Red Blood Cell Age, Pyruvate Kinase Activity, and Insulin Receptors

Evidence that Monocytes and RBCs May Behave Differently

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SUMMARY

Data emerging from insulin receptor studies performed on red blood cells (RBCs) and monocytes from the same subject are not always in agreement; dichotomy might occur since variations in mean RBC age are not taken into account or because insulin receptors on the two cell types behave differently.

In the present investigation RBCs from normal male subjects were separated into five populations of different mean age by means of centrifugation of RBCs on a discontinuous gradient of buffered Percoll for 10 min at 1000 × *g*. Insulin binding varied significantly depending upon the RBC population tested and was closely correlated to the activity of pyruvate kinase ($r^2 = 0.86$), a well-known marker of RBC age.

These data suggested that pyruvate kinase assay might be helpful in studies of RBCs. To confirm this hypothesis, RBCs from 10 normal male subjects and 13 male patients with hemolytic anemia were studied; insulin binding was correlated to pyruvate kinase activity. By adjusting insulin binding to 2×10^9 RBCs/ml the range of data was abnormally high, but it became acceptable after adjusting insulin binding to pyruvate kinase activity (0.75 U/2×10^9 RBCs). The overall data indicated that insulin binding was highly correlated to pyruvate kinase activity ($r^2 = 0.82$) but only slightly to reticulocyte number ($r^2 = 0.56$) since not only reticulocytes but also erythrocytes lose receptors during maturation.

Pyruvate kinase activity was measured in RBCs from normal men and from normally menstruating women at the seventh and twenty-fourth days of the cycle; results demonstrated that adjustment of data, according to mean RBC age, broadens dichotomy of monocyte and RBC data.

In conclusion, this paper demonstrates that (1) insulin binding experiments may be carried out on RBCs separated into populations of different mean age; (2) by assaying the pyruvate kinase activity, it is possible to study RBC samples of different mean age and to reveal insulin binding variations provoked by changes in mean RBC age; (3) RBCs lose insulin receptors physiologically and continuously with age; (4) RBC insulin receptors of patients with hemolytic anemia are normal; and (5) monocyte and RBC insulin receptors may behave differently. DIABETES *32*:1017–1022, November 1983.

During the last 4 yr numerous studies have been carried out in order to determine whether or not the insulin receptor present on red blood cells (RBCs) reflects the insulin receptor present on other cells. Early studies suggested that results obtained with RBCs are in agreement with those obtained using monocytes[1–5] but later studies carried out on monocytes and RBCs from the same patient showed that in some cases the two cell types behave differently.[6–11]

The reason for this dichotomy remains to be explained; it has, however, been reported that RBC samples containing a high percentage of reticulocytes and/or young erythrocytes bind more insulin than RBC samples with a normal mean age,[11–13] and studies to evaluate the influence of RBC age on insulin binding have indicated that RBCs lose receptors with age.[14,15] Thus, it might be suggested that the dichotomy of monocytes and RBCs results from clinical studies being performed without paying attention to mean RBC age. A further explanation, on the other hand, may be that monocytes and RBCs display a different behavior; in this case, the possibility exists that young and old RBCs react differently to stimuli affecting the insulin receptor.

Until now the question was still open since no simple methods were available to study the insulin receptor–avoiding artifacts provoked by mean RBC age. This article deals with the application of a simple method of RBC fractionation to insulin receptor studies and the relationship emerging between pyruvate kinase activity and insulin binding to RBCs.

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MATERIALS AND METHODS

Subjects. Twenty-nine men (23–42 yr of age) and 10 normally menstruating women (21–35 yr of age) were studied. Thirteen of the 29 men had hemolytic anemia. All were volunteers and normal with regard to body weight (Geigy table, 7th edit.), metabolic parameters, and family history of diabetes and metabolic diseases.

