A Glucose-Containing Fraction Extracted from the Young Erythrocyte Membrane is Capable of Transferring Glucose to Hemoglobin In Vitro

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SUMMARY

Red blood cell (RBC) membranes are rich in a glycoconjugate that is extractable in chloroform/methanol solutions (2/1, v/v) and contains several hexoses, such as glucose. Old and young RBC are separated and their respective glycoconjugates are prepared. HbA_0 is purified by column chromatography and incubated with solutions of this conjugate. After 24-h incubation, Hb is dialyzed and the amount of glycosylated Hb is measured by a method of column chromatography adapted from Trivelli. A very significant amount of HBA_{Ic} is formed when young RBC extracts are incubated: 3.6% of total Hb becomes HBA_{Ic} with the extracts, versus 3.2% with free glucose, and only 2.5% for controls. No increase in HbA_{Ic} is obtained when extracts of old RBC are incubated. Another difference between the action of the glycoconjugate and free glucose is that the former induces the increase of only the HBA_{Ic} fraction, whereas glucose induces the increase of all the minor Hb fractions. The evaluation of glucose contained in the conjugate before and after the glycosylation reaction demonstrates that it is due to an exchange of glucose units from the conjugate to Hb. The reaction is stereospecifically inhibited by p-nitrophenyl-β-D-glucoside. The nature of the formed HbA_{Ic} is demonstrated by isoelectric focusing. A slight increase of HbA_{Ic} observed in the incubated controls may be due to an internal migration of some residues of glucose primitively bound to lysyl residues in an unstable form and also to some degree of denaturation during the incubation. DIABETES *31*:371–374, April 1982.

Glycosylated hemoglobins (Hb) arise by post-translational nonenzymatic modifications of the globin chains. Many recent reports give evidence for the in vitro glycosylation of Hb by direct incubation with glucose.[1–5] The same process seems to occur in vivo: the patients suffering from diabetes mellitus exhibit an increase of glycosylated Hb. Nevertheless, the demonstration of a direct glucose fixation does not rule out the possibility for other mechanisms of Hb glycosylation. The finding by two of us[6–8] that the amount of carbohydrates in the red blood cell (RBC) membrane decreases with the aging of erythrocytes and the fact that some of these glycoconjugates contain glucose in addition to other hexoses (galactose, mannose), were brought together with the well known increase of the glycosylated Hb in the aging RBC.[9] We hypothesized that one of the membrane conjugates was able to transfer a glucose unit to the neighboring Hb. To check this possibility, we incubated purified nonglycosylated Hb (HbA_0) with some of the glycoconjugates extracted from the membranes of young and old RBC. We actually found that such an in vitro transfer is possible.

MATERIAL AND METHODS

The RBC obtained from normal blood donors were separated by centrifugation into young and old ones.[7] The ghosts were extracted by a phosphate buffer according to Hamaguchi,[10] giving an RBC membrane extract. This extract was, in turn, extracted by 2 vol of a chloroform/methanol solution (2/1, v/v). All the extracts were evaporated to dryness under vacuum and dissolved in a suitable amount of 0.15 M phosphate buffer of Schneck (developer no. 4).[11] The amount of glucose contained in the conjugate was measured according to Schachter.[12]

HbA_0 was prepared by two successive ion-exchange chromatographies on a large column of Biorex 70 resin, according to Allen.[13]

After a preliminary kinetic experiment performed in quadruplicate from 0 to 48 h, all the incubations were performed in quadruplicate in sterile capped test tubes at 37° for 24 h without stirring. Each tube received 75 nmol of HbA_0 in 0.1 ml of 0.15 M phosphate buffer. The control tubes received, in addition, 0.1 ml of the same buffer. The "glucose control" tubes received 0.1 ml of a 27.5 mM solution of glucose in the same buffer. The sample tubes received 0.1 ml of com-

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plete membrane extracts from old or young RBC, or chloroform/methanol extracts from these membranes from old or young RBC, or a membrane extract previously heated at 100°C for 2 min. In another experiment, HbA_0 was heated at 65°C for 1 h prior to the incubation.

In some cases, simple hemolyzates were directly used to demonstrate that the preparation of HbA_0 made according to Allen in the presence of cyanide, did not introduce artifactual reactions. Blood donor RBC, after 3 washes in saline, were hemolyzed by adding 1 vol of distilled water and 0.4 vol of toluene, centrifuged, and the supernatant was dialyzed against 100 volumes of 0.15 M phosphate buffer, pH 7.4 for 18 h at + 4°C. In this type of experiment, each incubation test tube received 75 nmol of total Hb (without previous separation of the glycosylated Hb). Control tubes and glucose-control tubes were prepared as above and the sample tubes received 0.1 ml of complete membrane extract from young RBC.

