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Research Article

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Hematologic and Clinical Responses in Patients with Sickle Cell Anemia after Chronic Extracorporeal Red Cell Carbamylation

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ABSTRACT In eight patients with sickle cell anemia, weekly extracorporeal carbamylation of about 20% of the circulating red cell mass was carried out for 2 yr or longer. At each visit, a mean of 1.3±0.2 mol of cyanate were incorporated per mole of hemoglobin in the carbamylated erythrocytes. Within 3 mo, a stable level of about 35–50% of the circulating erythrocytes was carbamylated. This quantity and degree of hemoglobin carbamylation produced a decrease in mean whole blood P_{50} from 33 to 26 mm Hg. During the first 3 mo of carbamylation, the mean hemoglobin increased from 6.4 to 9.1 g/100 ml, while mean absolute reticulocytes decreased by 58% and circulating irreversibly sickled erythrocytes decreased by 65%. The mean red cell life span increased from 13 days before treatment to 21.6 days after 3 mo of carbamylation. Beyond the 3rd mo of carbamylation, blood P_{50}, hemoglobin, and reticulocytes remained quite stable. No toxic effects of extracorporeal carbamylation of erythrocytes were noted. The capacity of blood to release oxygen at 30 mm Hg P_{O_2} increased from 4.3 to 5.0 cm^3/100 ml blood during carbamylation.

The overall frequency of severe painful crises decreased by about 80% during carbamylation. Before carbamylation, 34% of the crises were induced by a concomitant illness, usually an infection. During carbamylation, the incidence of induced crises decreased 50% while spontaneous crises virtually disappeared. The marked improvements in hematologic parameters and the decreased frequency of severe painful crises observed during this study offer sufficient promise to warrant further exploration, hopefully using more efficient techniques, of the clinical efficacy of extracorporeal erythrocyte carbamylation in sickle cell anemia.

INTRODUCTION

Incubation of erythrocytes from patients with sickle cell anemia (Hb SS)[1] with cyanate (NCO) or carbamyl phosphate inhibits in vitro sickling upon deoxygenation of these cells (1, 2). NCO and carbamyl phosphate react irreversibly with the amino-terminal valine residues of hemoglobin in a reaction termed carbamylation (1–6). Carbamylation increases the oxygen affinity (7–9) and inhibits the gelation of sickle cell hemoglobin (Hb S) (1, 4). Because of the increase in oxygen affinity of carbamylated Hb SS erythrocytes, greater saturation of hemoglobin with oxygen occurs at physiological oxygen tensions. The increase in oxygen affinity produced by carbamylation appears to be the major mechanism responsible for the antisickling effect of NCO (4, 7–9).

In vitro carbamylation of Hb SS erythrocytes prolongs the survival of such erythrocytes returned to the

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[1] *Abbreviations used in this paper:* DFP, diisopropylfluorophosphate; Hb S, sickle hemoglobin; Hb SS, sickle cell anemia; ISC, irreversibly sickled cells; MLS, mean cell life-spans; NCO, cyanate.

TABLE I

Base-Line Information and Observations on Patients before Beginning Carbamylation

Patient	Sex	Age	Hemoglobin electrophoresis S	F	A_2	Hemoglobin mean (range)*	Reticulocytes mean (range)*	Major clinical problems‡	Length of observation
		yr	%	%	%	*g/100 ml*	%		*yr*
1	M	36	82.0	14.9	3.0	8.6 (7.9–8.9)	18 (16–20)	PC	12
2	F	18	91.8	4.1	4.1	6.6 (6.4–6.9)	18 (15–20)	PC	§
3	F	27	90.0	3.5	3.8	6.2 (5.9–6.9)	27 (25–28)	PC, ND, A	12
4	F	19	90.4	5.2	4.3	6.5 (6.3–6.9)	26 (24–27)	PC	§
5	M	17	90.5	5.2	4.3	6.2 (6.0–6.3)	27 (23–30)	PC	§
6	M	22	9.04	3.5	4.3	6.0 (5.9–6.5)	27 (25–28)	PC	2
7	F	18	86.8	8.9	4.3	4.2 (3.8–4.7)	24 (23–26)	PC	5
8	M	24	88.0	5.7	4.1	6.8 (6.6–6.9)	23 (20–25)	ND, MU, A	3
9	M	26	93.9	4.0	2.1	7.8 (7.6–8.1)	17 (16–19)	PC, MU	3
10	F	42	85.2	9.8	4.2	8.1 (7.7–8.5)	16 (14–19)	PC, MU	5

* Mean values and range for a minimum of seven determinations during the 3 mo before start of carbamylation.

‡ Abbreviations: PC, painful crises; ND, neurological defict; MU, indolent malleolar ulcerations; A, anemic symptoms.

§ Previous medical care outside of this hospital.

patient (6, 7, 10–12). This observation suggests that the antisickling effects observed in vitro persist in vivo and also that there is no overall deleterious effect of incubating erythrocytes with NCO or carbamyl phosphate. With this information, pilot studies employing repeated extracorporeal red cell carbamylation with sodium cyanate were undertaken in informed symptomatic patients with sickle cell anemia at this institution in 1972. This report describes hematologic and clinical responses observed in these patients.

METHODS

10 patients with sickle cell anemia participated in this study. Pertinent base-line hematologic and clinical information on each patient is presented in Table I. All of the patients were examined weekly for 2–3 mo before beginning weekly carbamylation. During this period, control values for hemoglobin, reticulocytes, irreversibly sickled cells (ISC), and whole blood P_{50} (oxygen tension at which $(HbO_2/Hb) = 1$ or 37°C, pH 7.40) were established for each patient. Reticulocyte and ISC counts are reported as percent of circulating erythrocytes (uncorrected).