RBC sample preparation. Blood samples were collected at 0800 h, after overnight fasting, by collecting venous blood from an antecubital vein in sodium citrate (3.8%). Samples were repeatedly washed in saline and passed through columns of microcrystalline cellulose- α cellulose (1:1; wt:wt) (Sigma Chemical Co., St. Louis, Missouri) according to the method described by Beutler et al.[16] Elution on cellulose column does not alter the insulin receptor[17] and isolate RBCs in a pure population.[16,17] In this article we will call these samples "whole RBCs" for practical purposes.

RBCs were separated in populations of different mean age by means of centrifugation on Percoll as previously described in detail.[18] Briefly, whole RBCs (4 ml, 20–24 × 10^9 cells) were applied at the top of a discontinuous gradient of Percoll and gradients were centrifuged in a Sorvall centrifuge (RC 2B) using an angle rotor (SS-34) at 1000 × *g* for 10 min at 20°C. After centrifugation cells were recovered in five well-distinguished layers, each of which was aspirated with a peristaltic pump, and washed twice in Hepes buffered isotonic saline and twice in the buffer used in the insulin binding experiments. Using this procedure, six samples of RBCs from each subject were prepared: one from whole RBCs and five from fractions recovered after centrifugation on Percoll.

RBC characterization. RBCs were characterized either by staining films of samples with new methylene blue for 15 min at 37°C in order to visualize reticulocytes or by measuring pyruvate kinase activity according to the method described by Beutler et al.[19] The coefficient of interassay variation was 4.2% and that of intra-assay was 3.0%.

Preparation of discontinuous gradients. The following reagents were used: (1) Hepes buffered stock solution (HBS): 2.66 M NaCl, 0.09 M KCl, 0.2 M Hepes (pH 7.4); (2) A solution: BSA Hepes buffered solution (pH 7.4), π 265 mosm/kg H_2O at 3.5% BSA final concentration. This solution is obtained by adding 19 vol BSA in water (pH 7.4) to 1 vol HBS; (3) B solution: BSA Percoll Hepes buffered solution (pH 7.2) π 330 mosm/kg H_2O at 3.5% BSA final concentration. This solution is obtained by adding 19 vol BSA in Percoll (Pharmacia Fine Chemicals, Sweden) to 1 vol HBS.

TABLE 1
Pyruvate kinase activity (PK), reticulocyte content, and insulin binding (B/T%) to RBCs of different mean age obtained from six normal men

	PK (IU/g Hb)	Reticulocytes (% of RBCs)	Insulin binding (2 × 10^9 cells/ml)
Whole RBCs	13.95 ± 0.65	0.5 ± 0.1	2.99 ± 0.26
Fraction 1	29.35 ± 1.36	65.0 ± 1.6	9.54 ± 0.83
Fraction 2	20.98 ± 2.03	20.3 ± 1.0	5.75 ± 0.71
Fraction 3	15.75 ± 0.80	4.1 ± 0.9	4.58 ± 0.34
Fraction 4	13.35 ± 0.66	Undetectable	3.04 ± 0.27
Fraction 5	9.75 ± 0.70	Undetectable	2.65 ± 0.31

Mean ± SE. Whole RBCs and fractions 1–5 were obtained as described in MATERIALS AND METHODS.