In all cases, after incubation, the solutions were transferred together with 0.1 ml of saline rinse, into small dialysis bags (Union Carbide 8 × 100 FT) and dialyzed against the chromatographic buffer developer 6[11] for 18 h at 4°C to eliminate any glucose bound to Hb in an unstable form. The dialyzed Hb was analyzed by quantitative chromatography on a small column of Biorex 70 resin thermostated at 24°C, according to modification[14] of the technique of Trivelli.[15] This method permits the separation of 3 fractions containing HbA_{1a+b}, HbA_{1c}, and $HbA_0 + A_2$, respectively. The results are expressed in percentages of total Hb.

The nature of the Hb derivatives obtained after incubation was checked by isoelectric focussing with a FBE 3000 Pharmacia apparatus, in a pH range of 6.5–9.0 (Pharmalyte, Pharmacia, Piscataway, New Jersey).

RESULTS AND DISCUSSION

When HbA_0 is incubated with extracts prepared from young RBC membranes for periods ranging from 0 to 48 h, the amount of HbA_{1c} formed increases during the first 24 h and, then, remains constant (Figure 1). Surprisingly, the amounts of HbA_{1c} in the controls also increase during the incubation despite the fact that HbA_0 had been purified twice from any minor Hb prior to incubation. This unexpected increase may depend on a slight denaturation process of HbA_0 occurring during the incubation period and/or on the internal migration of glucose units (see below). When the increase of controls is deducted from the increase of the samples incubated with young RBC membrane extracts the difference is still largely significant. In contrast, there is no increase of HbA_{1a+b} over that of the corresponding control.

Table 1 shows the results of experiments performed in quadruplicate, in which HbA_0 was incubated for 24 h with various preparations of membrane glycoconjugates or with free glucose. The controls are HbA_0 without incubation, or after 24-h incubation without addition of glycoconjugate. Again, an unexpected increase of HbA_{1c} is found when HbA_0 is incubated alone. We find, as demonstrated by other authors[2] that when HbA_0 is incubated with free glucose, the amounts of HbA_{1a+b} and HbA_{1c} are significantly increased. On the other hand, in the presence of extracts prepared from membranes of young RBC without any free glucose added, the percentage of formed HbA_{1c} is very significantly increased whereas HbA_{1a+b} is not. The extracts prepared from old RBC have no effect. If the amount of glucose present in the preparation is used as a reference, the necessary amount of glycoconjugate is 100 times less than that of free glucose required for forming the same amount of HbA_{1c}.

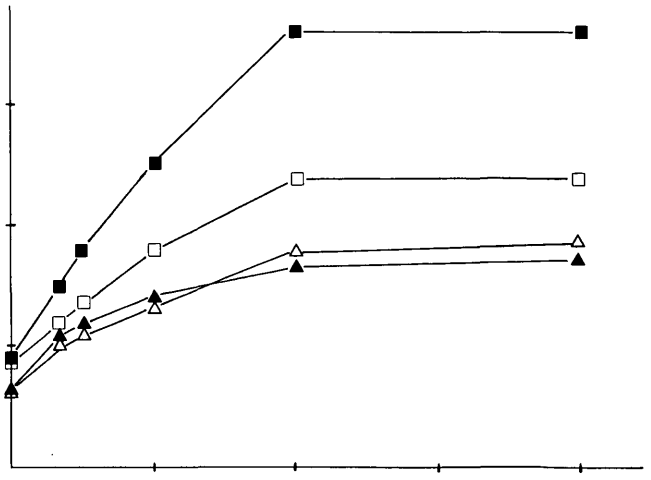

FIGURE 1. Kinetics of the incubation of 75 nmol of purified HbA_0 with a young RBC membrane extract (controls = incubation of 75 nmol purified HbA_0 with 0.15 M phosphate buffer alone). ■ Percentage of HbA_{1c} formed by incubation with young RBC membrane extract. □ Percentage of HbA_{1c} spontaneously formed by incubation in the buffer alone. ▲ Percentage of HbA_{1a+b} formed by incubation with young RBC membrane extract. △ Percentage of HbA_{1a+b} spontaneously formed in the buffer alone.

When the extracts prepared from young RBC are incubated for 24 h with HbA_0 in the presence of 2.75 μmol of glucose, the amount of HbA_{1c} formed is not larger than that obtained by incubation with either the membrane extract or glucose alone: there is not additivity of the effects.