Criteria for selection of patients for this study were: (*a*) frequent, severe painful manifestations with at least two hospitalizations for acute painful crises in the previous 6 mo (patient 1–7, 9); and (*b*) chronic indolent malleolar ulcerations interfering with ambulation (patients 8–10). Patients 3 and 8 had sustained permanent neurological damage during adolescence, resulting in hemiparesis. The neurological deficit became more pronounced in both patients during more pronounced anemia and cardiac decompensation.

Seven of the patients had been followed by physicians involved in this study for 2–12 yr before entering this study. Three of the patients were referred to this center for participation in the study; medical records from referring centers were reviewed in each to ascertain the frequency of painful crises requiring hospitalization for management.

The technique employed to carbamylate erythrocytes at each weekly visit was as follows. After placement of a no. 19 scalp vein needle in a forearm vein, 500 ml of blood was collected by gravity in a plastic bag containing 1,500 U of heparin and 100% O_2. After centrifugation, the plasma was reinfused while the red cells were diluted to a hematocrit of 50% with 0.15 M NaCl, after which a 0.15 M solution of NaNCO, recrystallized from 65% ethanol and forced through 0.22 μm Millipore filters,[2] was added to the red cells to a final concentration of 30 mM. 50 cm^3 of 95% O_2–5% CO_2 was added to the blood bag to maintain greater than 95% oxygenation of the hemoglobin and a reproducible pH (7.45±0.05 at 37°C) during incubation. Under these conditions, 1.2±0.4 mol of [^{14}C]NCO[3] were incorporated per tetrameric mole of hemoglobin after a 1-h incubation at 37°C. The red cells were washed twice with 0.15 M NaCl and reinfused. A second 500-ml unit of blood drawn just before the reinfusion of the first unit of carbamylated cells was handled similarly. The procedure was repeated weekly unless whole blood P_{50} decreased to less than 24 mm Hg or the hematocrit exceeded 32%; in these circumstances carbamylation was carried out biweekly.

At each weekly visit, hemoglobin, hematocrit, white blood count with differential, platelet count, reticulocyte, ISC count, and whole blood P_{50} were determined. Whole blood P_{50} was measured with the IL 217 blood gas system[4] to achieve progressive stepwise deoxygenation of the hemoglobin. ISC counts were determined on blood equilibrated with 95% O_2–5% CO_2 by criteria previously described (13).

The mean cell life-spans (MLS) of control and of carbamylated erythrocytes were determined simultaneously with separate isotopes of diisopropylfluorophosphate (DFP), [^{3}H]-DFP,[5] [^{32}P]DFP,[6] or [^{14}C]DFP,[7] according to recommended methods for radioisotopic erythrocyte survival studies (14).

[2] Millipore Corp., Bedford, Mass.

[3] [^{14}C]KNCO, Amersham/Searle Corp., Arlington Heights, Ill.

[4] Instrumentation Laboratory, Inc., Lexington, Mass.

[5] New England Nuclear Corp., Boston, Mass, sp act 5 μCi/μg.

[6] Amersham/Searle Corp., sp act 0.23 μCi/μg.

[7] New England Nuclear, sp act 0.6 μCi/μg.

TABLE II

Measurement of Hemoglobin Carbamylation

Sample	Incubation conditions		Hemoglobin carbamylation	
	[NCO]	Time	^{14}C incorporation	Valine hydantoin
	mM	*min*	*mol NCO/mol Hb*	
Hb S blood:				
1	25	15	0.46	0.49
		30	0.87	0.88
		60	1.22	1.17
2	30	60	1.29	1.20
3	30	60	1.27	1.19
4	30	60	1.30	1.10
Hb A blood:				
1	10	180	1.29	1.0
	20	180	1.90	1.7
	30	180	2.54	2.0
	50	180	3.84	3.0
2	25	60	1.35	1.25
3	25	60	1.20	1.10

Circulating untreated red cells were labeled in vivo by the intravenous infusion of [^{32}P]DFP, 0.5 μCi/kg; carbamylated erythrocytes were labeled in vitro by the addition of [^{3}H]-DFP, 5 μCi/kg, or of [^{14}C]DFP, 0.5 μCi/kg of body wt, to 100 ml of red cells. Before the addition of DFP, the red cells had been resuspended to a packed cell volume of 50% in 0.15 M NaCl and carbamylation, initiated by the addition of sodium cyanate, had been allowed to proceed for ½ h. 2 h after the addition of DFP, the red cells were washed three times with 0.15 M NaCl to remove unreacted DFP and then reinfused into the patient. In three experiments, from 46 to 58% of the total DFP radioactivity added to label the erythrocytes was removed and discarded in the NaCl washing steps. Heparinized blood samples were obtained at 30 min after reinfusion and then three times weekly for the subsequent measurement of radioactivity with a liquid scintillation counter[8] by the procedure described by Alter et al. (12). Radioactivity in blood, expressed as disintegrations per minute per milligram of hemoglobin, measured after 4–6 wk of sampling, ranged from 20–45 dpm/mg hemoglobin for each isotope, in samples drawn on day zero, to 4–20 dpm on samples drawn on day 35. The results were analyzed as described elsewhere (11, 15) to determine the MLS of each population of cells. The MLS in the normal adult by this method is 120 days (11). A red cell survival study was carried out on all patients before beginning repeated weekly carbamylation and was repeated in five patients after 3–6 mo of weekly carbamylation.

