic stress lose significant amounts of membrane lipids as they mature in the vasculature[6,8]. What has remained less clear is to what extent these events might also occur during maturation of normal reticulocytes, especially in man. Indeed early studies have shown significant differences in the maturation and survival of stressed reticulocytes compared to normal cells[7,16]. Morphological evidence obtained in human subjects subjected to mild erythropoietic stress indicated that similar membrane remodeling does occur under such conditions[9]. However, the extent of membrane loss and the time course over which it takes place have not been previously documented. The present results show clearly that in primates subjected to mild erythropoietic stress, significant membrane loss occurs over a period of 24-48 hours in the circulation. Presuming that the fitted distributions accurately distinguish between reticulocytes and mature cells in the reticulocyte population, the measurements show that on average, approximately 20% of the reticulocyte membrane area and 15% of the reticulocyte volume are lost during the first 24 to 48 hours in the circulation.

An alternative interpretation of the change in the distribution of cell area between the preinfusion sample and the day 1 sample might be that the larger cells in the preinfusion sample are selectively removed from the circulation, leaving only labeled mature cells to circulate. This interpretation is inconsistent with measurements of the circulating fraction of cells on day 1. These fractions are not significantly different from the fraction expected to be in circulation based on measurements of the volume of cells reinfused into the animal and the blood volume of the animal. Thus, the change in the distribution of surface areas of the labeled cells appears to reflect a change in the areas of individual cells and not selective removal of large cells.

There is considerable evidence that the loss of transferrin receptor and other membrane proteins during late stage reticulocyte maturation occurs via the formation and release of exosomes, vesicular bodies with diameters on the order of 100 nm[5,9]. The release of these vesicles necessarily involves a concomitant loss of membrane area. It would take, on average, a loss of approximately 1,000 such vesicles from each cell to account for the reduction in surface area that we have measured (~ 30 μm^2). Whether or not this is a reasonable number is difficult to judge, but certainly large numbers of these exosomes have been observed both within reticulocytes and in the circulation of hematopoietically stressed animals. Interestingly, this large number of exosomes corresponds only to a relatively small loss of cell volume (~ 0.5 μm^3). Thus, exosome loss by itself can not account for the decrease in cell volume that has been observed. (This also suggests that even if the exosomes contain hemoglobin, relatively little hemoglobin would be lost from the cells by this pathway[17].) The processing of exosomes is believed to involve the formation of internal, multivesicular bodies where the exosomes are collected prior to release from the cell[9,18]. These bodies are larger than the exosomes, having diameters of ~ 0.5 μm. Exosome release does not involve the loss of the encapsulating membrane area of the multivesicular body, since this membrane presumably fuses with the plasma membrane of the cell when the exosomes are released. However, the volume of the multivesicular body would be lost. Based on a diameter of 0.5 μm, it would take approximately 200 of these structures to account for the 15% volume loss that we have measured. This is not a realistic possibility, because the formation of that many multivesicular bodies would require more membrane than the total surface area of the cell. We conclude therefore, that the loss of surface area during late stage maturation could be explained by the formation and release of exosomes, but the volume loss must involve an additional mechanism such as the loss of solutes and water by volume regulatory pathways.

Previous investigations have also indicated that reticulocytes from the marrow[19,20], and reticulocytes from hemorrhagically stressed animals[21] exhibit higher than normal membrane shear rigidity. The present studies indicate that under conditions of mild erythropoietic stimulation, that such elevated membrane rigidity is not typically observed in circulating reticulocytes, and that under conditions in which some increased membrane rigidity may exist, this increased rigidity is short-lived. These findings are consistent with those of Chasis and coworkers[20] who found that immature reticulocytes were considerably more rigid than more mature reticulocytes, and that the more mature reticulocytes were not significantly different from mature cells. Either the rigid cells in the blood are preferentially removed from the circulation, or the increase in rigidity is transient, and cells attain normal deformability within a short time in the circulation.

Conclusions

Reticulocytes of baboons subjected to mild erythropoietic stimulation lose approximately 20% of their surface area and 15% of their volume as they undergo late stage maturation in the circulation. The amount of surface loss varied somewhat, even within the same animal subjected to similar treatments. In mildly stressed animals, increased membrane rigidity of circulating reticulocytes is not generally observed, and in cases for which there was some indication of increased rigidity, these indications were not evident within 24 hours after reinfusion.

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Table 1

Hematological Values.

Expt.	Animal	% Reticulocytes	% Labeled Cells Circulating			
No.	Desig.	in Cohort	Day 0[a]	1	13-14	>21
1	A	54	1.84[a]	1.93	1.30	0.6[d]
2	A	50	1.40[a]	1.15	1.32[b,c]	1.18[e]
3	B	64	1.02[a]	0.82	0.76[b]	--
4	A	50	1.59[a]	1.74	~0.0	--
5	B	60	0.88[a]	0.61	0.64	--

a. Estimated from volume of cells infused and the blood volume of the animal

b. A second reticulocyte rich cohort was prepared and reinfused after the day 1 sample

c. Day 7 sample

d. Day 43 sample

e. Day 22 sample

Table 2

Mean Surface Area and Volume Data for Reticulocyte-rich Cohorts Normalized to Matched Control.

