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PROTECTION OF ERYTHROCYTE MEMBRANE AMINO GROUPS FROM REACTION WITH METHYL ACETIMIDATE BY PYRIDOXAL 5′-PHOSPHATE SCHIFF BASE FORMATION

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Pre-equilibration of erythrocytes with the membrane-impermeable aldehyde, pyridoxal 5′-phosphate, for 30 min at 22°C, prior to the addition of methyl acetimidate to the incubation mixture has been shown to prevent agglutination of acetamidinated cells which were resuspended in immune serum (Chao, T.L. and Berenfeld, M.R. (1981) J. Biol. Chem. 256, 5324–5326). This observation led to the possibility that the immune reaction, observed in some sickle cell anemia patients to reinfused cells which had been reacted with methyl acetimidate, could be prevented. The present communication further evaluates that reaction sequence and shows that while the pre-equilibration of cells with pyridoxal 5′-phosphate does protect membrane amines from reaction with methyl acetimidate, the protection is not extensive enough to prevent an immune response in a sickle cell anemia patient who had already been sensitized against acetamidinated cells. It is apparent that the design of antisickling agents which covalently modify hemoglobin must take into account protection of functional groups in the erythrocyte membrane, modification of which could produce an immunogenic response.

Introduction

Methyl acetimidate, a monofunctional imido ester which reacts specifically with primary amino groups, has been shown to be an effective antisickling agent [1,2]. Cells reacted in vitro with methyl acetimidate had a prolonged life span when reinjected in sickle cell anemia patients [2]. However, an immune reaction against acetamidinated cells was observed in some of these patients following multiple reinfusions of treated cells [3]. The in vitro test for antigenic surface constituents was performed by resuspending the reacted cells in immune serum containing agglutinating antibodies directed against acetamidinated cells [3]. Pre-equilibration of erythrocytes with pyridoxal 5′-phosphate, a membrane-impermeable aldehyde, for 30 min at 22°C, prior to the addition of methyl acetimidate to the reaction mixture has been shown to prevent agglutination of acetamidinated cells which were resuspended in immune serum [4]. This observation led to the possibility that the immune reaction could be prevented by protecting amines on the outer surface of the erythrocyte membrane from reacting with methyl acetimidate by prior Schiff base formation with pyridoxal 5′-phosphate. This communication further evaluates that reaction sequence for its applicability towards the potential use of methyl acetimidate as a therapeutic extracorporeal antisickling agent.

Preliminary accounts of this work have been published [16].

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Materials and Methods

Washed erythrocytes obtained from sickle cell anemia patients were pre-equilibrated with 40 mM pyridoxal 5′-phosphate (Sigma) in Tris-HCl, pH 8.8 buffer, prior to the addition of methyl acetimidate to the incubation mixture, as previously described [4]. The incubated cells were washed with isotonic Tris-HCl, pH 8.8 buffer followed by 0.15 M potassium phosphate, pH 7.4 buffer and isotonic saline in order to hydrolyze both the Schiff base and any excess methyl acetimidate.

The in vitro test for antigenic surface constituents was performed by resuspending the methyl acetimidate reacted cells in autologous immune serum containing agglutinating antibodies directed against acetamidinated cells [3]. Quantitation of the indirect Coombs test was performed according to the procedure of Cines and Schreiber [5] by incubating the acetamidinated cells with ^{125}I-anti-IgG after the reacted cells were resuspended in immune serum and washed.

Methyl [2-^{14}C]acetimidate was synthesized from [2-^{14}C]acetonitrile (Amersham) in methanolic HCl according to the procedure of Hunter and Ludwig [6]. The specific activity was determined directly by measurement of the radioactivity of serial dilutions of a weighed dry sample [7]. Washed intact cells and leaky erythrocyte ghosts [8] were incubated with methyl [^{14}C]acetimidate at various pH values in the presence and absence of pyridoxal 5′-phosphate as described above. The acetamidinated cells and ghosts were washed three times with 225 mM Tris-HCl, pH 8.8 buffer, once with 0.15 M potassium phosphate, pH 7.4 buffer, and resuspended in 0.9% NaCl. Erythrocyte membrane ghosts were then prepared from the intact cells according to the method of Tsukamoto et al. [9]. Hemolysate obtained from the first 10 mM Tris-HCl wash was reserved for determination of the extent of radioactivity incorporated in hemoglobin. The hemolysate obtained was exhaustively dialyzed against 0.15 M potassium phosphate, containing 1 mM EDTA, pH 7.4 buffer; the ghosts were exhaustively dialyzed against 10 mM Tris-HCl, pH 7.8 buffer. Samples containing hemoglobin were prepared for liquid scintillation counting as previously described [3]. Erythrocyte ghosts (100–400 μl) were counted in 15 ml Biofluor (New England Nuclear). Quench corrections were calculated from calibration curves made with the use of the external standard of the spectrometer.