Hemoglobin was measured as cyanmethemglobin with Van Kampen and Kijlstra's solution (16); hematocrits on oxygenated samples of blood were measured to a precision of 0.5% in microcapillary tubes examined on an expanded scale reader.[9]

Hemoglobin carbamylation was estimated by measuring the $N^{14}CO$ or ^{14}CP[10] incorporation per tetrameric mole of hemoglobin in a small sample of incubation mixture handled parallel to the unit of red cells. In selected instances, in vitro hemoglobin carbamylation as well as circulating levels of carbamylated hemoglobin were determined chemically by the valine hydantoin method.[11] The valine hydantoin method measures the group-specific carbamylation of amino-terminal valine residues of hemoglobin, whereas the radioactive incorporation method measures total hemoglobin carbamylation, including carbamylation of the epsilon NH_2 group of lysine residues. To facilitate analysis of the data presented, Table II presents for comparison measurements of hemoglobin carbamylation as determined by both methods in the same samples of blood containing hemoglobin A or S incubated with NaNCO at pH 7.45±0.05 and at 37°C. Under the carefully controlled conditions for carbamylation used during the patient studies, 90% or more of the ^{14}C incorporated into the A or S hemoglobin reacted with the aminoterminal valine residues. With prolongation of the incubation and increasing NCO concentrations, progressively greater carbamylation of nonvaline residues occurred. Other investigators (4) have demonstrated progressive carbamylation of epsilon NH_2 groups of lysine residues under these latter conditions.

RESULTS

Effects of repeated carbamylation on oxygen affinity, in vivo sickling, and on the rate of hemolysis. 8 of the 10 patients underwent weekly extracorporeal carbamylation of about 20% of their circulating red cell mass for 24–38 mo (mean 31 mo); 2 patients underwent carbamylation for shorter periods of time (patient 7 for 3 mo, patient 6 for 3 mo on two separate occasions). Base line values (mean as well as the range) for the hematologic data obtained before starting carbamylation of all 10 patients is shown in Tables I and III. Hemoglobin, reticulocytes, ISC counts, and whole blood P_{50} values measured before each weekly carbamylation are presented for the first mo on all 10 patients in Table III. A graphic presentation of similar data for the first 24 mo of carbamylation on the eight patients who underwent long-term carbamylation is shown in Fig. 1. Hemoglobin rose progressively and reticulocytes, ISC, and P_{50} decreased progressively in all patients during the first 3 mo of carbamylation. By the 3rd mo of carbamylation, whole blood P_{50} decreased from a mean value of 33 to 26 mm Hg, reticulocytes and ISC decreased from means of 22 and 20 to 11 and 7%, respectively, and mean hemoglobin rose from 6.7 to 8.8 g/100 ml. Absolute decreases in reticulocytes and ISC of 58 and 65%, respectively, occurred. Hemoglobin, reticulocyte, ISC, and P_{50} values remained stable beyond the 3rd month in those eight patients who continued carbamylation. The hemoglobin increased from 6.4 g/100

[8] Packard Tri-Carb, model 3385; Packard Instrument Co., Inc., Downers Grove, Ill.

[9] C. R. Reader, Damon/IEC, Div., Damon Corp., Needham Heights, Mass.

[10] [^{14}C]carbamyl phosphate, New England Nuclear Corp.

[11] Performed by A. Cerami, Ph.D., The Rockefeller University, New York.

TABLE III

Hemotologic Responses to Red Cell Carbamylation

Patient	Length of carbamylation	Hemoglobin	Reticulocytes	ISC	Whole blood P_{50}	MLS
	mo	*g/100 ml*	%	%	*mm Hg*	*days*
1	0	8.6	18	18	30	16.6
	1	9.6	12	16	28	
	3	10.8	8	10	26	
2	0	6.6	18	20	31	16.2
	1	7.6	14	20	29	
	3	9.0	8	10	24	
3	0	6.2	27	18	32	12.4
	1	7.7	16	15	30	
	3	9.1	15	9	26	18.5
4	0	6.5	26	21	33	8.4
	1	7.4	22	20	29	
	3	8.0	11	4	27	
5	0	6.2	27	20	34	14.3
	1	6.9	18	13	31	
	3	7.7	13	8	27	23.2
6	0	6.0	27	17	33	15.4
	1	7.9	14	12	31	
	3	9.2	8	5	24	
7	0	4.2	24	21	34	9.1
	1	5.4	18	14	30	
	3	6.2	15	9	26	
8	0	6.8	23	27	37	9.1
	1	6.9	18	17	32	
	3	8.3	11	8	26	16.5
9	0	7.8	17	18	34	13.3
	1	8.5	15	11	32	
	3	8.8	13	5	28	23.3
10	0	8.1	16	15	32	16.8
	1	9.7	11	11	29	
	3	10.7	7	6	25	27.7
Mean	0	6.7±0.36	22.3±1.86	19.5±1.5	33±0.47	13.2
±SEM	1	7.7±0.61	16±1.07	14.9±1.5	30±0.43	
	3	8.8±0.33	11±1.19	7.4±0.8	26±0.60	(21.8)

ml before treatment to 9.8 g/100 ml after 9 mo of carbamylation in these eight patients.

Direct measurements of MLS demonstrated prolongation of the MLS of erythrocytes after carbamylation. Before beginning weekly carbamylation, the mean MLS of untreated erythrocytes for all 10 patients was 13.2 days (range 8.4–16.8 days). The simultaneously measured mean MLS of erythrocytes containing 1.3±0.4 mol of NCO/mol of hemoglobin [estimated by ^{14}NCO incorporation in separate aliquots of blood] was 25 days (columns 1 and 2, respectively, Fig. 2). In a given patient, prolongation of the MLS of carbamylated erythrocytes was related overall to the degree of hemoglobin carbamylation obtained (Table IV). In analyzing the actual prolongation of MLS of carbamylated erythrocytes among the 10 patients, the incorporation of a mean of 1.3 mol of NCO/mol of hemoglobin roughly doubled the MLS value (Fig. 2). In five patients a repeat dual survival study was carried out after 3–6 mo of repeated carbamylation. Again, [^{32}P]DFP was infused slowly intravenously to label circulating erythrocytes; about 100 ml of erythrocytes were labeled with [^{3}H] DFP in vitro after carbamylation. The MLS of [^{32}P]DFP-labeled cells ranged from 16.5 to 27.7 days, mean 21.8 days; the mean MLS of the carbamylated cells was 31.5 days (columns 3 and 4 of Fig. 2). The mean MLS of untreated cells in these same patients before repeated carbamylation was 13.2 days (Table III, last column). The prolongation of erythrocyte survivals observed in these five patients strongly suggests a decrease in the rate of destruction of erythrocytes during carbamylation.

