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Decreased deformability of erythrocytes in hyperglycaemic non-inbred ob/ob mice

K. G. Engström and I.-B. Täljedal

Department of Histology and Cell Biology, University of Umeå, Umeå, Sweden

Summary. The deformability of erythrocytes from non-inbred ob/ob mice and lean controls was analyzed by filtration through Nuclepore polycarbonate under constant pressure. At the age of 1-2 months there was no difference in erythrocyte filtrability between the two types of mice, whereas from 3 months the ob/ob mouse erythrocytes exhibited a markedly decreased deformability. The filtrability of erythrocytes was sensitive to osmotic pressure (NaCl or glucose). However, the difference between normal and ob/ob mouse erythrocytes was not due to acute osmotic effects of the hyperglycaemia in the ob/ob mice. When filtration was performed in the same glucose concentration as that recorded in the blood of the erythrocyte-donor animal, the difference in filtrability between adult normal and ob/ob mice remained large and significant ($p < 0.01$). Moreover, the most pronounced hyperglycaemia occurred in young ob/ob mice with normal erythrocyte filtrability. It is suggested that non-inbred ob/ob mice are a useful model for studying the damaging influence of diabetes on erythrocyte deformability.

Key words: Diabetic mice, hyperglycaemia, hyperinsulinaemia, ob/ob mice, erythrocyte deformability.

The deformability of erythrocytes is essential to normal micro-circulation. Deformability decreases during erythrocyte ageing, a phenomenon that may play a role in the elimination of old erythrocytes from the blood stream. A decrease in erythrocyte deformability has also been reported to occur in patients with diabetes mellitus [1-5]. This pathological change may contribute to the increase in whole blood viscosity in diabetes [1], adding to the effects of elevated plasma viscosity and the enhanced tendency of diabetic erythrocytes to aggregate. Rheological factors may contribute to diabetic microangiopathy [6].

Several reports have indicated that intensive diabetic care, with normalization of the metabolic state, can reverse the impaired erythrocyte deformability [1, 3, 5]. However, the mechanisms underlying the decreased deformability of diabetic erythrocytes and the therapy-induced improvement are unclear. The elevated blood glucose concentration and hyperosmolarity have been implicated [1], but there is also evidence for direct insulin effects on the erythrocytes. Insulin added to erythrocytes in vitro has been reported to increase the erythrocyte deformability [4, 5, 7] and their membrane fluidity [7, 8]. The deformability also improved in vivo in response to insulin infusion under conditions of a hyperglycaemic glucose clamp [4].

In the present study we have used a filtration method to investigate the erythrocyte deformability in non-inbred ob/ob mice. This animal model exhibits glucose intolerance early in life [9]. Because the pancreas is capable of responding to the glycaemic challenge by developing hyperplastic islets with actively secreting B cells, adult non-inbred ob/ob mice are characterized by both a mild hyperglycaemia and a 10- to 100-fold increase in the circulating concentration of insulin [10, 11]. Attention is here paid to the development of any erythrocyte deformability difference between non-inbred ob/ob mice and lean control mice of the same ages. The effect of glucose in vitro on erythrocyte filtrability, as distinct from effects of the long-standing diabetes, is also examined.

Materials and methods

Animals and preparation of erythrocytes

Female non-inbred ob/ob mice and lean controls were taken from a local colony. The pancreatic islets of these Umeå ob/ob mice exhibit adequate insulin secretory responses to glucose and other secretagogues and have been extensively used for studies of insulin secretory mechanisms [12]. The non-inbred Umeå ob/ob mice should not be confused with strains of some other genetic backgrounds in which the ob gene may cause grave degenerative dysfunctions of the B cells.