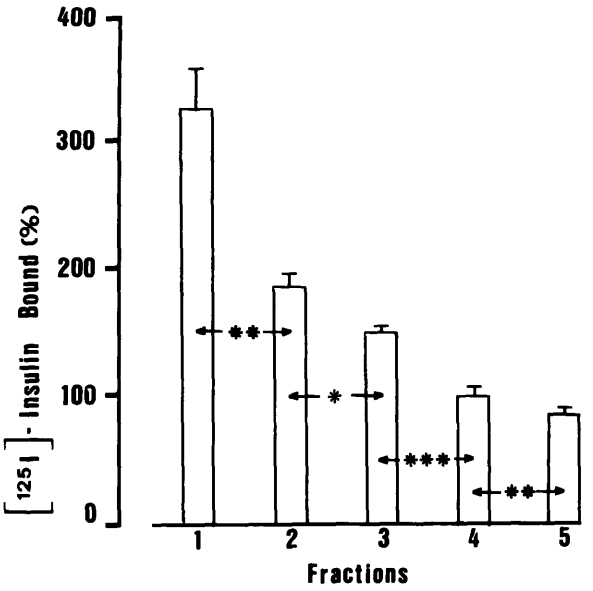

FIGURE 1. Effect of RBC age on insulin binding. RBCs from six normal men were isolated from blood (whole RBCs) and separated into five populations of different mean age; fraction 1 contained the youngest cells, whereas fraction 5 contained the oldest cells (see Table 1). 2 × 10^9 RBCs/ml were incubated with labeled insulin (0.2 ng/ml) in the presence or absence of porcine insulin (20 μg/ml); ~15% of total binding was nonspecific and was subtracted. Data are given as percent of insulin binding to whole RBCs from the same subject and were analyzed according to Student's *t* test for paired data. ***P < 0.001; **P < 0.01; *P < 0.02.

A and B solutions were appropriately mixed to prepare four solutions at final Percoll concentration of 60%, 63%, 66%, and 69%, respectively, (density 1.075–1.100, pH 7.3, π 310–320 mosm/kg H_2O). Discontinuous four-step gradient was prepared by superimposing 6 ml of each Percoll concentration.

Insulin binding studies. The binding of ^{125}I-insulin (260–300 μCi/μg; CEA-IRE, Sorin, Italy) was performed in Hepes-Tris buffer (pH 7.8) as previously reported in detail.[1] Experiments were carried out by incubating 2 × 10^9 cells/ml for 3.5 h at 15°C. RBCs were counted using a Coulter Counter Mod S Plus.

Statistical analysis. Regression analysis was used to evaluate the relationship between insulin binding and the number of RBCs, reticulocytes, or pyruvate kinase activity.

RESULTS

RBCs from six normal male subjects were isolated by means of elution on cellulose column (whole RBCs); then, a part was centrifuged on a discontinuous gradient of buffered Percoll (see MATERIALS and METHODS). After centrifugation, RBCs were recovered and divided into five well-distinguished fractions. Using this procedure it was thus possible to prepare six samples of RBCs for each subject; one from whole RBCs and five from the fractions recovered after centrifugation.

Whole RBCs and fraction 4 had similar pyruvate kinase values (marker of RBC age); furthermore, pyruvate kinase activity decreased progressively and significantly (P < 0.01–0.001) from fraction 1 to fraction 5 (Table 1). This would suggest that whole RBCs and fraction 4 had a similar mean age, and that fraction 1 contained the youngest and fraction 5 the oldest cells. It is noteworthy that the reticulocyte

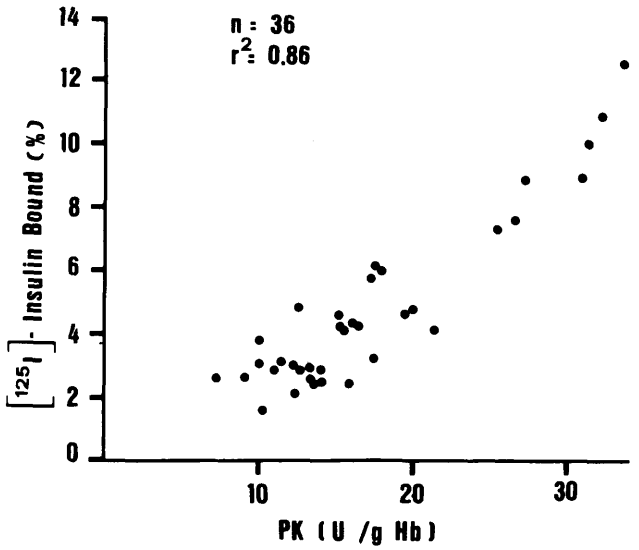

FIGURE 2. Relationship between insulin binding and pyruvate kinase activity. RBCs from normal men were isolated from blood (whole RBCs) and a part separated into five populations of different mean age. Insulin binding and pyruvate kinase activity were measured on 2 × 10^9 RBCs/ml from whole RBCs and each population. Measuring pyruvate kinase as U/2 × 10^9 RBCs/ml (r^2 = 0.86) or measuring both insulin binding and pyruvate kinase activity as a percentage of values obtained on whole RBCs from the same man (r^2 = 0.85) did not affect the data obtained.