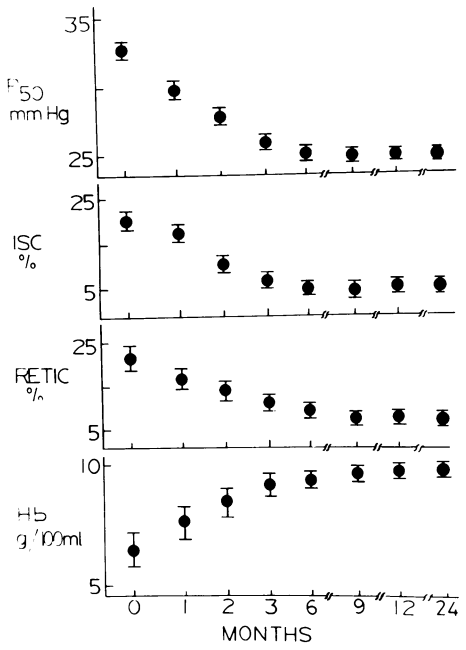

FIGURE 1 Hematologic responses of eight patients with sickle cell anemia maintained on weekly extracorporeal carbamylation of about 20% of the red cell mass. The mean values±SEM are given for P_{50}, ISC, reticulocytes, and hemoglobin. Carbamylation was begun at time 0. The changes in values from month 0 to month 3 for all parameters measured are significant ($P < 0.001$). The absolute decrease in reticulocytes and in ISC at month 3 was 58 and 65%, respectively.

In addition, the increase in hemoglobin and concomitant decrease in reticulocytes observed in all patients also suggests decreased destruction rather than increased production of erythrocytes in these patients during carbamylation.

Worsening of the hemolytic anemia invariably occurs upon stopping repeated carbamylation. The hematological responses of two patients during and after 12 wk of carbamylation are presented in Figs. 3 (patient 7) and 4 (patient 6). Both patients demonstrated an improvement in their anemia during carbamylation and return of hemoglobin and reticulocytes to pretreatment levels within 5–8 wk after stopping carbamylation. It is important to note the slower increase in hemoglobin during carbamylation in patient 7, who presented both a more severe but stable anemia and shorter MLC than patient 6 (MLS 9.1 vs. 15.4 days, respectively). During the initial visits, only 130–140 ml of erythrocytes were carbamylated weekly in patient 7, as opposed to 180–200 ml of erythrocytes in patient 6, because of the more severe anemia in patient 7. In addition the MLS of carbamylated erythrocytes was 15.6 days in patient 7 and 27 days in patient 6.

Levels of circulating carbamylated hemoglobin. In six patients maintained on weekly carbamylation for 6 mo, hemoglobin carbamylation of circulating erythrocytes was measured on two separate occasions by the valine hydantoin method. On each occasion carbamylation was measured on three erythrocyte samples: (*a*) circulating blood removed for the weekly carbamylation, (*b*) erythrocytes carbamylated in the usual manner, and (*c*) circulating erythrocytes removed 30 min after rein-

TABLE IV
Increase in MLS of Carbamylated Red Cells

Patient	Additive	Hemoglobin carbamylation*	MLS	Increase in MLS
		mol/mol	*days*	*days*
2	—	—	16.2	—
	NCO	0.9	20.3	4.1
	NCO	3.1	29.5	13.3
4	—	—	8.4	—
	NCO	0.4	8.7	0.3
	NCO	1.7	21.7	13.3
5	—	—	14.3	—
	NCO	0.4	21.0	6.7
	NCO	1.7	32.2	17.9
7	—	—	9.1	—
	NCO	1.3	15.6	6.5
	NCO	2.0	18.0	8.9
9	—	—	13.3	—
	NCO	1.3	22.0	8.7
	NCO	2.3	27.0	13.7
10	—	—	16.8	—
	NCO	1.5	27.6	8.8
	CP	1.3	23.8	7.0
3	—	—	7.0	—
	NCO	1.4	16.8	9.8
	CP	1.3	12.6	5.6

* Measured by [^{14}C]NCO or [^{14}C]carbamyl phosphate incorporation per mole of hemoglobin.

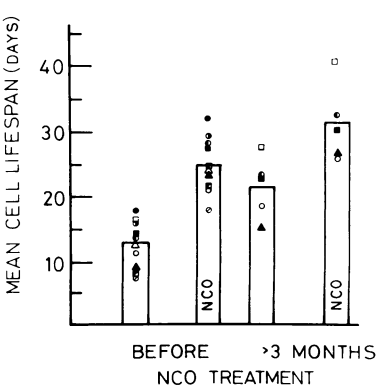

FIGURE 2 Mean life-span of untreated and of carbamylated sickle erythrocytes measured simultaneously before beginning weekly carbamylation in 10 patients; the same study was repeated after 3 mo of weekly carbamylation in 5 of the patients. The mean values for the group determinations are depicted by the height of the bars.