	Day	Experiment No. 1	2	3	4	5
ΔA	0	13.6	16.5	9.1	11.7	9.9
(% of Control)	1	3.6	-2.4	-7.5	7.1	5.5
	13-14	-1.4	2.1	1.5	-3.1	1.0
	>21	-0.4	-0.3	--	--	--
ΔV	0	8.8	12.7	2.1	11.2	7.9
(% of Control)	1	6.5	-4.3	-7.5	2.6	3.6
	13-14	-4.1	-2.8	0.1	-8.1	1.0
	>21	0.1	-2.0	--	--	--
ΔSpher.	0	-6.7	-7.2	-7.0	-4.2	-4.2
(% of Control)	1	0.7	0.1	2.8	-5.1	-3.2
	13-14	-1.3	-3.9	-1.4	-2.7	-0.3
	>21	0.4	-1.1	--	--	--
a_{ex}	0	47.7/37.7	43.5/33.2	46.8/36.6	39.7/33.9	41.0/35.1
(Retic-rich	1	35.3/36.2	31.6/31.8	31.1/34.8	35.7/28.9	35.7/31.4
Cohort/Contr.)	13-14	34.6/32.8	36.1/30.7	29.4/27.6	34.2/30.5	36.6/36.2
(%/%)	>21	31.8/32.3	35.3/33.9	--	--	--

Table 3

Estimated Areas and Reticulocyte Fractions for Day 0 Populations

Expt.	% Reticulocytes		Mean Surface Area ± S.D. (μm^2)		
No.	Staining	Fitted	Control	Reticulocyte	All Labeled Cells
1	54	62	131 ± 12	159 ± 12	150 ± 18
2	50	60	116 ± 13	150 ± 11	137 ± 20
3	64	85[a]	123 ± 12	143 ± 10	135 ± 16
4	50	50	125 ± 12	140 ± 19	142 ± 23
5	60	52[a]	135 ± 13	157 ± 18	148 ± 21

a Statistical confidence on the fit was low (95% confidence interval of the F parameters exceeded 20% of the parameter value)

Figure Legends

Figure 1. The distribution of surface areas within the reticulocyte-rich cohort prior to reinfusion (A, D), 24 hours after reinfusion (B, E) and two weeks after reinfusion (C, F) for two series of experiments. In one series (Expt. 1, panels A-C) there were two distinct populations of cells prior to reinfusion but only one population 24 hours after reinfusion. In the other series (Expt. 5, panels D-F) there appeared to be two distributions both prior to and 24 hours after reinfusion. Distributions of areas within the labeled cohort two weeks after infusion were indistinguishable from control (C and F).

Figure 2. Mean values for changes in cellular dimensions. A. The ratios of the mean area (□), volume (○) and sphericity (Δ) of the labeled, reticulocyte-rich cohort normalized with respect to the matched control sample are shown as a function of time in the circulation. Values are averaged for all five series of experiments in the study. (Because no measurements were performed on day 14 during experiment 2, the day 14 value for experiment 2 was taken to be the average of the measurements made on days 7 and 22.) B. The mean excess area expressed as a percentage of the area of a sphere with the same volume for the reticulocyte rich cohorts (●) and matched controls (○). Plotted values are the averages of the mean values for all five experimental series.