Hemoglobin concentrations were determined using the Hb^+CN^- ε_{mM} 540 nm = 10.99 [10]. Erythrocyte membrane protein concentration was measured using a modification of the Bio-Rad protein microassay procedure [11]: 0–5 μl of erythrocyte membranes in 0.9% NaCl were added to 1.0 ml H_2O, 100 μl of the Bio-Rad protein assay dye concentrate were added to each test tube in sets of three, the absorbance at 595 nm was determined after vortexing the three samples. The remaining samples were read in the same manner. A standard curve using bovine serum albumin (Bio-Rad) in the 1–10 μg/μl range was performed with each assay. The absorbance at 595 nm was plotted against the standard protein concentration, and the slope (m_{std}) of the resulting straight line was calculated by the least-squares method. The absorbance at 595 nm was plotted against the volumes (1–5 μl) used for each membrane sample, the slope (m_x) of the resulting straight line, the y-intercept (b_x), and the absorbance at 595 nm of the sample volume counted (A_x) was calculated by the least-squares method. The protein concentration in μg (y_x) was calculated using the following equation: $y_x = (A_x - b_x)/m_{std}$.

Osmotic fragilities of normal human and rabbit erythrocytes were performed on the incubated erythrocytes as previously described [2]. The washed erythrocytes were pre-equilibrated for 30 min with 40 mM pyridoxal 5′-phosphate in 20 mM Tris-HCl, pH 8.8 buffer containing 100 mg% glucose, at a hematocrit of 20%, prior to the addition of 2.5 mM methyl acetimidate, or equilibrated with pyridoxal 5′-phosphate alone. ^{51}Cr-erythrocyte survival studies of autologous cells incubated with pyridoxal 5′-phosphate/methyl acetimidate were performed in rabbits [2] and in a sickle cell anemia patient [3] who had previously developed an immune response to acetamidinated cells. The autologous cells were pre-equilibrated for 30 min with 100 mM pyridoxal 5′-phosphate in 50 mM Tris-HCl, pH 8.6: Plasmalyte (Travenol) containing 200 mg% glucose (1 : 1) at a hematocrit of 20% and final pyridoxal 5′-phosphate/Tris-HCl concentration of 40/20 mM, prior to the 20 min

incubation with or without 2.5 mM methyl acetimidate. Sterile conditions were maintained throughout the procedure.

Radioactivity incorporated in the samples was measured using a Packard Tri-Carb model 3320 or a Nuclear Chicago model 1085 spectrometer. Visible spectra were determined using a Gilford 2400 recording spectrophotometer.

Results

The extent of acetamidination of both hemoglobin and erythrocyte membrane protein was determined by incubating cells with varying concentrations of methyl [^{14}C]acetimidate (specific activity 15.5 μCi/mmol) at different pH values. As anticipated, the incorporation of [^{14}C]acetamidine increased in both hemoglobin and membrane protein as both the imido ester concentration and pH of the incubation mixture were increased from 2.5 to 10 mM and pH 7.2 to 8.8 (Fig. 1). In order to determine whether pre-equilibration of erythrocytes with pyridoxal 5'-phosphate blocked access of methyl acetimidate to membrane amines, the aldehyde/imido ester reaction sequence was performed on both intact cells and leaky erythrocyte ghosts. In the leaky ghosts, where pyridoxal 5'-phosphate can react with amino groups on both sides of the membrane, the aldehyde was shown to decrease the subsequent incorporation of [^{14}C]acetamidine by 36% when compared to ghosts which had been pre-equilibrated in buffer only (Table I). However, no difference in the amount of radioactivity incorporated in membrane amines or hemoglobin was detected between intact cells which had been pre-equilibrated with or without the aldehyde (Table I). The results of this experiment also show that the aldehyde did not interfere in the reaction of methyl acetimidate with hemoglobin. There are at least 15–20-times as many reactive protein amino groups and nearly 100-times as many reactive lipid amines on the inner surface as on the outer surface of the erythrocyte membrane [12]. Therefore, the decrease in [^{14}C]acetamidine incorporation due to Schiff base formation with only amino groups on the outer surface of the membrane is apparently too small to be detected. For this reason a more sensitive method of detecting the extent of amine protection by Schiff base formation was required.