Chloroform/methanol extraction leaves a hydrosoluble fraction completely devoid of activity (data not shown). On the other hand, the chloroform extract retains most of the RBC membrane glycosylating activity. Some of the activity appears to be lost in the extraction step. The incubation of the chloroform extract together with the hydrosoluble fraction does not improve the glycosylating activity of the former.

Table 1 also shows the results when HbA_0 had been heated at 65°C for 1 h prior to the incubation. The glycosylation reaction proceeds to a limit extent. On the other hand, when the membrane extracts have been heated for 2 min at 100°C prior to the incubation, the reaction occurs to a normal extent. These experiments show that the glycoconjugate is stable to heat but that the glycosylation reaction necessitates the three dimensional structure of Hb to be preserved.

For demonstrating the occurrence of a glucose exchange from the glycoconjugate to Hb, we measured in a separate experiment the amount of glucose in the glycoconjugate before and after incubation with HbA_0 (the conjugate was easily recovered from the dialyzate of the incubation medium, lyophilized and extracted into chloroform/methanol). The concentrations of glucose in the conjugate were 4.4 nmol per sample prior to incubation and 2.5 nmol after (difference 1.9 nmol), while the amount of HbA_{1c} increased by 1.2% (control deducted). The total concentration of HbA_0 in the sample was 75 nmol. The 1.2% of 75 nmol corresponds to 0.9 nmol. If 1 mol of Hb binds 2 mol of glucose, the glucose

TABLE 1
Percentages of glycosylated Hb obtained after 24-h incubation of HbA_0 with various extracts from young RBC membranes

Fractions incubated with 75 nmol of HbA_0	Glucose content in free or bound form per tube	Corresponding RBC counts	HbA_{1a+b} % of total Hb (mean of quadruplicate experiments ± 1 SD)	HbA_{1c} % of total Hb (mean of quadruplicate experiments ± 1 SD)
Zero time controls	0	0	0.6 ± 0.1	0.9 ± 0.1
Controls (24-h incubation)	0	0	2.3 ± 0.3	2.5 ± 0.2
Glucose controls (24-h incubation)	2750 nmol	0	2.7 ± 0.1*	3.2 ± 0.2†
RBC membranes extracts (old cells)	16.7 nmol	6.9×10^8	2.2 ± 0.3	2.4 ± 0.1
RBC membranes extracts (young cells)	8.3 nmol	6.0×10^8	2.4 ± 0.3	3.6 ± 0.3‡
RBC membranes extracts (young cells) + glucose	8.3 nmol + 2750 nmol	6.0×10^8	2.4 ± 0.1	3.7 ± 0.2‡
Chloroform/methanol extracted fractions (old cells)	5.5 nmol	18.6×10^8	2.3 ± 0.1	2.6 ± 0.2
Chloroform/methanol extracted fractions (young cells)	7.1 nmol	12.4×10^8	2.4 ± 0.2	3.2 ± 0.1‡
RBC membranes extracts (young cells) + HbA_0 heated for 1 h at 65°C	8.3 nmol	6.0×10^8	2.3 ± 0.3	2.9 ± 0.2*
RBC membranes extracts (young cells) after heating 2 min at 100°C	8.3 nmol	6.0×10^8	2.3 ± 0.3	3.5 ± 0.2‡

Every data, expressed in percentage of the total Hb, is the mean of quadruplicate determinations. The statistical significance of the differences between sample tubes and controls after 24 h incubation was analyzed by the Student's *t* test.
* $P < 0.05$. † $P < 0.01$. ‡ $P < 0.001$.
In all cases, the extracts from young RBC are active and the extracts from old RBC inactive.

amount bound to the incubated HbA_0 should be 1.8 nmol. The amount of glucose lost by the conjugate is actually equal to the amount bound to Hb.

To study the stereospecificity of the reaction, we incubated the same RBC membrane extracts in the presence of α- and β-para-nitrophenyl-D-glucosides. Table 2 shows that p-nitrophenyl-α-D-glucoside has no inhibiting effect, whereas p-nitrophenyl-β-D-glucoside completely inhibits the reactions in all the studied cases. It even causes a decrease in the blank value, giving a clue for the occurrence of a glycosylation process in the absence of added free or bound hexose. Bunn and Shapiro[16–18] demonstrated that HbA_0 contains residues of glucose bound to lysyl side chains of globin. We propose that the incubation is capable of inducing the transfer of these residues toward the N-terminal end of the β-chains of Hb, forming HbA_{1c}, which becomes detectable by chromatography.