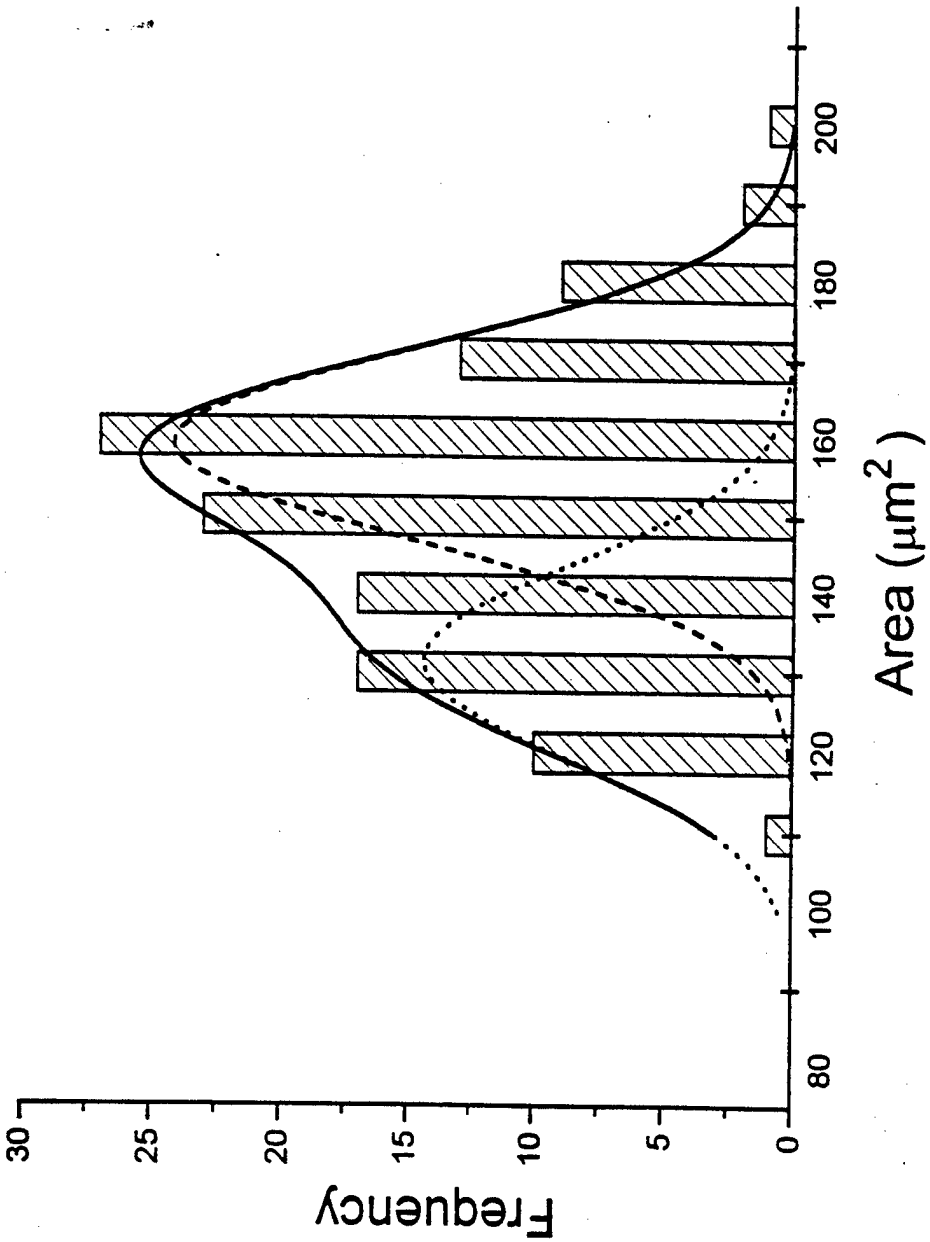

Fig. 1A
(Expt. 1, day 0, cohort)