number decreased progressively from fraction 1 to fractions 4 and 5, the latter two failing to show the presence of reticulocytes, and that whole RBCs had more reticulocytes than fraction 4 (Table 1). Insulin binding experiments were carried out on 2 × 10^9 cells/ml from each sample.

The time-course association at 15°C between a tracer amount of ^{125}I-insulin (0.2 ng/ml) and RBCs did not differ from sample to sample (data not shown).

Insulin binding to 2 × 10^9 cells/ml decreased progressively from fraction 1 to fraction 5 (Figure 1), thus demonstrating that the younger cells bound more insulin than the older ones. Furthermore, fraction 4 and whole RBCs showed similar insulin binding (Table 1). Data from competition-inhibition studies were analyzed according to Scatchard[20] and showed parallel plots, suggesting that insulin binding variation was due mainly to changes in receptor concentration rather than receptor affinity (data not shown).

Studies on the relationship between insulin binding and the number of RBCs, reticulocytes, or pyruvate kinase activity showed no correlation with the number of RBCs, a fairly good correlation with the percentage of reticulocytes (r^2 = 0.78), and a very good correlation with pyruvate kinase activity (r^2 = 0.86) (Figure 2). Thus, pyruvate kinase might be a good marker of insulin binding variation provoked by changes in mean RBC age.

Dilution experiments were then carried out in order to reveal the effect of the cell concentration chosen on insulin binding and pyruvate kinase values. All fractions were used at different cell dilutions (from 2 × 10^9/ml to 0.5 × 10^9/ml for fractions 1, 2, and 3; from 2 × 10^9/ml to 1 × 10^9/ml for fractions 4 and 5). In each fraction both insulin binding and pyruvate kinase activity decreased proportionately to cell concentration dilution, and the former varied proportionately to the latter (data not shown). It was thus concluded that results were not affected by the cell concentration chosen.

TABLE 2
Pyruvate kinase activity (PK), reticulocyte content, and insulin binding (B/T %) to whole RBCs obtained from male patients with hemolytic anemia (G6PD deficiency or drug-induced)

Patient	PK (IU/g Hb)	Reticulocytes (% of RBCs)	Insulin binding (2 × 10^9 cells/ml)
1	11.2	1.6	3.41
2	11.0	1.5	3.12
3	11.3	1.0	3.50
4	17.9	4.5	5.60
5	11.2	2.0	3.63
6	19.9	2.0	8.50
7	21.3	6.6	7.69
8	12.9	1.0	5.87
9	13.0	2.0	3.54
10	36.2	4.6	9.52
11	17.9	0.6	5.75
12	15.9	2.0	6.48
13	18.3	3.6	5.62

Investigations were then performed to determine the relationship between insulin binding and pyruvate kinase activity in samples of whole RBCs of different age with insulin binding experiments being carried out on whole RBCs obtained from normal male subjects and male patients with hemolytic anemia. As expected, some patients with hemolytic anemia showed high insulin binding; furthermore, the same patients showed high pyruvate kinase activity and slight reticulocytosis (Table 2). It is noteworthy that RBCs from patients 6, 7, 10, 11, and 13 showed similar insulin binding and pyruvate kinase values to those obtained using RBCs from fractions 1 and 2, whereas the percentage of reticulocytes differed considerably (Tables 1 and 2).