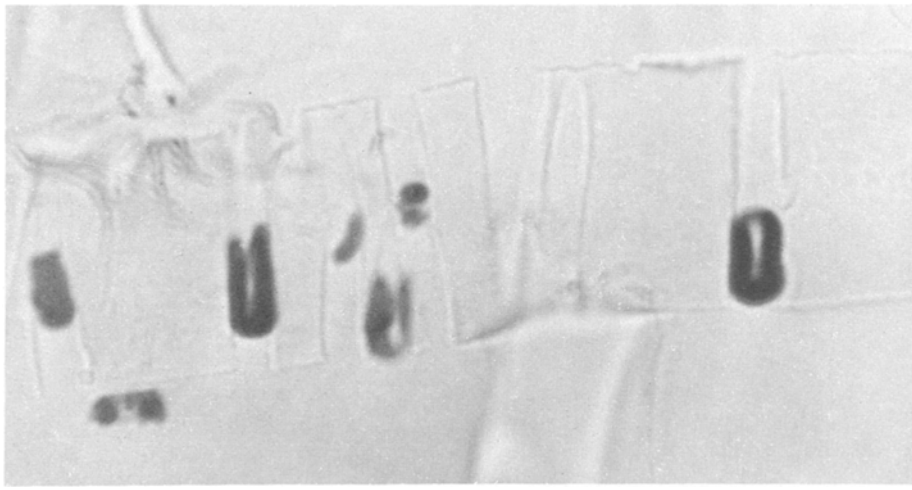

Fig. 1. Light microscopic photograph of a Nuclepore polycarbonate filter with ob/ob mouse erythrocytes in the pores. Note profiles indicative of erythrocyte folding. 2,000×

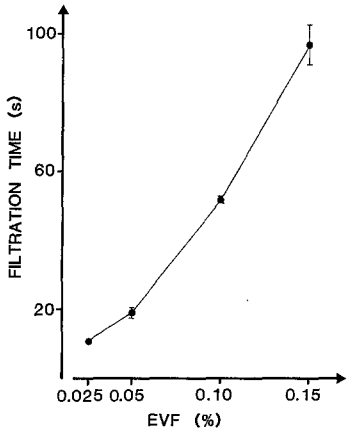

Fig. 2. Filtration time as a function of the erythrocyte volume fraction (EVF). Mean values ± SEM for 3-4 filtrations of 1-ml erythrocyte suspensions from a normal mouse

Mice with free access to food and water were anaesthetized by intraperitoneal pentobarbital. Blood was obtained by puncturing the right cardiac ventricle with a heparin-treated syringe. After centrifugation at 1,500 *g* for 15 min the erythrocyte volume fraction (EVF) was determined. Plasma, buffy coat, and the uppermost layer of erythrocytes were carefully removed by a pipette. The wall of the tube above the erythrocyte column was then washed with saline. Erythrocytes were immediately resuspended in Hepes-buffered medium (see below). To avoid unnecessary trauma to the erythrocytes, they were not washed in saline but instead heavily diluted to yield a final EVF of 0.05%.

Glucose determinations

A portion of uncentrifuged blood was used for glucose determinations. Rapid measurements, allowing adjustment of the glucose concentration in imminent erythrocyte filtration experiments, were performed with the aid of strips and a reflectometer (Reflolux type 716235, Boehringer-Mannheim, Mannheim, FRG). In five cases the validity of the rapid reflectometer measurements was checked by subsequent analysis of frozen and stored plasma samples. These samples contained glucose, 9-26 mmol/l, as assayed by a luciferin-luciferase method [13]. The individual data correlated with those obtained by the reflectometer with $r = 0.99$.

Measurements of erythrocyte filtrability

The basal medium in filtration experiments was a salt-balanced medium of the same composition as Krebs-Ringer bicarbonate buffer, except that bicarbonate was replaced by 20 mmol/l 2-(N-hydroxyethylpiperazine-N′-yl)ethanesulphonic acid (Hepes) and that bovine serum albumin was added at a concentration of 2 g/l. The medium was equilibrated with ambient air and pH adjusted to 7.40. In some experiments glucose was added to the medium to yield the same concentration as the blood glucose concentration recorded for the erythrocyte-donor mouse. In such cases, erythrocytes from the same mouse were also suspended and analyzed for deformability in glucose-free medium.