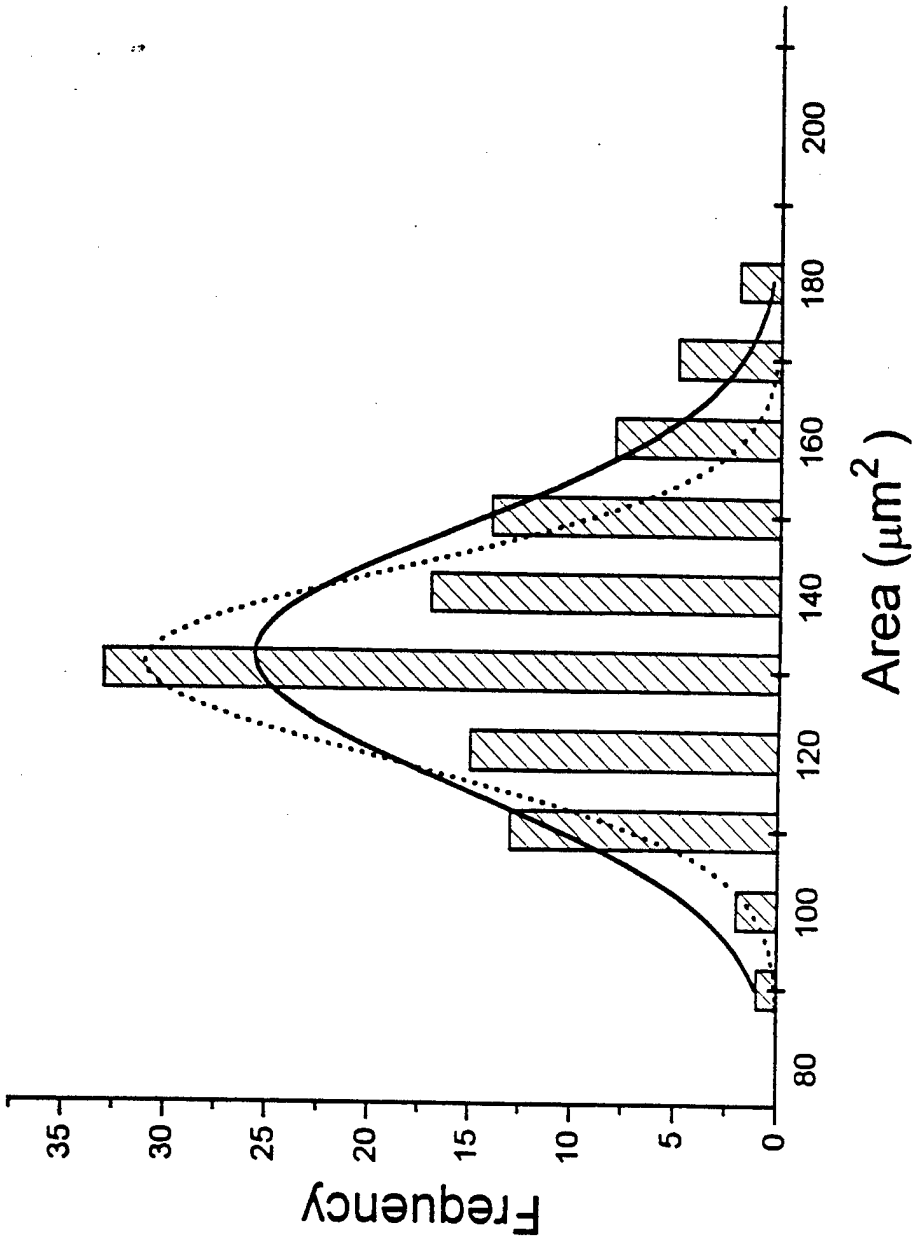

Fig. 1B
(Expt. 1, day 1, cohort)

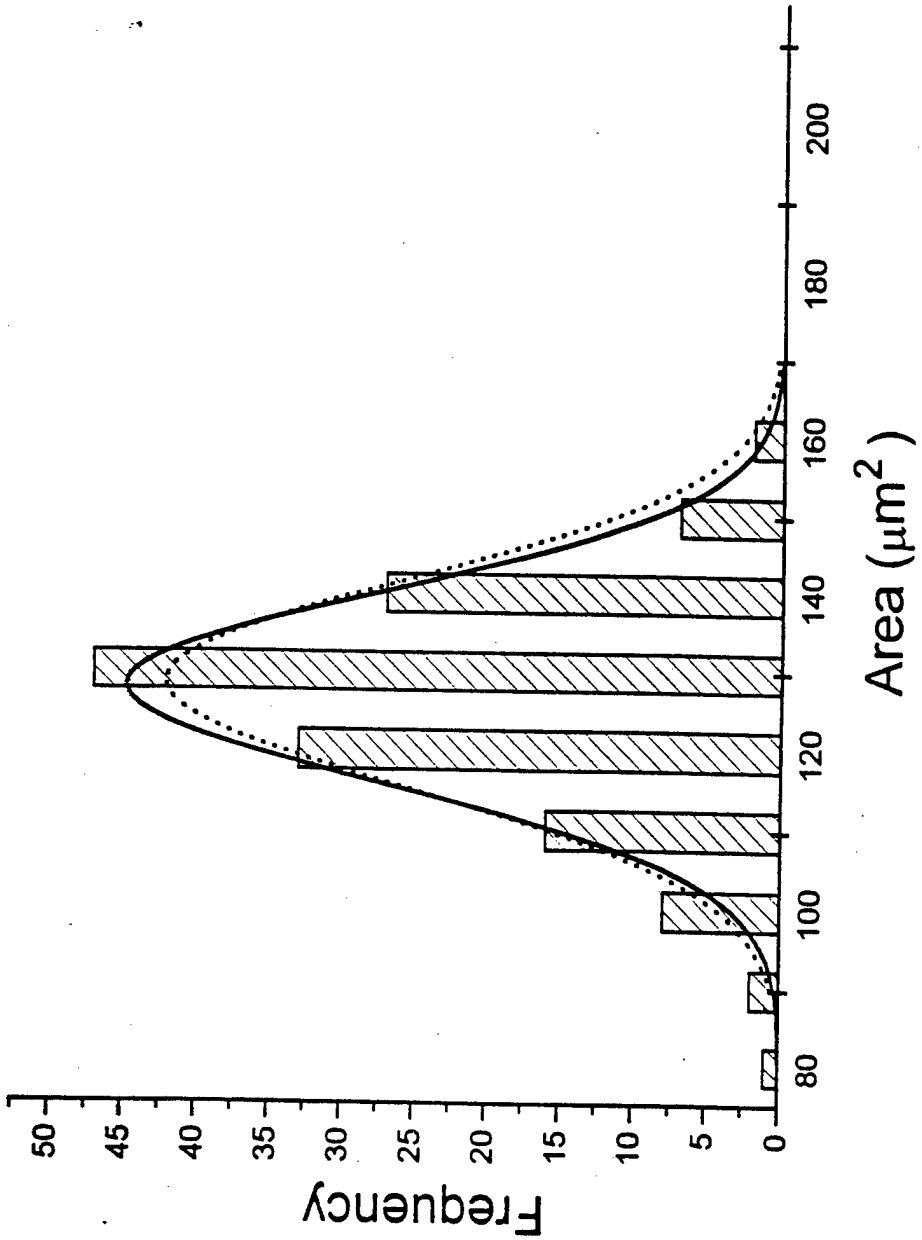

Fig. 1C
(Expt. 1, day 14, cohort)