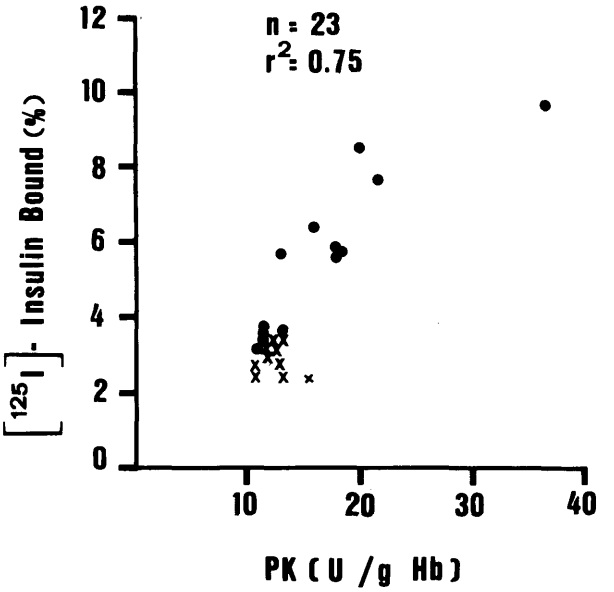

FIGURE 3. Relationship between insulin binding and pyruvate kinase activity in whole RBCs of different mean age. Whole RBCs were obtained from normal males (X) and patients with hemolytic anemia (•); insulin binding and pyruvate kinase activity were measured on 2 × 10^9 RBCs/ml.

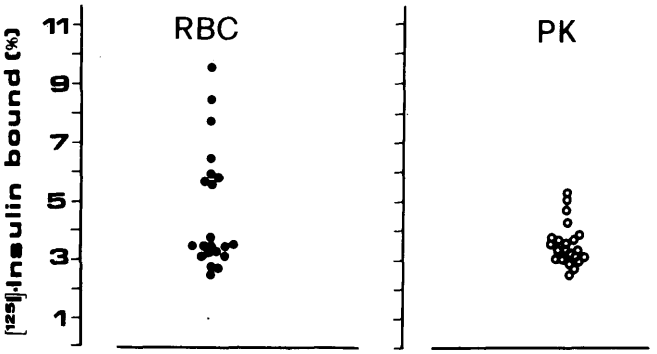

FIGURE 4. Insulin binding adjusted to the same amount of RBCs (2×10^9/ml) or the same pyruvate kinase activity (0.75 U/2 × 10^9 RBCs). U/RBCs may be obtained using the formula U/10^9 RBCs = U/g Hb × MCH × 10^{-3}.

A good relationship was found between insulin binding and pyruvate kinase activity ($r^2 = 0.75$) (Figure 3) and no change was observed when only data from patients with hemolytic anemia were considered.

In another series of experiments in which whole RBCs from patients 1, 2, 7, 10, and 11 were used at different cell dilutions (from 2×10^9/ml to 1×10^9/ml) insulin binding and pyruvate kinase activity were seen to vary proportionately to cell concentration. No change was observed in the data reported whether expressing pyruvate kinase as U/g Hb or U/number RBCs.

Insulin binding data from patients with hemolytic anemia and normal patients were adjusted to the same pyruvate kinase activity in order to evaluate the practical application of this pyruvate kinase assay. The range of insulin binding values was abnormally high when referring data to 2×10^9 RBCs/ml, but became acceptable when referring data to the same enzyme activity (Figure 4). The same phenomenon was found when adjusting the insulin binding data reported in Table 1 to the same enzyme activity (0.75 U/2 × 10^9 RBCs). In fact, the mean ± SD of the 36 values became 3.50 ± 0.66, in keeping with the range of values for normal subjects reported in the literature. Furthermore, RBCs from a patient with pernicious anemia were studied several times during the course of treatment with cyanocobalamin. Reticulocytes were 23.6% at the beginning of the study and 1.2% after 4 mo; the mean ± SD of 10 insulin binding values adjusted to 0.75 U pyruvate kinase/2×10^9 cells was 3.62 ± 0.41.

