equivalent to the ADCC of purified human blood at the same ratio. This suggests that only a few microliters of whole blood are needed to measure the ADCC, an amount easily obtained from blood drawn for other diagnostic tests in clinical settings. For animal studies, since only a few microliters of blood are needed for testing, one can serially bleed the same small animal without having to kill it to obtain cells.

Recent studies have shown that up to 1×10^6 mononuclear cells can be extracted from human tumor biopsy samples (*10*); with conventional techniques, functional studies of these cells would be impossible. With the FCM assay, functional studies of extremely small cell subpopulations obtained by cell sorting or gradient density techniques can be envisioned. Circulating immune complexes are found in several disease states such as cancer and parasitic infections (*11*). Since ADCC is mediated by a subpopulation of Fc receptor-bearing cells (*1*), it is extremely sensitive to inhibition by immune complexes. We have shown that the ADCC assay by FCM is more sensitive than conventional techniques for detecting immune complexes (*12*); when the number of target cells is reduced to 1000, the amount of serum needed for immune complex detection becomes minimal. These potential applications of ADCC assay by FCM demonstrate the utility and practicality of flow cytometry in clinical immunopathology in terms of prognosis and diagnosis.

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5. Procedures have been described in detail in (*4*). Briefly, the CRBC, with and without antibody to CRBC, were mixed with varying amounts of effector cells to give the desired *E:T* ratio. With 200,000 target cells, the total culture volume was 200 μl; with 1000 target cells, it was 4 μl. At the end of 1 to 2½ hours of incubation at 37°C in a humidified atmosphere, the cultures were harvested by aspirating the contents of a culture into 1 ml of dye solution containing 50 μg of propidium iodide (Calbiochem), 0.1 percent trisodium citrate, 0.01*M* NaCl, and 0.1 percent Nonidet P-40 (Bethesda Research Laboratories). After equilibrating in the dye for at least 15 minutes at 4°C, the samples were analyzed by flow cytometry on an Ortho Cytofluorograf, model FC-200. In this instrument cells are individually illuminated by an argon ion laser (488 nm) at a rate of about 500 cell/sec; as each cell passes the laser beam it emits red fluorescent light in proportion to its nuclear DNA content [P. Noguchi and W. Browne, *J. Histochem. Cytochem.* **26**, 761 (1978)]. The signal generated by each cell is converted to a digital value between 1 and 511 by an analog-to-digital converter and stored in an Ortho 2102 multichannel analyzer. The resulting DNA histogram showed two distinct peaks corresponding to CRBC and effector cells (*4*). The number of each cells in each peak was obtained by integration and the *E:T* ratio for each sample calculated from those values. Percent of killing due to ADCC was calculated as $100 \times [1 - (E:T \text{ control})/(E:T \text{ antibody})]$.
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9 August 1979; revised 17 October 1979

Carboxyhemoglobin Levels in an Unstable Hemoglobin Disorder (Hb Zürich): Effect on Phenotypic Expression

Abstract. *The affinity of Hb Zürich for carbon monoxide is approximately 65 times that of normal hemoglobin. The carboxyhemoglobin content in serum from individuals with Hb Zürich ranged from 3.9 to 6.7 percent in nine nonsmokers and from 9.8 to 19.7 percent in six smokers. Rates of hemolysis and hemoglobin denaturation were less in smokers than in nonsmokers, effects that may be secondary to the stabilization of Hb Zürich by carbon monoxide.*

Unstable hemoglobins constitute approximately one-fourth of the 350 human hemoglobin variants. The hallmark of these disorders is the propensity of the abnormal hemoglobin to denature and precipitate when intact red blood cells or hemolyzates are exposed to heat, redox dyes, or isopropanol (*1*). The first of the unstable hemoglobins to be analyzed structurally, Hb Zürich (HbZ), is a mutant in which the histidine distal to the heme at position 63 in the β chain is replaced by arginine. Since the original description of the hemoglobinopathy (*2*) two additional and unrelated families have been described in Maryland (*3*). Affected individuals are asymptomatic, with normal blood findings except for mild reticulocytosis. Clinical recognition of the disorder most often depends on the occurrence of anemia and jaundice during infections or following the administration of sulfonamides.

A review of clinical and blood findings in 15 individuals with HbZ, observed by us for 20 years, showed variation in the phenotypic expression of the disorder. One subject did not become anemic after exposure to sulfonamides (*3*); serum concentrations of haptoglobin (an α_2-globulin that binds free hemoglobin) and of hemopexin (a β-globulin that binds free heme) ranged from zero to normal (*4*); and thermally induced denaturation of HbZ in intact red cells was slightly increased in some and markedly increased in others (*4*). These observations led to further investigations to identify factors that may alter the stability of the abnormal hemoglobin and, in turn, its clinical presentation.