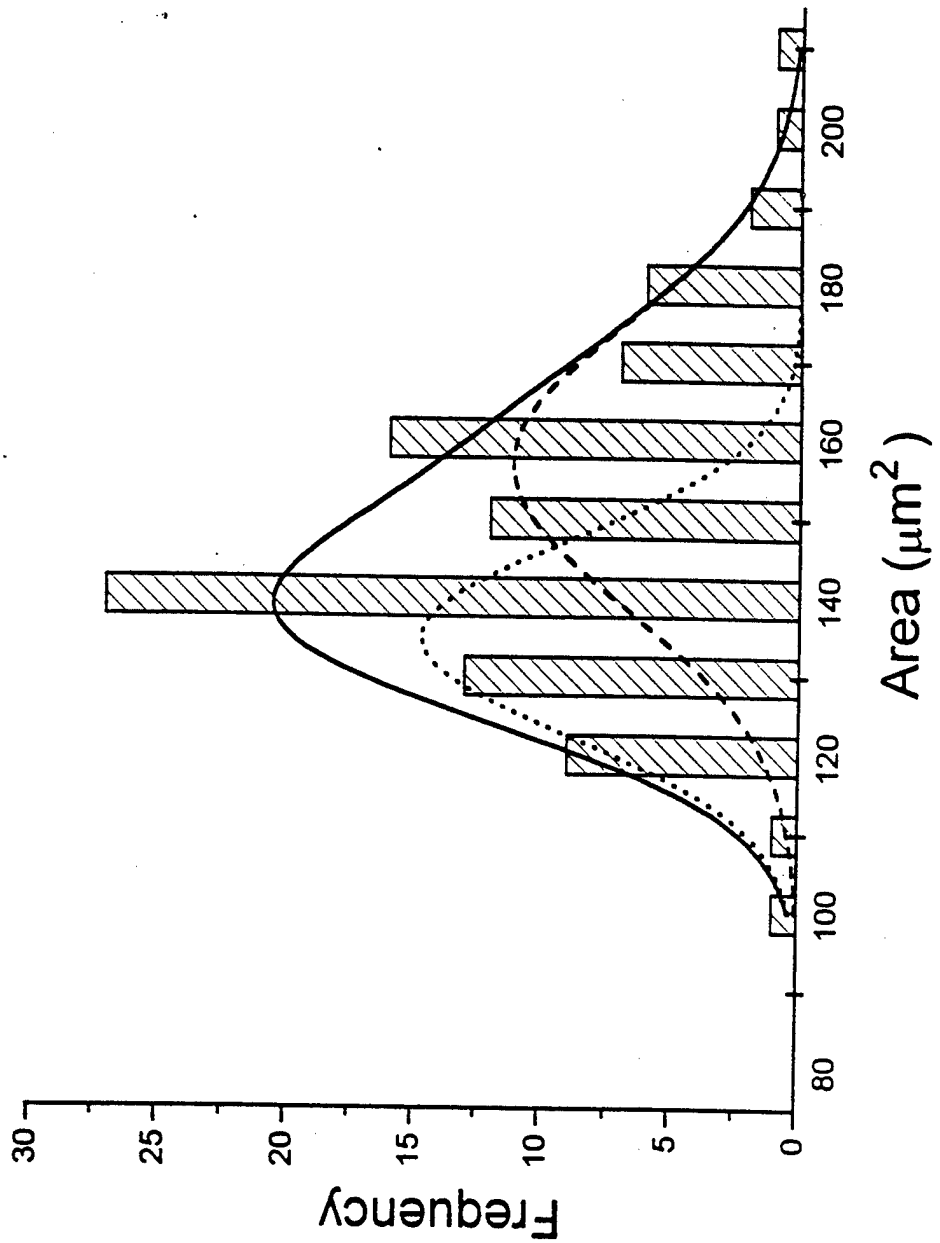

Fig. 1D
(Expt. 5, day 0, cohort)