Deformability was determined by recording the time required for passing 1 ml of the erythrocyte suspension through a Nuclepore polycarbonate filter with a nominal pore diameter of 3 μm. Filtration was performed under the constant pressure of 120 mm water (1.2 kPa). The erythrocyte suspension was sucked into a horizontal pipette by means of a loading syringe fitted to a three-way valve. Via the same valve the pipette was also connected with a pressure-delivering system consisting of a large air reservoir, a pump syringe and a manometer. The pipette was encased in an outer glass tube filled with water. Exact horizontal positioning was achieved with the aid of an air bubble in the outer tube. The distal end of the pipette was connected with a Nuclepore filter holder, the outlet from which was channelled back to a glass tube kept in horizontal position parallel and level with the filtering pipette. A measurement was started by opening the valve to the pressure-delivering system. The time required for the column of erythrocyte suspension to move a distance corresponding to a fixed volume (usually 1.0 ml) in the graded filtering pipette was determined with a stopwatch. Each erythrocyte suspension was analyzed in 3-4 repeated experiments. A fresh clean filter was used in each measurement. Before filtering the erythrocyte suspension in some experiments, a blank value was obtained by recording the time required for 1.0 ml of medium alone to pass the filter. This blank value showed a systematic variation between two lots of filters supplied by the manufacturer during the course of the study. It was 6.06 ± 0.07 s (mean ± SEM for 63 observations) unless otherwise stated and 7.41 ± 0.05 s (mean ± SEM for 99 observations) for the filters used in Figure 7. This increase in blank value corresponded to a markedly decreased velocity in the filtering of erythrocytes. The experiments in Figure 7 were therefore based on filtrations of 0.5 ml suspensions.

In five control experiments, excess pentobarbital (24 μg/ml) or heparin (0.5 mg/ml) was added to normal mouse erythrocyte suspensions which were then immediately filtered. These high concentrations of drugs did not alter the filtrability in comparison with erythrocytes that were only exposed to the minimum concentrations of pentobarbital and heparin inherent in the standard sampling and preparation procedure (not shown).

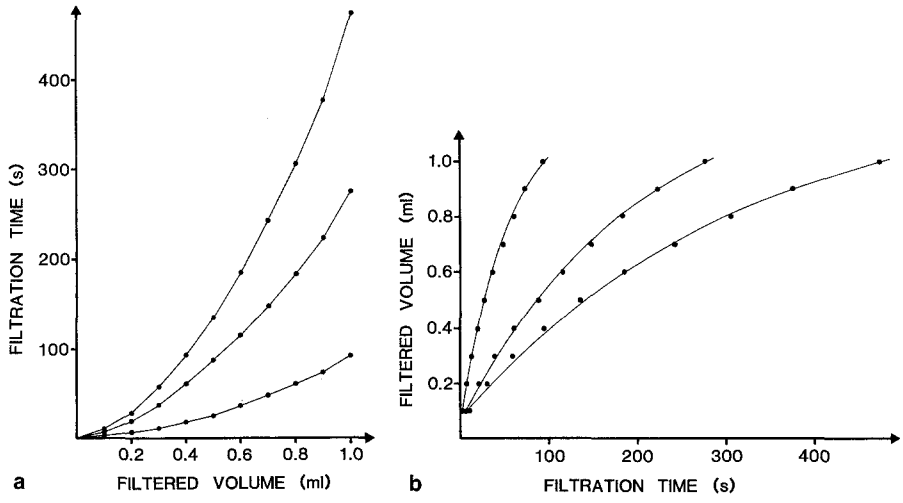

Fig. 3 a and b. Relationships between filtration time and volume filtered. Three individual experiments with ob/ob mouse erythrocytes (EVF = 0.05%) are shown. **a** The accumulated time for filtering successive 0.1 ml portions are given as recorded in the experiments; the points are connected by straight lines. **b** The same data have been fitted to theoretically derived exponential functions of time as the independent variable; the curve-fitting was done by an iterative method of least squares