The rate of heat denaturation of normal hemoglobin (HbA) and some of the unstable hemoglobins is decreased by the addition of carbon monoxide (CO), a ligand that stabilizes the linkage between heme and globin (*1*, *5*). Because the affinity of HbZ for CO is approximately 65 times that of HbA, an indication that most of the CO is complexed with the abnormal hemoglobin (*6*), excessive exposure to CO of subjects with HbZ may be a factor in modifying the phenotypic expression of the hemoglobinopathy.

The amount of CO bound to hemoglobin in packed erythrocytes from persons with and without HbZ was determined by infrared spectroscopy (*7*, *8*). Normal hemoglobin has one infrared absorption maximum for bound CO at 1951 cm^{-1}. In contrast, purified HbZ has two absorption maxima, one at 1951 cm^{-1} and another, which arises from the abnormal β subunit, at 1958 cm^{-1}. The degree of saturation by CO in vivo was measured as the ratio of integrated CO stretch band intensities at 1951 and 1958 cm^{-1} initially and after saturation with CO.

Percentages of carboxyhemoglobin were measured in 15 asymptomatic HbZ subjects from two unrelated families, together with simultaneous determinations of blood counts, serum concentrations of haptoglobin and hemopexin, and the effects of heat and isopropanol on rates of precipitation of the abnormal hemoglobin in intact red cells and hemolyzates (*9*). Carboxyhemoglobin levels are markedly increased in HbZ individuals who smoke and moderately elevated in those who do not smoke; hematocrit values are higher in the smokers than in the

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nonsmokers (Table 1). In addition, HbZ subjects who smoked had a marked reduction in absolute reticulocyte counts, together with normal or moderately reduced serum concentrations of haptoglobin and hemopexin, findings that suggest a decreased rate of hemolysis in these individuals.

The effects of CO in vivo on the stability of hemoglobins in vitro were determined by simultaneous measurements of carboxyhemoglobin in whole blood and rates of hemoglobin precipitation in heated red cells and hemolyzates exposed to isopropanol. In HbZ individuals with high levels of carboxyhemoglobin (subjects I-2 and III-1), formation of hemoglobin precipitates (Heinz bodies) in heated red cells proceeded at significantly slower rates than those observed in nonsmokers (Fig. 1).

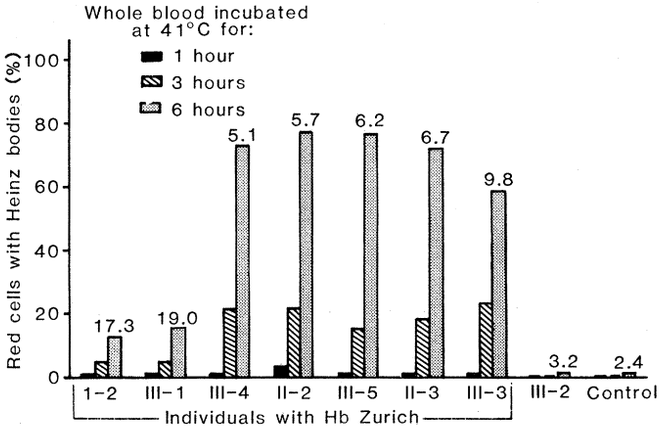

Fig. 1. Relationship between carboxyhemoglobin levels and rate of formation of hemoglobin precipitates (Heinz bodies) in whole blood. Venous blood was collected in EDTA-Vacutainer tubes; 0.1 ml of 5 percent glucose was added to 1.0 ml of blood; and the mixture was incubated for 1, 3, and 6 hours at 41°C. Percentage of Heinz bodies was determined by a previously described method, normal values for 25 normal adult smokers and nonsmokers at 1, 3, and 6 hours being 0, 0, and 8.6 ± 3.1 (standard deviation) percent (*4*). The numbers at the top of the bars represent the percentage of carboxyhemoglobin determined simultaneously in the same samples of blood. The designations of the subjects are identical to those used in previous reports (*3*).