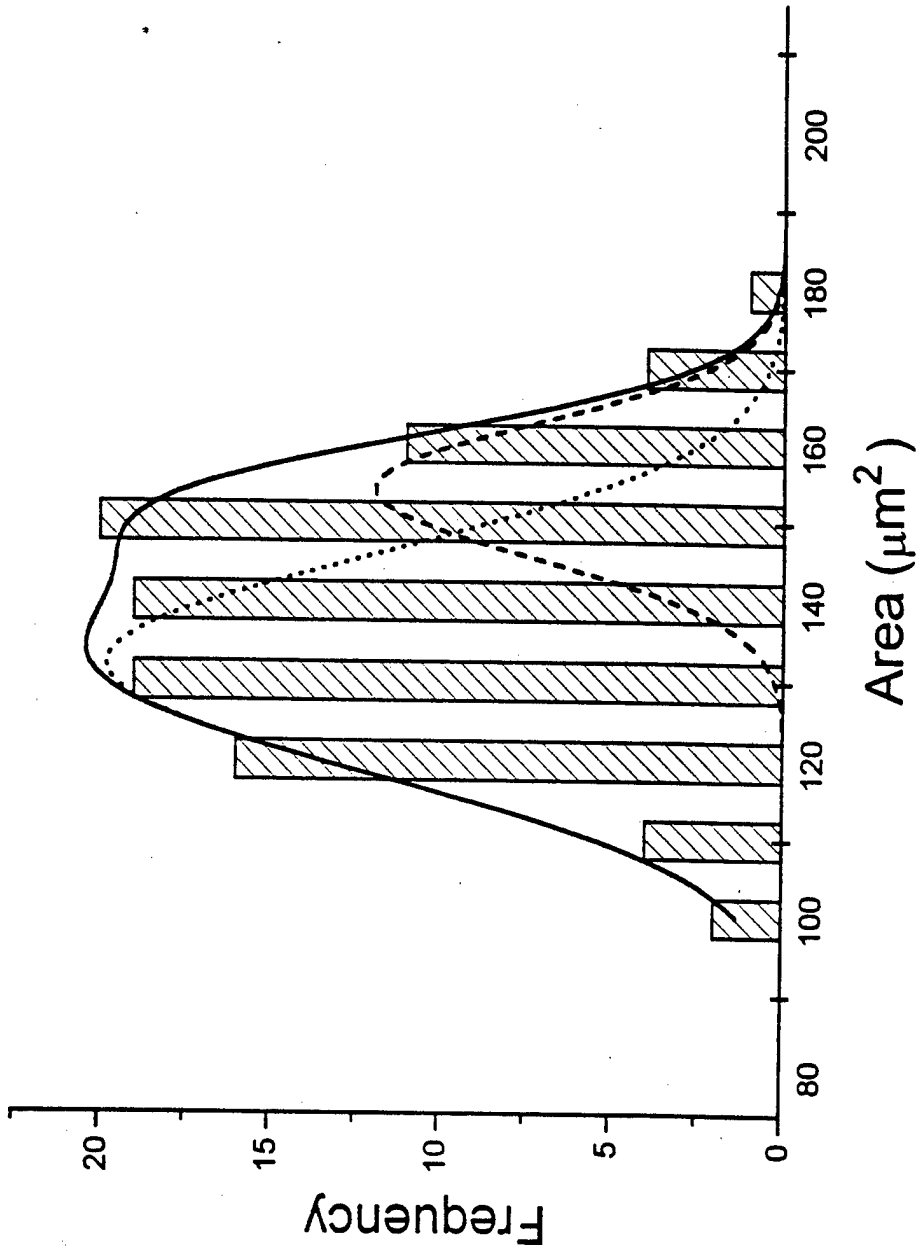

Fig. 1E
(Expt. 5, day 1, cohort)