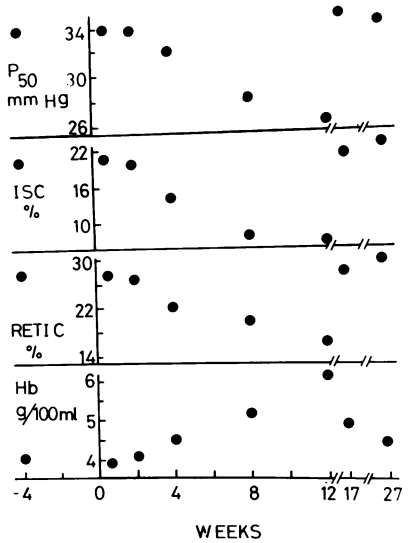

FIGURE 3 Hematologic response of patient 7 during 3 mo of weekly carbamylation of the erythrocytes from 1 liter of blood. Carbamylation was stopped at wk 12.

fusion of carbamylated cells at that visit (Table V). Hemoglobin carbamylation of cells removed for carbamylation (zero time, column 1) ranged from 0.35 to 0.71 mol of valine hydantoin (mean 0.51)/mol of hemoglobin. Erythrocytes after carbamylation contained a mean of 1.3 mol of valine hydantoin/mol of hemoglobin for the group (column 2). With the exception of patient 5, the degree of hemoglobin carbamylation obtained during the weekly NCO incubation was quite uniform (1.3±0.2 mol/mol). 30 min after the reinfusion of the in vitro-carbamylated erythrocytes, circulating erythrocytes contained 0.71 mol of valine hydantoin/mol of hemoglobin. Because the degree of hemoglobin carbamylation obtained during each weekly incubation of erythrocytes with NaNCO is quite uniform and reproducible, it is possible to estimate the percent of circulating erythrocytes carbamylated by determining the "specific activity" (moles of valine hydantoin per mole of hemoglobin) of circulating erythrocytes before and after weekly carbamylation. Such estimates can be performed for either the individual patient or for the entire group. Considering group data, 39% (0.51 ÷ 1.3) of the erythrocytes in circulation before the weekly carbamylation had previously been carbamylated. Similarly, about 55% (0.71 ÷ 1.3) of the erythrocytes in the circulation 30 min after the weekly carbamylation were carbamylated.

Effects of repeated carbamylation upon clinical manifestations. The clinical manifestations of sickle cell anemia are extremely variable, both among patients and in the individual patient. Painful manifestations experienced by the patient share this wide variability in frequency, duration, character, and severity. Acute painful crises, defined as severe painful episodes longer than

TABLE V

Levels of Circulating Carbamylated Hemoglobin Measured in Six Patients Maintained on Repeated In Vitro Red Cell Carbamylation

Patient	Circulating red cells zero time*	Red cells reinfused‡	Circulating red cells 30 min after reinfusion
	mol valine hydantoin/mol hemoglobin		
3	0.55	1.1	—
	0.60	1.3	0.73
4	0.59	1.5	—
	0.58	1.3	0.68
5	0.65	1.9	—
	0.71	1.6	0.93
8	0.39	1.2	—
	0.49	1.3	0.68
9	0.37	1.2	—
	0.43	1.1	0.56
10	0.36	1.1	—
	0.35	1.1	0.69
Mean	0.51	1.3	0.71
±SE	0.04	0.07	0.05

* Red cells were drawn for the weekly incubation with NaNCO and measurements made on two separate occasions in each patient.

‡ The same red cells in column 1 (*) after incubation with NaNCO.

12 h, which required hospitalization for management are more amenable to objective assessment than painful manifestations of lesser severity. Such painful crises may occur with no recognized underlying precipitating cause(s), in which case they are referred to herein as spontaneous crises, as opposed to induced crises. To assess a potential effect of chronic extracorporeal red cell

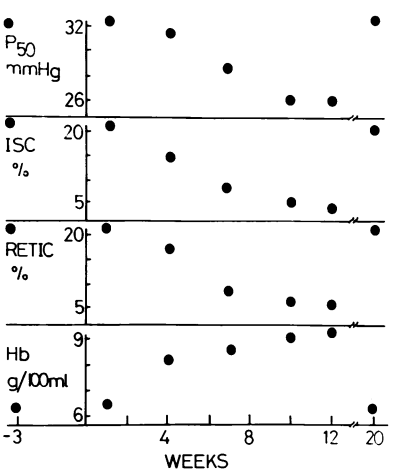

FIGURE 4 Response of patient 6 upon discontinuing weekly carbamylation after the 12th wk.

TABLE VI

Summary of Hospitalizations for Acute Painful Crises before and during Carbamylation

Patient	24 mo before carbamylation				24 mo during carbamylation			
	Total number	Frequency of crises	Spontaneous crises	Total duration of crises	Total number	Frequency of crises	Spontaneous crises	Total duration of crises
		mo^{-1}		*days*		*mo*$^{-1}$		*days*
1	24	1.0	17	200	9	0.375	1	49
2	9	0.37	5	63	1	0.04	0	5
3	8	0.33	4	58	1	0.04	0	5
4	13	0.54	4	75	2	0.08	0	8
5	4	0.57	3	35	—			
9	4	0.17	2	34	—			
Total	62	(0.52)	35 (56%)	461	14	(0.11)	1 (7%)	71