The percentage of hemoglobin precipitated in hemolyzates incubated with 17 percent isopropanol for 20 minutes at 41°C was less in HbZ subjects who were smokers than in those who were nonsmokers. In four nonsmokers with carboxyhemoglobin percentages of 5.5, 5.4, 5.4, and 5.4, the amount of hemoglobin precipitated was 20.8, 21.6, 19.4, and 22.5 percent, respectively. Values for three smokers with carboxyhemoglobin percentages of 14.8, 19.7, and 19.0 were 12.3, 12.0, and 10.7 percent. The amount of hemoglobin precipitated in hemolyzates from six normal nonsmoking adults ranged from 1.7 to 2.1 percent.

The increased content of carboxyhemoglobin in nonsmoking HbZ subjects is probably secondary to endogenous production of CO (4 moles of CO formed for each mole of hemoglobin catabolized). Carboxyhemoglobin levels in subjects with more severe degrees of hemolysis than those observed in HbZ subjects ranged between 1.5 and 2.6 percent (*10*). Thus the higher percentages observed in nonsmoking HbZ subjects provides additional evidence for the increased affinity of CO for HbZ.

Several factors may contribute to the elevated hematocrit values in HbZ subjects who smoke. A portion of the increase could be secondary to the leftward shift of the oxygen-hemoglobin dissociation curve that occurs in normal adults with carboxyhemoglobinemia secondary to smoking—0.4 torr for each 1 percent increase in carboxyhemoglobin (*11*). Although a previous study showed that the affinity of HbZ-containing red cells for oxygen is increased, it is not known whether this phenomenon was secondary to elevation of carboxyhemoglobin (*12*). Any increase in oxygen affinity, whatever the cause, would result in tissue hypoxia and thereby stimulate erythropoesis, a mechanism that has been proposed for other unstable hemoglobins with altered oxygen affinity (*1*). Presumably the hypoxic stimulus is greater in HbZ subjects who are smokers than in those who are nonsmokers.

Another mechanism for hypoxia could be the effects of smoking on pulmonary function. Spirograms and lung volumes in subjects III-1 (smoker) and II-2 (nonsmoker) were normal. Subject I-2 (a smoker for many years) has mild obstructive airway disease, possibly secondary to chronic bronchitis. Since pulmonary function values were normal in subject III-1, a smoker for 3 years with a carboxyhemoglobin percentage of 19.0, other causes for the elevated hematocrit value have to be explored.

Additional findings suggest that the severity of the hemolytic process in HbZ subjects is less in smokers than in nonsmokers; absolute reticulocyte counts (a measure of the rate of erythropoiesis) are reduced, and serum concentrations of

Table 1. Hematologic data and carboxyhemoglobin content in 15 subjects with Hb Zürich from two unrelated families.

Subject	Age (years)	Sex	HbZ	Hematocrit* (%)	Absolute reticulocyte count† (10^9 per liter)	Haptoglobin‡ (mg/dl)	Hemopexin‡ (mg/dl)	Carboxyhemoglobin (%)	Smoking habit§
					Family M				
I-2	62	F	+	48	78	154	50	17.3	2-3
II-2	43	F	+	36	247	0	0	5.7	0
II-3	30	F	+	38	182	9	12	6.7	0
III-1	24	M	+	52	75	132	30	19.0	2-3
III-3	19	M	+	45	206	0	0	9.8	0-1/2
III-4	18	F	+	41	164	0	0	5.1	0
III-5	6	F	+	36	163	0	0	6.2	0
III-2	22	M	−	45	45	140	66	3.2	1/4-1
					Family E				
I-1	55	F	+	46	42	65	49	19.0	1-2
II-3	37	F	+	49	96	98	42	19.7	1-2
II-5	35	F	+	44	87	68	20	14.8	1
III-10	16	M	+	46	182	0	0	5.4	0
III-11	15	M	+	46	217	0	0	5.4	0
III-12	11	M	+	41	173	0	0	3.9	0
III-13	13	M	+	37	229	0	0	5.4	0
III-14	11	M	+	42	254	0	0	5.4	0
					Normal				
1	55	M	−	43	50	182	95	2.4	1-1 1/2
2	24	M	−	44	45	150	69	2.6	1/2
3	26	M	−	46	52	145	72	0.9	0

*Normal values: adult males, 46 ± 3.1; adult females, 40.9 ± 3.0. †Normal values: 10 × 10^9 to 100 × 10^9 per liter. ‡Normal values: for haptoglobin, 100 to 300 mg per deciliter of serum and for hemopexin, 50 to 100 mg per deciliter of serum. §Unit: packages of cigarettes per day.

haptoglobin and hemopexin (sensitive indicators of the degree of hemolysis) are normal or only moderately reduced. Results of in vitro studies on the solubility of HbZ in whole blood and hemolyzates exposed to heat and isopropanol suggest that in vivo elevations of carboxyhemoglobin secondary to smoking may be decreasing the rate of hemolysis by stabilizing the abnormal hemoglobin.