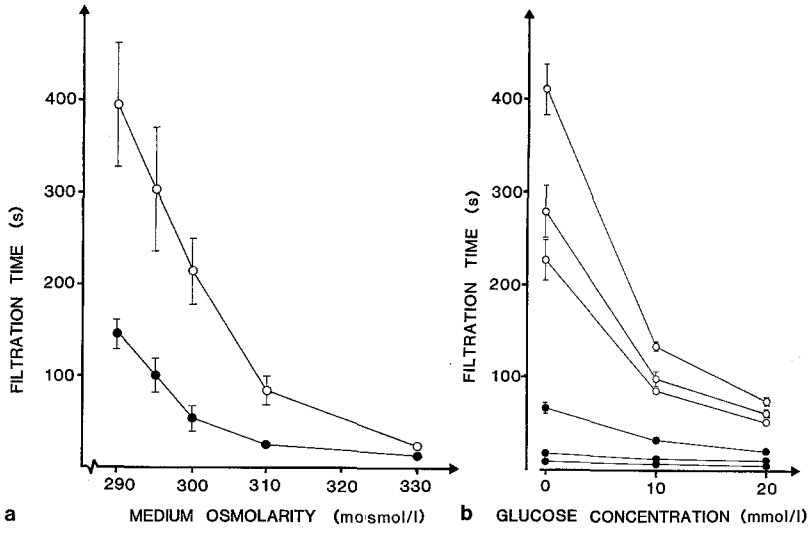

Fig. 4. **a** The influence of medium osmolarity on erythrocyte filtrability. NaCl was removed from or added to the basal medium as indicated. The time required to filter 1-ml suspensions (EVF = 0.05%) of adult normal (solid circles) or ob/ob mouse (open circles) erythrocytes is given as mean values ± SEM for 3–4 filtrations. Two mice of either type were studied in parallel. **b** The influence of glucose on erythrocyte filtrability. Glucose at the concentrations indicated was added to the basal medium without compensating for the increase in osmotic pressure. The time required to filter 1-ml suspensions (EVF = 0.05%) of adult normal (solid circles) or ob/ob mouse (open circles) erythrocytes is given for 3 different mice of either type. Mean values ± SEM for 3–4 filtrations

Microscopy

In some experiments a more concentrated erythrocyte suspension (EVF = 1%) was run through the filtering apparatus to produce a great number of erythrocyte interactions with the filter pores. The filter was then removed, fixed in 2.5% glutaraldehyde, dehydrated in ethanol, and embedded in Epon 812. Semi-thin sections, perpendicular to the filter surface, were cut on an LKB Ultratome, stained with Toluidine Blue, and analyzed in a light microscope.

Statistical analysis

Results are given as mean values ± SEM. The significance of group differences was evaluated by the two-tailed Student's t-test. Because the frequency distribution of filtration times in a population of mice exhibited some skewness (not shown), the non-parametric Wilcoxon rank sum test was also employed.

Results

Basic factors influencing the passage of erythrocytes through polycarbonate filters

Figure 1 shows the profiles of erythrocytes penetrating a Nuclepore polycarbonate filter. When located in the filter pores, the erythrocytes appeared folded, forming an elongated plug of the same diameter as the pores.

The passage of erythrocytes through the filter caused a retardation of flow which expressed itself in two types of interaction phenomena. As shown in Figure 2, the time required to filter 1 ml erythrocyte suspension was not linearly related to the EVF, but increased as some power function of the initial concentration of erythrocytes in the suspension. Similarly, the rate of flow decreased progressively during filtration (Fig. 3 a). The empirical data fitted closely to theoretical exponential curves derived on the assumption that the flow rate at any time point was related to the suspension volume that had already passed through the filter (Fig. 3 a).