carbamylation upon the clinical manifestation of these patients, we determined the frequency of acute painful crises experienced by the patients before and during carbamylation by review of the hospital records of each patient. Patients 2, 4, and 5 obtained their medical care at two local hospitals before beginning carbamylation; the frequency and nature (spontaneous versus induced) of crises were ascertained from a record review for each patient. The admission of a patient to a hospital before or during carbamylation was at the discretion of an emergency room physician not involved in the carbamylation protocol. Interim as well as in-patient medical care precarbamylation for patients 1, 3, 6–10 and all patients during carbamylation was provided by physicians involved in this study. The frequency of acute painful crises during the 12 mo before and 24 mo after the initiation of carbamylation in all 10 patients is portrayed in Fig. 5. Carbamylation in patient 2 was interrupted when she was discovered to be pregnant and was then resumed 4 mo after a successful pregnancy. Patient 7 underwent carbamylation for 3 mo before electing to stop carbamylation. In patient 7, carbamylation was carried out for 3 mo on two occasions. Carbamylation was reinstituted to control recurrent episodes of severe priapism that had disappeared during the first 3 mo of carbamylation. The priapism disappeared a second time during carbamylation. Carbamylation was again stopped when the patient went elsewhere for job training. 8 of the 10 patients underwent repeated carbamylation for a minimum of 24 mo; severe painful crises were a significant or major clinical problem in six of these eight patients. A more detailed analysis of the painful crises in these six patients is presented in Table VI. In the 24 mo before beginning carbamylation, a total of 62 hospitalizations for painful crises occurred among these six patients. The frequency of painful crises ranged from 0.17 to 1 crisis/mo; the average duration of each crisis ranged from 4 to 8 days. Overall, one-half of these crises were spontaneous, with no underlying precipitating event detected. Some quantitation of the crisis-induced disability experienced by the patients can be appreciated by the total cumulative duration of crises for each patient, arbitrarily defined as the length of hospitalization for all admissions prompted by acute painful manifestations. Infrequently, the length of hospitalization was prolonged by therapy of underlying and/or superimposed infectious processes. One-half

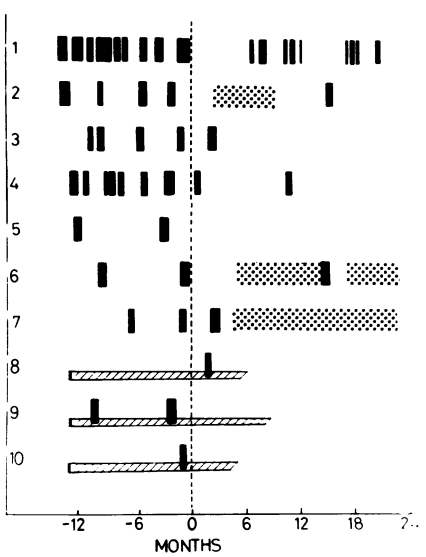

FIGURE 5 Frequency and duration of acute painful crises in 10 patients with sickle cell anemia before and during extracorporeal carbamylation. Each crisis is depicted by a bar; the width of each bar is proportionate to the duration of the crisis. Weekly carbamylation was begun at week 0; subsequent interruptions in carbamylation are shown by the dotted areas in patients 2, 6, and 7. The existence of open, active malleolar ulcerations in patients 8–10 is shown by the horizontal lines.

(27) of the crises occurred in the wake of a precipitating event, in 19 instances a febrile respiratory tract infection and in 4 instances a urinary tract infection. On four occasions, patient 1 presented clinical and radiological manifestations of pulmonary embolism with infarction.

All six patients experienced significantly fewer crises during the 24 mo of carbamylation; a total of 14 admissions for painful crises occurred during carbamylation. The average duration for each crisis during carbamylation was 5 days. As before carbamylation, the frequency of crises in patient 1 grossly exceeded that of the other patients. Only 1 of the 14 crises appeared to be a spontaneous crisis. Respiratory and urinary tract infections ushered in 10 and 3 of the crises, respectively. The rarity of spontaneous crises during carbamylation was striking; the total number of induced crises during carbamylation was one-half that before carbamylation (13 versus 27).

A second major objective clinical response noted during carbamylation was the complete healing of chronic malleolar ulcerations in three patients (8–10). Bilateral, painful ulcerations accompanied by adjacent induration, brawny edema, and recurrent episodes of superficial thrombophlebitis of surrounding areas were present for 2 yr in patient 8, for 4 yr in patient 9, and for 6 yr in patient 10. Transfusions followed by a skin graft in 1969 were of only temporary benfit in patient 10. Local supportive measures, consisting of twice-daily saline soaks followed by dilute hydrogen peroxide debridement, were continued before and during carbamylation. The induration and tenderness of the area adjacent to the ulcerations disappeared after 2–3 mo of carbamylation after which gradual circumferential closure of the open ulcerations occurred. After 5–8 mo of carbamylation (as depicted in Fig. 5), irregular areas of depigmentation of skin persisted as the only residual of the previous ulcerations in the three patients.

We have had the opportunity to follow the clinical response of patient 1 upon stopping carbamylation for 11 mo in 1975. Gaining ready access to the venous circulation was a formidable technical problem throughout the previous 2 yr of carbamylation in this patient. In January 1975, a bovine carotid artery arteriovenous fistula was created in the forearm, but it functioned only temporarily. The consequent loss of forearm veins after this unsuccessful procedure made phlebotomies for red cell carbamylation extremely difficult for several months thereafter. Carbamylation of 10–20% of the red cell mass was accomplished on only 9 of 24 wk between February and June 1975. No further attempts at red cell carbamylation were made until November, when weekly carbamylation was successfully resumed. During the 11 mo ending in November 1975, the patient was hospitalized on nine occasions for a total of 61 days

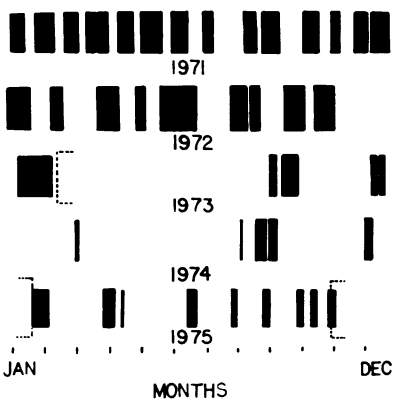

FIGURE 6 5-yr record of hospitalizations for severe painful crises (indicated by bars) in patient 1. Carbamylation was begun in February 1973 and continued to January 1975, when regular carbamylation was interrupted. See text for details. Weekly carbamylation was successfully resumed in November 1975.

because of painful crises. The frequency and total duration of crises during 1975 was essentially doubled that observed during carbamylation in 1973 or 1974 (Fig. 6). Unlike the crises during carbamylation, four of the nine crises in 1975 were spontaneous.