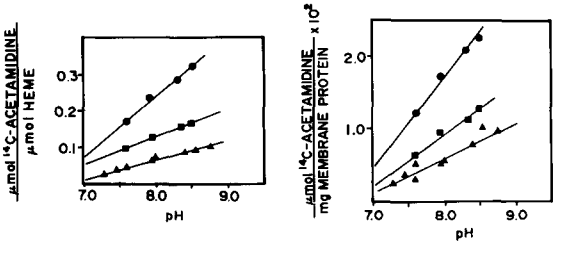

Fig. 1. Incorporation of [^{14}C]acetamidine in hemolysate and membrane protein as a result of the reaction of methyl [2-^{14}C]acetimidate (▲———▲, 2.5 mM; ■———■, 5.0 mM; ●———●, 10.0 mM) with intact erythrocytes. The solid lines were calculated by the least-squares method.

TABLE I

INCORPORATION OF [^{14}C]ACETAMIDINE IN INTACT ERYTHROCYTES (A) AND LEAKY GHOSTS (B)

	Pyridoxal 5'-phosphate concn. (mM)	Methyl [^{14}C]acetimidate concn. (mM)	μmol [^{14}C]acetamidine per μmol heme	μmol [^{14}C]acetamidine per mg ghost protein
A	0	2.5	0.110	$5.43 \cdot 10^{-4}$
	40	2.5	0.105	$5.83 \cdot 10^{-4}$
	0	10.0	0.396	$1.40 \cdot 10^{-3}$
	40	10.0	0.386	$1.48 \cdot 10^{-3}$
B	0	10.0	–	0.064
	0	10.0	–	0.064
	40	10.0	–	0.041
	40	10.0	–	0.041

Radioimmunoassays are among the most sensitive methods for detecting altered proteins. For this reason the indirect Coombs test was employed in an effort to determine if pyridoxal 5'-phosphate was efficiently protecting membrane amines. Incubation of acetamidinated erythrocytes in ^{125}I-anti-IgG showed a linear increase in the binding of ^{125}I-anti-IgG as the methyl acetimidate concentration increased. For methyl acetimidate concentrations of 0, 0.25, 2.5, 5.0, and 10.0 mM the radioactivity incorporated was 1008, 987, 1054, 1141, and 1311 cpm/10^8 cells, respectively. This indicated an increase in the number of antigenic sites on the outer surface of the erythrocyte membrane as the extent of acetamidination increased. Pre-equilibration of cells with 40 mM pyridoxal 5'-phosphate was shown to decrease the subsequent binding of

^{125}I-anti-IgG after acetamidination of the cells. For example, cells which had been incubated in 0.25 or 2.5 mM methyl acetimidate prior to resuspension in immune serum, washing and ^{125}I-anti-IgG were found to incorporate 258 and 361 cpm/10^8 cells, respectively. Pre-equilibration of these cells with 40 mM pyridoxal 5′-phosphate decreased the ^{125}I-anti-IgG binding to 223 and 300 cpm/10^8, respectively. Control cells, untreated with either imido ester or aldehyde, which were incubated in immune serum followed by ^{125}I-anti-IgG, incorporated 228 cpm/10^8 cells. Cells from another individual, treated in the same manner incorporated 282 and 482 cpm/10^8 cells for 0.25 and 2.5 mM methyl acetimidate, respectively. Pre-equilibration with the aldehyde before the addition of methyl acetimidate decreased the binding of ^{125}I-anti-IgG to 226 and 265 cpm/10^8 cells, respectively. The binding of ^{125}I-anti-IgG to control cells from this individual was 269 cpm/10^8 cells. Thus, the pyridoxal 5′-phosphate pre-equilibration appeared to provide sufficient protection for amines on the exterior surface of the erythrocyte membrane as shown by the reduction of immunoreactive sites.

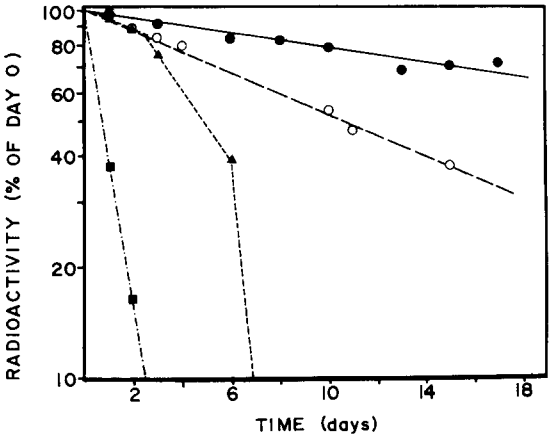

Fig. 2. Multiple ^{51}Cr-erythrocyte survival studies in a single sickle cell anemia patient. O— —O, control study performed on 6/5/78, $t^1_{1/2}$ = 10.5 days; ●———●, 2.5 mM methyl acetimidate incubation, 2/20/79, $t^2_{1/2}$ = 29.5 days; ■–·–■, 2.5 mM methyl acetimidate incubation, 5/22/79, $t^3_{1/2}$ < 1 day; ▲– – –▲, 40 mM pyridoxal 5′-phosphate pre-equilibration followed by 2.5 mM methyl acetimidate incubation 9/15/81, extrapolated $t^4_{1/2}$ values of 11.4, 3.3 and less than 1 day (the last point for this survival curve occurs at 1.9% remaining radioactivity on the 8th day).