To test whether the rate of filtration through 3 μm pores would sensitively reflect subtle differences in erythrocyte geometry, the influence of osmotic pressure was studied. Figure 4 a shows that removal of NaCl from the basal medium drastically decreased the filtration rate for erythrocytes from both ob/ob mice and normal controls. Conversely, addition of NaCl enhanced the filtration rate. A similar effect was obtained by adding glucose to the medium (Fig. 4 b). When erythrocytes from ob/ob mice were placed in glucose-free basal medium, their filtrability rapidly decreased and

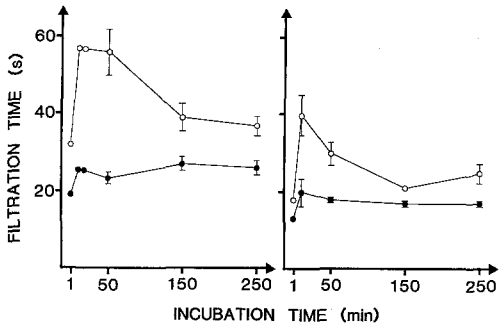

Fig. 5. Time course of the effect of glucose on erythrocyte filtrability. Erythrocyte suspensions (EVF = 0.05%) were prepared from two adult ob/ob mice (left and right panel) and incubated at room temperature (22 °C) with 0 mmol/l (solid circles) or 20 mmol/l (open circles) glucose. After various periods of incubation, the time required to filter a 1 ml portion was determined in 19 (left panel) or 15 (right panel) filtrations with each suspension. The results of these filtrations were pooled in 4 groups with the approximate average incubation times indicated on the abscissa. The points represent mean values of 1-7 filtrations. SEM is indicated by bars for mean values comprising more than 2 filtrations

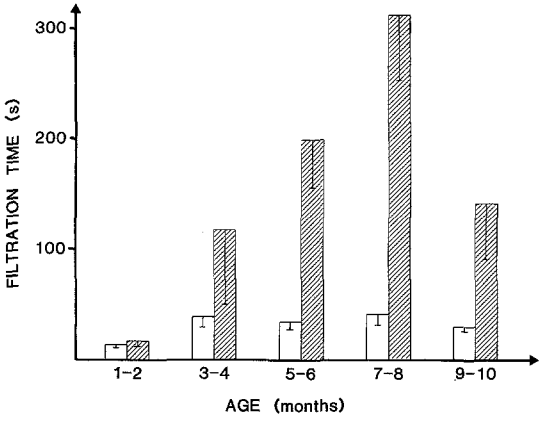

Fig. 6. The influence of animal age on erythrocyte filtrability in normal (open bars) and ob/ob (hatched bars) mice. The time required to filter 1-ml suspensions (EVF = 0.05%) in the absence of added glucose is given as mean values and SEM for each group of animals. The number of mice in each group was 5 (1-2 months), 2-3 (3-4 months), 8 (5-6 months), 9 (7-8 months), and 2 (9-10 months). At 7-8 months, the difference between normal and ob/ob mice was significant with $p < 0.01$ according to both the Student's t-test (with correction for unequal variance) and the non-parametric Wilcoxon rank sum test

Table 1. Body weight, erythrocyte volume fraction (EVF), and blood glucose in young (1-4 months) and old (5-10 months) ob/ob mice and lean controls

Age	Mice	Body wt. (g)	EVF (%)	Blood glucose (mmol/l)
Young	ob/ob	37 ± 2.9 (13)	45.4 ± 1.2 (14)	20.1 ± 2.7 (7)
Young	lean	20 ± 0.7 (13)	45.7 ± 0.7 (14)	10.4 ± 0.5 (7)
Old	ob/ob	61 ± 1.2 (26)	44.7 ± 1.2 (22)	14.5 ± 2.3 (4)
Old	lean	22 ± 0.6 (8)	45.2 ± 0.8 (22)	10.5 ± 0.5 (4)

Blood glucose was measured by Reflolux reflectometer. Results are given as mean values ± SEM for the numbers of animals stated in parentheses. Blood glucose in young ob/ob mice was significantly different from than in young lean mice ($p < 0.02$, with correction for unequal variance)

then only very slowly increased during an observation period of several hours (Fig. 5). Glucose, 20 mmol/l, inhibited the initial decrease in filtrability (Fig. 5).