All data reported were combined in order to evaluate the relationship between pyruvate kinase activity or reticulocytes and insulin binding. Pyruvate kinase activity ($r^2 = 0.82$), but not reticulocytes ($r^2 = 0.56$), was found to be well correlated to insulin binding (Figure 5).

Pyruvate kinase assay was performed on samples of whole RBCs from 10 normally menstruating women at the seventh and twenty-fourth days of the menstrual cycle. While no differences were observed between the seventh (PK = 15.53 ± 0.88; mean ± SE) and the twenty-fourth days (PK = 16.63 ± 0.68), these values differed from those in whole RBCs from the 16 normal men (PK = 13.53 ± 0.43) ($P < 0.01$).

DISCUSSION

Many investigators consider RBCs the model of choice for studies on insulin receptors in man since only a small amount of blood (10–15 ml) is necessary to perform experiments, thus avoiding reluctance on the part of the patients as occurs when the monocyte model (requiring large amounts of blood) or the adipocyte model (requiring biopsy) is used. Nevertheless, this model cannot be used freely since reticulocytes bind more insulin than erythrocytes,[11–13] and discrepancies between monocyte and erythrocyte data have been reported.[6–11] Two main problems therefore need to be resolved: (1) to find a method by which to study RBCs, avoiding the problem of mean RBC age; and (2) to establish the influence of RBC age on data previously reported.

The first series of experiments was carried out on RBCs from normal subjects separated on a discontinuous gradient of buffered Percoll. After centrifugation, RBCs were recovered in five distinguished fractions characterized by different pyruvate kinase activity (i.e., five RBC populations of different mean age).

Insulin binding decreased progressively and significantly from the younger (fraction 1) to the older cells (fraction 5) (Figure 1) and changes in binding were due mainly to variations in receptor concentration. These data are in agreement with those of other authors using other techniques.[11,14,15]

Insulin binding was correlated to pyruvate kinase activity, thus indicating the possible usefulness of this method in the

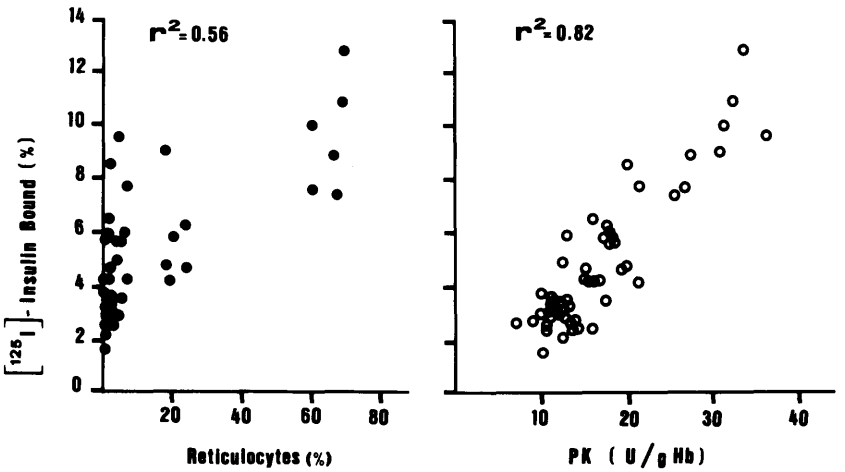

FIGURE 5. Relationship between reticulocyte number or pyruvate kinase activity and insulin binding. Data from Tables 1 and 2 and Figure 3.

evaluation of insulin binding to RBCs, i.e., to correlate insulin binding to pyruvate kinase activity instead of the number of RBCs. The problem of mean RBC age would thus become negligible. To support this hypothesis, samples of whole RBCs (pure RBC samples not separated in subpopulations) from normal male subjects, male patients with hemolytic anemia, and one male patient with pernicious anemia on cyanocobalamin treatment were studied.