Although the Schiff base formed between pyridoxal 5′-phosphate and membrane amino groups should be entirely hydrolyzed in subsequent washing steps; nevertheless, before proceeding to further human studies, it was important to demonstrate that the viability of cells treated with pyridoxal 5′-phosphate and methyl acetimidate were within normal limits. Cell viability was analyzed by osmotic fragility and ^{51}Cr-erythrocyte survival in rabbits. Equilibration of cells with 40 mM pyridoxal 5′-phosphate alone was shown to have no effect on the osmotic fragilities of normal human and rabbit cells. The osmotic fragilities of normal human and rabbit erythrocytes pre-equilibrated with 40 mM pyridoxal 5′-phosphate followed by 2.5 mM methyl acetimidate were also within the normal limits.

^{51}Cr-erythrocyte survival studies of autologous cells pre-equilibrated with 40 mM pyridoxal 5′-phosphate prior to the incubation with or without 2.5 mM methyl acetimidate were performed in 6 rabbits. The $t_{1/2}$ for cells incubated with only the aldehyde was 12, 13, and 15 days, while that for cells pre-equilibrated with the aldehyde prior to addition of the imido ester was 15.2, 15, and 16.5 days. These $t_{1/2}$ values were within the normal range established for rabbits in a previous study [2]. A ^{51}Cr-erythrocyte survival study of cells pre-equilibrated with 40 mM pyridoxal 5′-phosphate prior to 2.5 mM methyl acetimidate was then performed in a sickle cell anemia patient who had already been sensitized against acetamidinated cells. In order to ensure that the aldehyde Schiff base did protect membrane amines from reacting with methyl acetimidate, the in vitro agglutination test was shown to be negative with both autologous immune serum and Coombs serum before the treated cells were reinfused into the patient. The resulting survival curve was triphasic, with extrapolated $t_{1/2}$ values of 11.4, 3.3, and < 1 day (the last point for this survival curve occurs at 1.9% remaining radioactivity on the 8th day) ($t^4_{1/2}$, Fig. 2). This result showed that while the initial extrapolated half-life of the treated cells (11.4 days) was longer than the half-life of the third consecutive study ($t^3_{1/2}$ < 1 day) performed in this patient, the immune reaction was nevertheless activated by the treated cells. It is therefore clear that while pyridoxal 5′-phosphate protects most of the amino

groups on the outer surface of the erythrocyte membrane from reacting with methyl acetimidate, the small number left unprotected are sufficient to elicit an immune response in vivo.

Discussion

The results presented here demonstrate that although pre-equilibration of erythrocytes with pyridoxal 5′-phosphate does protect exterior membrane amines from reaction with methyl acetimidate, the protection is not complete and some of the amino groups are acetamidinated. This lack of complete protection was responsible for the delayed immune response observed in the previously sensitized sickle cell anemia patient who received cells that had been treated with both aldehyde and imido ester.

The bifunctional imido ester, dimethyl adipimidate, has also been shown to be an effective antisickling agent [13] with potential therapeutic applications [14]. However, red cells reacted with other imido esters, including dimethyl adipimidate, also agglutinate when resuspended in immune serum containing agglutinating antibodies directed against acetamidinated cells [3]. It has been subsequently reported that an immunogenic reaction against red cells treated with dimethyl adipimidate was observed in three out of five sickle cell anemia patients who received a second infusion of treated autologous cells [15]. It is therefore apparent that multiple infusions of cells treated with any imido ester will very likely lead to an immune response in some individuals.

Other aldehydes where the equilibrium involving Schiff base formation is further to the right than it is with pyridoxal 5′-phosphate might provide sufficient protection to completely prevent external membrane amines from reacting with methyl acetimidate. It is also possible that if the reaction sequence with pyridoxal 5′-phosphate/methyl acetimidate as outlined in this communication were to be used in patients who have never been exposed to acetamidinated cells, the incidence of the immune response might be significantly lower. In a previous study, it was shown that only 5 out of 13 sickle cell anemia patients exposed to multiple reinfusions of acetamidinated cells developed an immune reaction [3].

In conclusion these results suggest that essentially all of the exterior membrane amino groups must be protected from reaction with imido esters in order to eliminate the possibility of an immunogenic reaction against the treated cells. Thus, membrane protection now appears to be a prerequisite for the clinical application of antisickling agents which chemically modify both hemoglobin and membrane protein.

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