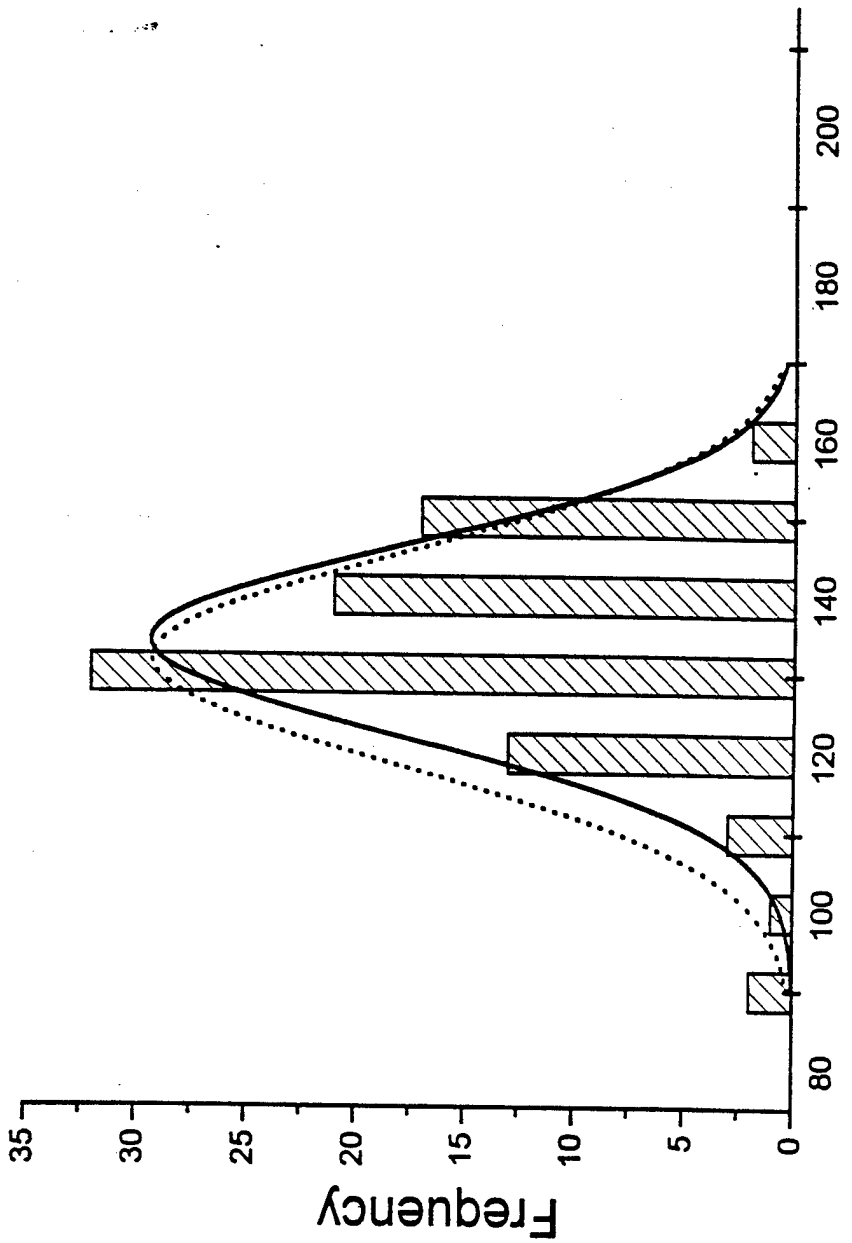

Fig. 1F
(Expt. 5, day 14, cohort)

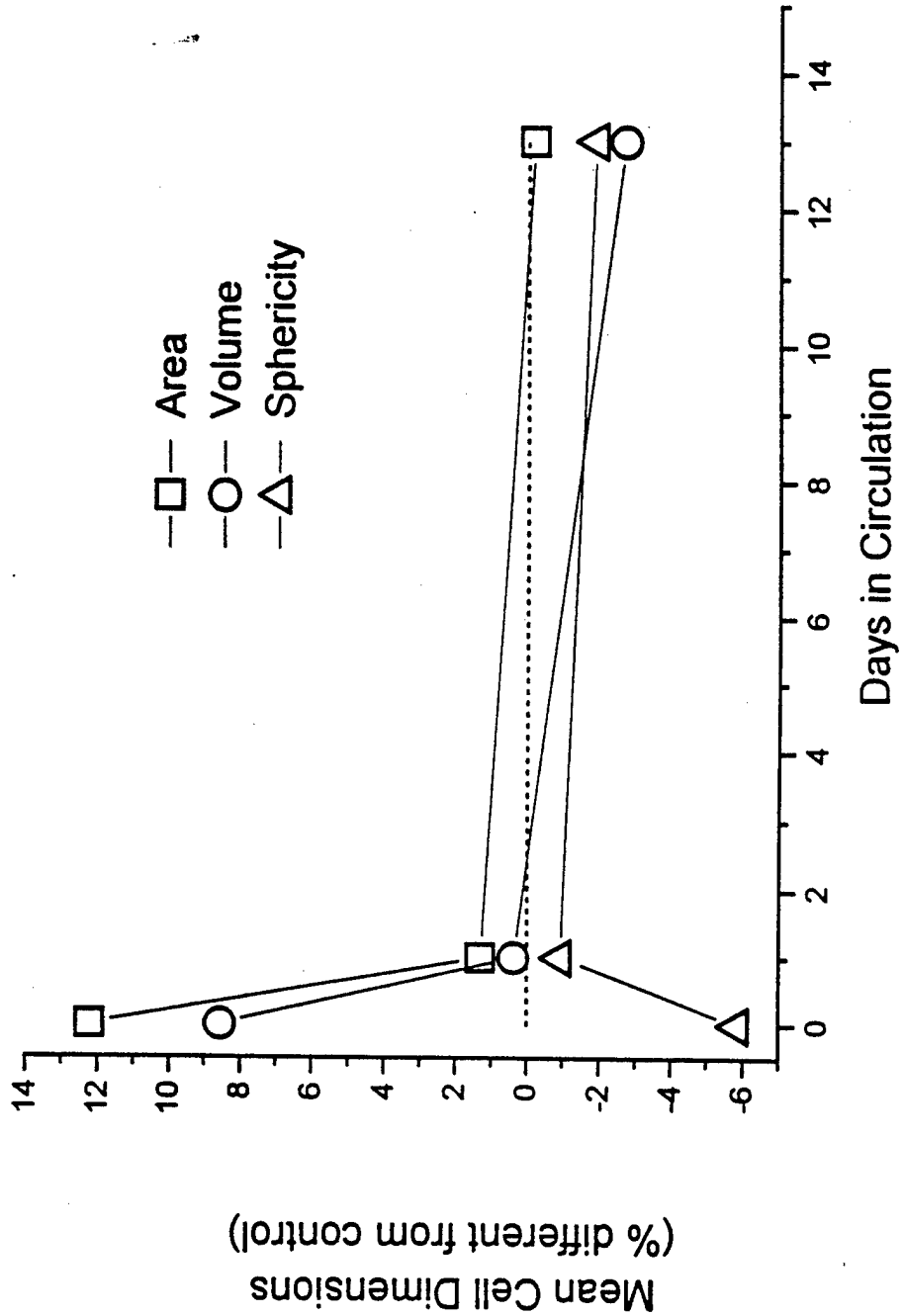
Area
Volume
Sphericity
Mean Cell Dimensions
(% different from control)
Days in Circulation
14
12
10
8
6
4
2
0
-2
-4
-6
0
2
4
6
8
10
12
14