In vitro studies have shown that the binding of CO to hemoglobin in place of O_2 prevents reactions of O_2 that give superoxide and peroxide as well as methemoglobin (*13*, *14*). Exposure of red blood cells with HbZ to certain "oxidant" drugs, especially those of the primaquine or sulfonamide class, results in the ready formation of hemoglobin precipitates (*15*). This hemoglobin denaturation-precipitation process is proposed to follow autoxidation of heme iron from Fe^{2+} to Fe^{3+} (*16*, *17*). The autoxidation can proceed via a two-electron reduction of Hb-bound O_2 to peroxide. One electron is donated by heme Fe^{2+} and one by the drug to give methemoglobin (Fe^{3+}), H_2O_2, and oxidized drug (usually a potentially destructive free radical) (*13*, *18*, *19*). Such a reaction has recently been shown to be much faster for O_2 bound to the abnormal β subunit of HbZ than for O_2 bound to normal subunits (*19*, *20*). Carboxyhemoglobin does not undergo these reactions because CO, unlike O_2, cannot serve as an electron acceptor. Therefore, replacement of O_2 by CO or the prevention of O_2 binding by bound CO can prevent the initiation of a series of destructive processes.

Further studies are necessary to determine whether carboxyhemoglobin in vivo in the range of 20 percent alters the susceptibility of HbZ subjects to the hemolytic effects of drugs or chemicals. Even if it is true, the overall risks of smoking outweigh the benefits. There appears to be no difference in the lifestyle of the smokers and nonsmokers, and one of the long-term smokers has mild chronic bronchitis. Of greater significance is the discovery that CO can modify the characteristics of an unstable hemoglobin in vivo and in vitro and that studies on the function and physicochemical properties of other unstable hemoglobins should include measurements of carboxyhemoglobin levels.

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21. This research was supported by grants HL 07143 and 15890 from the National Institutes of Health. We thank S. M. Dixon and J. Hutchison for their technical assistance.

21 January 1980

Plasminogen Is Synthesized by Primary Cultures of Rat Hepatocytes

Abstract. *The accumulation of rat plasminogen in the medium of primary monolayer cultures of adult parenchymal hepatocytes was detected with a quantitative immunological assay. These primary cultures synthesized and secreted both circulating isozymic forms of plasminogen at rates sufficient to account for the majority of the in vivo plasminogen turnover.*

Plasminogen is the circulating glycoprotein which, upon proteolytic modification, yields the serine protease, plasmin. Circulating plasminogen is composed of two (*1*) non-interconvertible (*2*) isozymic forms that differ in their state of glycosylation (*3*). While the chemistry of the plasminogen molecule and the processes of its activation have been characterized in great detail, much less is known about its metabolism. The zymogen's in vivo site of synthesis has remained controversial and undemonstrated despite decades of investigation. The earliest effort at localizing plasminogen stores was the direct immunofluorescence study of Barnhart and Riddle (*4*). On examination of human biopsy specimens, these investigators found positive fluoresence (interpreted as plasminogen stores) associated primarily with the granules of immature eosinophilic leukocytes. This led them to postulate a role for the eosinophil in the synthesis of plasminogen, but there has been no direct demonstration of plasminogen synthesis by these cells.

Kline and Highsmith (*5*) demonstrated an apparent involvement of the kidney in the restoration of plasminogen levels after a streptokinase-induced "acute depletion." The infusion into cats of large doses of streptokinase resulted in an immediate decrease in the circulating level of plasminogen activity. After a lag period, this activity was found to return to the normal predepletion level. The return to normal could be prevented, or halted in midcourse, by ligating the kidneys of the depleted animal. This finding, coupled with the detection of a gradient of plasminogen activity across the kidney (*5*), led these investigators to conclude that the kidney was the source of plasminogen after "acute depletion." However, their data preclude the conclusion that the kidney is the site of routine plasminogen synthesis, since partially restored animals with ligated kidneys were able to maintain a constant circulating plasminogen level for more than 24 hours [see figure 3 in (*5*)]. The circulating half-life of plasminogen in the rat and rabbit is approximately 12 hours (*2*). The fact that the kidney has no role in the routine synthesis of plasminogen was clearly demonstrated by Siefring and Castellino (*6*), who showed (i) that kidney ligation of rats does not halt plasminogen synthesis and (ii) that anephric humans possess normal circulating levels of both forms of plasminogen. This

0036-8075/80/0718-0408$00.50/0