Untoward effects of long-term extracorporeal carbamylation of sickle erythrocytes. We have encountered no evidence of NCO toxicity in patients who have been maintained on weekly red cell carbamylation for up to 3 yr. Systemic toxicity resulting from cyanate administration at the weekly visit would be extremely unlikely because of (*a*) the irreversibility of the carbamylation reaction, (*b*) preferential red cell incubation (plasma removed before incubation), and (*c*) the removal of free NCO by saline washing before reinfusion of the carbamylated erythrocytes.

Several potential problems associated with the phlebotomy exist. Hypotension due to the phlebotomy, clotting, and contamination of the blood during in vitro manipulation are ever-present concerns. Of these potential problems, we have encountered only one. On two occasions 1 pint of blood was lost due to faulty plastic bags, which leaked during centrifugation. Saline infusion before initiating the phlebotomy and reinfusion of the plasma shortly after its collection effectively prevent any hypotension.

Carbamylation increases the affinity of hemoglobin for oxygen; the increase in oxygen affinity depends upon the degree of hemoglobin carbamylation. The increase in oxygen affinity noted during carbamylation, unless accompanied by increases in hemoglobin levels, will decrease the capacity of blood to release oxygen to tissues. We examined the alterations in the oxygen release capacity of blood during carbamylation. By knowing blood hemoglobin and P_{50} values, and assuming uniform equili-

TABLE VII

Alterations in the Capacity of Blood to Release Oxygen Produced by Hemoglobin Carbamylation

Period	Hemoglobin	P_{50}	Oxygen content		Oxygen release capacity
			Arterial*	Venous*	
	g/100 ml	*mm Hg*	*cm³/100 ml*		*cm³/100 ml*
A. Before carbamylation	6.5	33	8.4	4.1	4.3
B. During carbamylation:					
Mo 1	7.6	30	9.9	5.3	4.6
Mo 3	9.2	26	12.3	7.4	4.9
Mo 9	9.8	25	13.2	8.2	5.0
C. Hemodialysis carbamylation					
1.2 mol NCO/mol hemoglobin	6.5	26	8.7	5.2	3.5
2.3 mol NCO/mol hemoglobin	6.5	20	8.8	6.5	2.3
D. Cluster carbamylation (23, 24)	8.0‡	20	10.8	8.0	2.8

* Blood equilibrated with P_{O_2} tensions of 90 (arterial) and 30 (venous) mm Hg.

‡ Data based upon 25% increase in the pretreatment hemoglobin of 6.5 g/100 ml.

bration of arterial and mixed venous blood at oxygen pressures of 90 and 30 mm Hg, respectively, one can calculate the quantity of oxygen that can be released from blood equilibrated under these conditions (oxygen release capacity). Table VII (parts A and B) summarizes the alterations in the mean oxygen release capacity of blood in response to carbamylation in the eight patients who underwent long-term carbamylation. Because the hemoglobin level increased progressively as the P_{50} of blood decreased, the oxygen release capacity of these eight patients actually increased during carbamylation. It is important to note the minimum P_{50} during carbamylation was 25 mm Hg.

DISCUSSION

The ideal mode of approach for carbamylating sickle erythrocytes would be the daily oral administration of a nontoxic reagent which reacted selectively with only the sickle-cell hemoglobin. Unfortunately, such a reagent is currently not available. Widespread protein carbamylation occurs in the body after oral or parenteral administration of sodium cyanate (17–19). Toxicity arising from this indiscriminant protein carbamylation, particularly a dose-dependent polyneuropathy (20, 21), has in essence stopped further clinical trials with orally administered NCO. Our reservations concerning the oral use of sodium cyanate in patients led us to pursue the approach of repeated extracorporeal carbamylation of sickle erythrocytes to avoid systemic toxicity resulting from indiscriminant protein carbamylation.

The studies described in this report demonstrate a significant improvement in the hemolytic anemia of sicklemic patients in whom a mean of 1.3 mol of NCO was incorporated per mole of hemoglobin in about 20% of the circulating red cell mass on a weekly basis. A steady state was reached by the 3rd mo of weekly carbamylation, wherein 35–50% of the circulating erythrocytes were carbamylated. Mean hemoglobin increased 40% during the first 3 mo of weekly carbamylation. Coincident with the increases in hemoglobin, circulating reticulocytes decreased; mean absolute reticulocytes decreased by 58% by the 3rd mo. In addition, MLS increased after 3 mo of carbamylation. The improvement in the hemolytic anemia observed during carbamylation thus appears to result more from decreased destruction than from increased production of erythrocytes. Upon stopping carbamylation, reticulocytes promptly increased while hemoglobin values decreased, both returning to pretreatment levels. This observation lends additional support to the view that decreased destruction of erythrocytes during carbamylation was responsible for the improvement in the anemia.

In addition to the beneficial hematologic response, an obvious decrease in the frequency of severe painful crises and unexpected spontaneous healing of chronic malleolar ulcerations were also noted during carbamylation. What is not so obvious is the importance of the red cell carbamylation per se in effecting these clinical responses. By the nature of the protocol, an important additional variable entered into patient management during carbamylation, namely, more frequent physician-patient contact. Minor clinical manifestations such as a cough, low-grade fever, or laboratory findings such as leukocytosis, pyuria, an increase in blood P_{50} or ISC count, etc., were recognized earlier and more frequently during carbamylation. More aggressive medical management during carbamylation no doubt decreased the frequency of events capable of precipitating crises. The

50% decrease in the frequency of induced crises during carbamylation would seem in great measure due to more aggressive preventive medical management. Such an explanation for the virtual disappearance of spontaneous crises during carbamylation and their reappearance in the one patient after cessation of carbamylation seems much less tenable. Rather, we would attribute this disappearance of spontaneous crises to the carbamylation. Each patient will better serve as his or her own control when at least 1 yr of equally intense medical management without carbamylation is completed. Without first completing this latter type of observation in these patients, it appears medically unsound to embark upon a protocol that entails carrying out sham phlebotomies over several months. It is important to re-emphasize that upon stopping carbamylation, the anemia worsens, and ISC and blood P_{50} values increase impressively. Carrying out repeated 2-U phlebotomies on a patient with a hematocrit of 17–20%, a blood P_{50} of 34–38 mm Hg, and erythrocytes 25% of which are irreversibly sickled is quite a different undertaking than what we are describing in this report.