When viewed under the light microscope, erythrocytes that had passed through the Nuclepore filter were seen to maintain their binconcave discoid shape and in general showed little sign of morphological deterioration (not shown).

Comparison of ob/ob and normal mice

There was no significant difference in EVF between blood from non-inbred ob/ob mice and normal controls (Table 1).

When erythrocytes from adult mice were suspended in glucose-free basal medium, the filtrability of ob/ob mouse erythrocytes was considerably lower than that of normal mouse erythrocytes. This difference could be compensated for by adding NaCl or glucose to the medium, and corresponded to the effect of increasing the osmotic pressure by about 10-20 mosmol/l (Fig. 4). It was not observed in young animals but developed progressively with age after 2-3 months (Fig. 6).

The filtrability decrease in ob/ob mice could theoretically reflect swelling of the erythrocytes on their transfer from hyperglycaemic blood to basal suspension medium. Experiments were therefore performed in which glucose was added to the medium to yield the same concentration as that in the blood of the individual erythrocyte donor animal. As shown in Table 1, 1 to 4-month-old ob/ob mice were clearly hyperglycaemic, whereas at the age of 5-10 months there was but minimum difference in blood sugar between the ob/ob and control mice. In the presence of individually adjusted glucose concentrations the difference in erythrocyte filtrability between old ob/ob and normal mice was not eliminated, but remained striking (Fig. 7). In the young animals the rather high glucose concentration added to the ob/ob mouse erythrocytes tended to make these erythrocytes more filtrable than those of the similarly aged control mice.

Discussion

As the pressure was kept constant throughout each experiment, the progressive decline in flow rate reflects the interaction of erythrocytes with the polycarbonate filter. The pore size of the filter was chosen so as to provide a relative resistance to the erythrocytes. Therefore, one must assume that the number of occupied pores gradually increased as a function of the number of erythrocytes arrived at the filter, i.e. as a function of the suspension volume that had filtered through at any time point. In the simplest case the situation can be described by a non-homogeneous first-order differential equation:

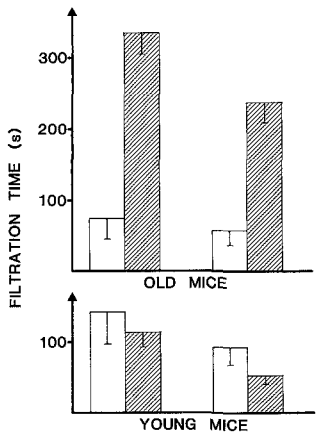

Fig. 7. Filtrability of normal (open bars) and ob/ob (hatched bars) mouse erythrocyte suspensions (0.5 ml, EVF = 0.05%) in the absence (left pairs of bars) and presence (right pairs of bars) of added glucose. For each mouse, one suspension of erythrocytes was prepared without glucose in the medium, while another was prepared by maintaining the same glucose concentration as that recorded in blood. The top panel shows results for old mice (5–8 months) and the bottom panel results for young mice (2–3 months). Mean values ± SEM for 3 (old mice) or 6 (young mice) animals are given. In the old animals, the difference between normal and ob/ob mice was significant ($p < 0.01$) both in the absence and presence of glucose

$$dV/dt = A - B \times V$$

and, upon integration,

$$V = C \times e^{-Bt} + A/B$$

where V is the filtered suspension volume, t the time, and A, B, and C are constants characterizing the properties of filter and erythrocytes. When the integral was fitted to data by an iterative method of least squares, excellent agreement was obtained in support of the model tested. In view of this analysis, we conclude that the time required to filter 1 ml is a simple, yet meaningful and experimentally stringent measure of the deformability of the erythrocytes.