Figure 2A

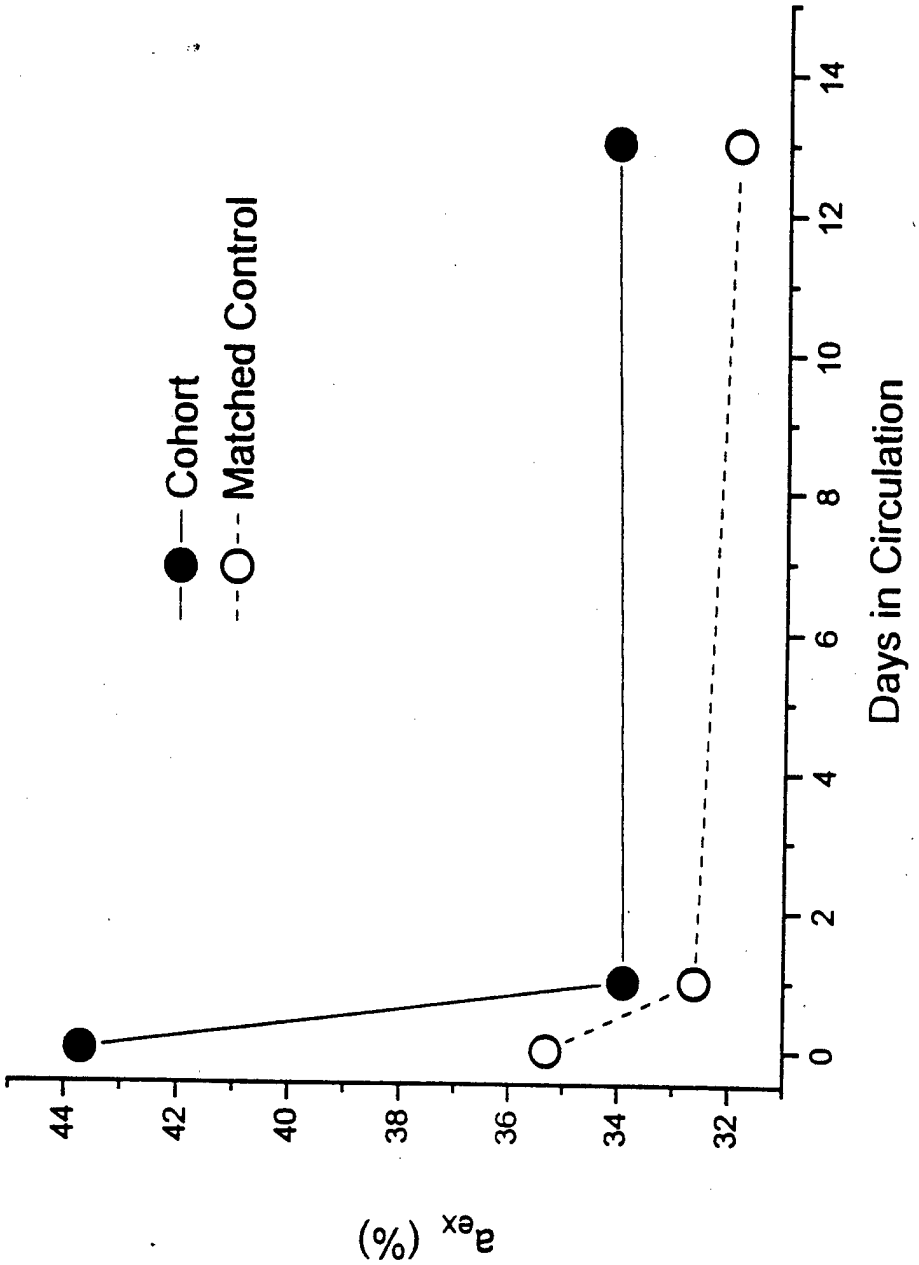
Cohort
Matched Control
a_{ex} (%)
44
42
40
38
36
34
32
0
2
4
6
8
10
12
14
Days in Circulation

Figure 2B