The findings of a separate study (22, 23) employing extracorporeal red cell carbamylation in four sicklemic patients were discordant with the observations of this report in two critical areas. During 6 mo of treatment in the separate study no decrease in the frequency or severity of painful episodes were noted in three of the four patients. Second, during carbamylation reticulocytes increased by an average of 22% over control period values, red cell production as measured by erythron iron turnover was 17% greater than during the pretreatment period, and approximate twofold elevations of erythropoeitin levels were noted after a carbamylation cluster (23). The protocol described for this study also differed with that reported herein in two important aspects. First, red cell carbamylation was carried out in clusters, usually one unit of blood a day until cyanation had reached two moles per mole of hemoglobin in the blood of the patient and until the P_{50} had dropped to about 20 mm of mercury. When the P_{50} rose to 24 to 26 mm of mercury the carbamylation series was repeated (23). Second, the degree of hemoglobin carbamylation achieved during incubation was much greater, averaging 2.34 mol of cyanate per mole of hemoglobin as opposed to 1.3 mol obtained with our protocol. An additional potentially important difference during the incubation of red cells with NaNCO was that partially oxygenated erythrocytes were incubated at a lower pH (acid citrate dextrose anticoagulant) and at 4°C for 24 h. More alpha chain, less beta chain amino-terminal valine carbamylation and possibly greater epsilon amino-lysine carbamylation of hemoglobin could be anticipated under these latter incubation conditions (4, 5).

A logical explanation exists for the discordant finding of increased red cell production during carbamylation observed in the Seattle study. Because of the extensive hemoglobin carbamylation and the carbamylation schedule (cluster approach) utilized, the ability of erythrocytes to release oxygen to tissues during carbamylation was significantly impaired in the Seattle study. It is informative to compare the alterations in the oxygen release capacity of blood during carbamylation in our protocol with alterations that would occur after a cluster of carbamylation of circulating erythrocytes (Table VII). Had we utilized the Seattle approach of daily (cluster) blood carbamylation until blood P_{50} decreased to 20 mm Hg, the oxygen release capacity of blood would have decreased to approximately half of the pretreatment value after a cluster series, if the hemoglobin increased by the 25% reported (23) (part D, Table VII). With a decrease in blood P_{50} to 20 mm Hg, the hemoglobin level would have to increase from 6.5 to 17.2 g/100 ml to maintain equal oxygen release capacity after carbamylation. In view of these considerations, it is not surprising that reticulocytes and erythrocyte iron turnover increased in response to carbamylation in the Seattle study (23).

It is tempting to speculate that the lack of clinical improvement during carbamylation in the Seattle study also was in part related to the carbamylation protocol. Because of the inordinate oxygen affinity of the carbamylated erythrocytes, enhanced deoxygenation of untreated erythrocytes would be necessary to meet tissue oxygen requirements. Enhanced deoxygenation would predispose the untreated erythrocytes to exaggerated sickling, offsetting any clinical benefit afforded by carbamylation.

The hematologic improvement, the healing of chronic sickle ulcerations of skin, and the decrease in the frequency of severe painful crises noted during carbamylation in this study offer sufficient promise to justify the development of more efficient and more physiological techniques for extracorporeal carbamylation of sickle erythrocytes. In considering future applications of extracorporeal red cell carbamylation in the management of patients with sickle cell anemia, it is essential that the quantity and quality (degree) of hemoglobin carbamylation be carefully controlled to avoid undesirable side effects, such as major decreases in the oxygen release capacity of blood. A potentially useful technique for uniform carbamylation of the entire circulating red cell mass in several hours is hemodialysis-carbamylation. Cyanate can be continuously added to blood, and after incubation, unreacted cyanate can be removed from the blood before its return to the patient by dialysis. During each carbamylation session, the oxygen affinity of blood increases acutely in proportion to the degree of hemo-

globin carbamylation. The increase in oxygen affinity, unaccompanied by an increase in hemoglobin during the carbamylation session, decreases the oxygen release capacity of blood (part C, Table VII). Until hemoglobin levels increase in the severely anemic patient, the degree of carbamylation must be limited to avoid serious embarrassment to tissue oxygenation.

Implicit in the data of this presentation are several important shortcomings of erythrocyte carbamylation as a potential therapy for sickle cell anemia. The degree of inhibition of sickling effected by carbamylation in large measure depends upon the increased oxygen affinity of the carbamylated hemoglobin. Carbamylated sickle erythrocytes will sickle when deoxygenated. Even extensive carbamylation does not restore the life-span of the SS erythrocyte to more than approximately 40–60 days (11, 23). The inordinate oxygen affinity of extensively carbamylated cells presents new problems to the anemic patient. It may be possible to better utilize sodium cyanate as an antisickling agent in future clinical studies by carrying out more selective hemoglobin chain carbamylation. Carbamylation of the amino-terminal valine residue of the beta chains increases (rather than decreases) oxygen affinity and, more important, increases the minimum concentration of hemoglobin required for gelation (24, 25). Alpha chain valine carbamylation increases the affinity of hemoglobin for oxygen. More selective beta chain carbamylation may well allow a greater antisickling effect while minimizing the increase in oxygen affinity.

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