Cyanmethemoglobin was used throughout the above described experiments. We demonstrated in separate experiments that the in vitro glycosylation proceeds at the same rate when Hb is used in its native form. To this end, we prepared a simple hemolyzate, dialyzed it against a 0.15 M

TABLE 2
Stereospecific inhibition of Hb glycosylation. Seventy-five nanomoles of HbA_0 were incubated in quadruplicate for 24 h with either 2.75 μmol of glucose or amounts of chloroform extracts from young RBC membrane containing 8.8 nmol of glucose per tube. In each case, the incubation was performed without addition, with addition of 0.5 μmol of p-nitrophenyl-α-D-glucoside, or with addition of 0.5 μmol of p-nitrophenyl-β-D-glucoside, respectively, to check for the stereochemical specificity of the glycosylation reaction

	HbA_{1a+b} % of total Hb (mean of quadruplicate experiments ± 1 SD)	HbA_{1c} % of total Hb (mean of quadruplicate experiments ± 1 SD)
Control (no incubation)	0.5 ± 0.1	0.4 ± 0.2
Control (24-h incubation)	1.3 ± 0.1	1.9 ± 0.1
Control + p-nitrophenyl-α-D-glucoside	1.3 ± 0.2	1.9 ± 0.1
Control + p-nitrophenyl-β-D-glucoside	1.1 ± 0.2	1.5 ± 0.1†
Glucose control (2.75 μmol)	1.8 ± 0.1*	3.2 ± 0.1*
Glucose + p-nitrophenyl-α-D-glucoside	1.9 ± 0.1*	3.2 ± 0.1*
Glucose + p-nitrophenyl-β-D-glucoside	1.4 ± 0.1	1.9 ± 0.1
Chloroform extract	1.3 ± 0.2	3.0 ± 0.1*
Chloroform extract + p-nitrophenyl-α-D-glucoside	1.4 ± 0.1	2.9 ± 0.1*
Chloroform extract + p-nitrophenyl-β-D-glucoside	1.2 ± 0.1	1.9 ± 0.2

The statistical analysis of differences between the various samples and the control was performed by Student's *t* test.
* Significant increase at $P < 0.001$.
† Significant decrease at $P < 0.01$.
The differences in the values of the incubated controls between Tables 1 and 2 depend on the fact that different batches of HbA_0 were used.

TABLE 3
Incubation of a fresh hemolyzate with RBC membrane fractions

Fraction incubated with 75 nmol of Hb in a fresh hemolyzate	HbA_{1a+b} expressed as % of total Hb (means of quadruplicate experiments ± 1 SD)	HbA_{1c} expressed as % of total Hb (means of quadruplicate experiments ± 1 SD)
Controls (no incubation)	1.6 ± 0.2	4.9 ± 0.3
Controls (24-h incubation)	2.2 ± 0.2	5.3 ± 0.1
Glucose controls (2750 nmol/tube)	2.7 ± 0.1*	6.5 ± 0.2†
RBC membranes (young cells) corresponding to 6.9×10^8 RBC	2.1 ± 0.1	6.9 ± 0.2†

The statistical significance of the differences between sample tubes and control tubes was analyzed by the Student's *t* test.
* Significant increase at $P < 0.01$.
† Significant increase at $P < 0.001$.

phosphate buffer for one night and incubated this solution with the RBC membrane extracts. The controls were either the hemolyzate incubated alone, or with free glucose. Table 3 demonstrates that the glycosylation reaction occurs with this native Hb as well as with cyanment-Hb.

It was verified that the derivative of Hb formed during its incubation with the glycoconjugate had the same mobility in isoelectric focusing as HbA_{1c} formed by incubation with glucose or purified from the RBC of diabetic patients.

The experiments reported in this paper[19] favor the hypothesis that, in vitro, there is the young RBC membranes a factor (or factors) capable of transferring a glucose unit to Hb. The factor disappears in old RBC. It is extracted in the organic phase by chloroform/methanol. It contains glucose which is partly lost during the exchange and its structure is under investigation. The mechanism of this transglycosylation reaction is still unknown but it must be noticed that it is strictly stereospecific as demonstrated by the complete inhibition caused by p-nitrophenyl-β-D-glucoside. The demonstration of such an effect in vitro does not provide certainty for its occurrence in vivo. Much additional work will be devoted to this demonstration. It may be hypothesized that two different pathways exist for glycosylation of Hb, one nonenzymatic pathway using free glucose as shown by Bunn[1] and the other one depending on a glycoconjugate of a still unknown structure which could be under enzymatic control.

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