The present results indicate that erythrocytes of non-inbred ob/ob mice are characterized by an impaired deformability. This animal may therefore be a valuable model for studying the decrease in erythrocyte deformability reported to occur in human diabetes mellitus [1–5]. Although the full penetrance of the homozygous ob gene load, the great proliferative capacity of the B cells, and the extreme hyperinsulinism and obesity make the picture of diabetes different from that in most human patients, the insulin resistance and protracted hyperglycaemia are basic factors of similarity with Type 2 (non-insulin-dependent) diabetes [10, 11]. It is also worthy of note that ob/ob mice have been reported to display certain glomerular lesions [14, 15] as well as arteriolar abnormalities [16].

The difference between ob/ob mice and normal mice increased with age and was considerable among the 7- to 8-month-old animals. Although glucose in the medium affected the filtrability of the erythrocytes, the diminished deformability in ob/ob mice did not merely reflect acute osmotic effects due to differences in blood sugar. The greatest hyperglycaemia occurred in the young ob/ob mice, whose erythrocytes showed the least difference from normal mouse erythrocytes even when filtered in a glucose-free medium. Moreover, when filtration was performed in the same glucose concentration as that existing in the blood of the erythrocyte donor animal, the marked difference between adult ob/ob and normal mice was only partially reduced and remained large and significant.

Insulin in vivo or in vitro has been found to reverse the deformability of human diabetic erythrocytes [4, 5]. In normal human erythrocytes, physiological concentrations of insulin caused a reduction of membrane micro-viscosity and filtration time, whereas supraphysiological concentrations reversed the effect of the lower concentrations of insulin [8]. The decreased erythrocyte deformability in non-inbred ob/ob mice cannot be due to a lack of insulin, as these animals have excessively elevated circulating levels of the hormone [10, 11]. It is not known, however, whether insulin exerts any physiological influence on mouse erythrocytes and whether there is any difference in insulin sensitivity between the erythrocytes of normal and ob/ob mice. At the moment it seems plausible that either the long-standing moderate hyperglycaemia or the excessive hyperinsulinaemia had induced material and morphological alterations in the ob/ob mouse erythrocytes so as to make them abnormally rigid.

Deformability in terms of filtrability could reflect several different properties of the erythrocytes, such as size, shape and material characteristics. The present technique does not distinguish between these properties. It is worthy of note, however, that light microscopy suggested the erythrocytes to be folded in the filter pores. Folding seems to be the initial event in the physiological deformation of erythrocytes before their entrance into a narrow capillary [17]. Increased glycosylation of the diabetic erythrocytes is readily envisaged as one of several material changes that could conceivable affect both the visco-elastic properties and the shape of the erythrocytes and hence their resistance to folding. However, there seems to be no studies on the geometry of diabetic erythrocytes, except for a few contradictory reports suggesting or questioning an increase in the mean corpuscular volume [18–21]. An interesting observation is that diabetic erythrocytes are characterized by a lowered surface electric charge [22]. This alteration may indicate a decrease in membrane area, and hence in erythrocyte shape. Such a phenomenon is encountered in old normal erythrocytes as the result of membrane loss by micro-vesiculation [23]. In an earlier paper [24] we have mathematically demonstrated that the rather small differences in geometry between young and old (with regard to the circulatory life-span) human erythrocytes can give rise to substantial effects on the resistance to initial deformation. The present results there-

fore encourage empirical measurements of the erythrocyte geometry in non-inbred ob/ob mice to be performed, both to further test the static-bending model of the role of shape for erythrocyte deformability [24] and to elucidate the mechanisms underlying the decreased erythrocyte deformability in diabetes mellitus.

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Dr. K. Gunnar Engström
Department of Histology and Cell Biology
University of Umeå
S-901 87 Umeå
Sweden