RBCs from patients with hemolytic anemia or the patient with pernicious anemia showed higher insulin binding than those from normal subjects in agreement with reports by others.[11,12,14,15] The high dispersion of data commonly found when studying these RBC samples[11,12,14,15] disappeared when insulin binding was related to pyruvate kinase activity instead of the number of RBCs. It is worthwhile to point out that dilution experiments demonstrated that pyruvate kinase activity varied parallel to insulin binding and cell concentration changes, thus demonstrating the applicability of this method.

Results obtained on RBCs from patients with hemolytic anemia demonstrated that some of those patients (nos. 6, 7, 10, 11, and 13; Table 2) had the same high insulin binding and pyruvate kinase activity as those found in fractions 1 and 2 but a considerably lower number of reticulocytes. Furthermore, considering results reported in Tables 1 and 2 and Figure 3, it was clear that insulin binding is related to pyruvate kinase activity ($r^2 = 0.82$) but not to reticulocyte number ($r^2 = 0.56$).

From these results it may be extrapolated that RBCs lose insulin receptors physiologically and continuously with age; furthermore, it is possible to confirm a commonly made but as yet unproven assumption, i.e., that the insulin receptors on RBCs from patients with hemolytic anemia are indeed normal since the high insulin binding is due only to variations in mean RBC age.

In the present investigation pyruvate kinase activity was measured as U/g Hb, according to the method recommended by various authors.[21] This method was used since the subjects studied showed no alteration in the mean corpuscular hemoglobin (MCH); therefore, when measuring pyruvate kinase activity as U/2 × 10^9 RBCs, the data did not change. On the other hand, it should be stressed that enzyme activity should be measured as U/RBCs in disease states affecting MCH (e.g., thalassemias); furthermore, it would be helpful to bear in mind that acquired pyruvate kinase activity deficiency has been rarely observed in patients with preleukemia, acute leukemia, and refractory anemia.[22]

These data demonstrate that (1) the problem of RBC age may be overcome by adjusting insulin binding to the same pyruvate kinase activity, and (2) insulin binding variations provoked by changes in RBC age may be revealed by evaluating pyruvate kinase activity. Dichotomy between the results of monocytes and RBCs have been reported in several conditions;[6-11] to evaluate the influence of RBC age on these results a physiologic situation was studied in order to avoid the possibility of patient-to-patient differences.

Moore et al.[23] and our group[6,24] have demonstrated a variation in insulin binding to monocytes and RBCs induced by the menstrual cycle, with binding values being higher in the follicular than in the luteal phase. Comparison of data obtained in men and women, however, revealed the dichotomy; in fact, monocytes from normal men bound the same percentage of insulin as monocytes from women in the follicular phase, whereas RBCs from the same men bound more insulin than RBCs from the same women in the follicular phase.[6] The fact that RBCs from men bind more insulin than RBCs from women was confirmed also by Hendricks et al.[25]

To suggest that RBC data were affected by cell age it should be demonstrated that RBCs from men had higher pyruvate kinase activity than RBCs from women and that RBCs from women in the follicular phase had lower enzyme activity than RBCs from women in the luteal phase. On the contrary, enzyme activity was lower in men than in women ($P < 0.01$) and no difference was found between the follicular and the luteal phase. Consequently, it must be concluded that adjustment of data according to RBC age broadens the difference between men and women, and indicates that dichotomy between monocytes and RBCs is not provoked by an artifact. Thus, monocyte and RBC insulin receptors may behave differently.

Several hypotheses may be offered to explain the different behavior of monocyte and RBC insulin receptors. In our opinion, dichotomy may be due to the fact that RBC insulin receptors behave differently depending upon the age of the cells, in keeping with the current opinion that the RBCs lose and change several biologic functions with age.[18,21,26] It is tempting to suggest that parts of RBCs (possibly reticulocytes and early mature erythrocytes) show a similar behavior to monocytes. Confirmation of this hypothesis should be possible by separation of RBC subpopulations according to the method described in this article.

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