Cytotoxicity of Chlorinated Hydrocarbons *in Vitro* Observations on Chloroform-Induced Hemolysis (34728)

FRANCESCO BELFIORE[1] AND HYMAN J. ZIMMERMAN

Department of Medicine, Boston University School of Medicine and Medical Service, Veterans Administration Hospital, Boston, Massachusetts 02130; and the Instituto di Patologia Medica E Metodologica Clinica, Universita di Catania, Catania, Italy

Previous studies (1) have shown that exposure of a cell suspension to a known hepatotoxin, carbon tetrachloride, leads to release of intracellular enzymes into the surrounding medium. The rapidity with which the adverse effect occurred in an *in vitro* model consisting of nonhepatic cells suggested that the carbon tetrachloride was able to exert this effect without first undergoing metabolic alteration. Studies in the intact organism, however, have led to the suggestion that the hepatotoxic effect of carbon tetrachloride depends on its being first converted to a toxic metabolite, perhaps a free radical (2).

The present study was designed to test the hypothesis that a hepatotoxic chlorinated hydrocarbon could exert a cytotoxic effect without first being metabolized. For this purpose, the effect of chloroform on a suspension of erythrocytes was selected for study. Erythrocytes were selected as the experimental model, since they do not contain endoplastic reticulum or mitochondria (3) and apparently lack the enzymatic machinery needed to convert the chlorinated hydrocarbon to the hypothetical toxic metabolite (4, 5). Chloroform was selected since it is more soluble than carbon tetrachloride. The data obtained indicate that chloroform induces lysis of erythrocytes and that this effect can be prevented by glutathione. These results have a bearing on the presumed mechanism of chloroform toxicity.

Materials and Methods. Human erythrocytes from normal, healthy adult volunteers were used. A total of 12 donors were used. Each supplied a number of specimens. The number of times each experiment was conducted is indicated for each experiment. Blood was drawn without anticoagulant and immediately diluted with 9 vol of 0.85% saline. This 10% stock suspension was diluted with appropriate amounts of saline solution to provide a concentration of erythrocytes of 2×10^6 cells/ml as determined by enumeration of cells in a hemocytometer. These erythrocytes were exposed to concentrations of chloroform ranging from 1.25×10^{-2} to 10×10^{-2} *M*. Each experiment included a control consisting of erythrocytes in saline without chloroform. In experiments conducted to evaluate the effects of reduced (GSH) or oxidized (GSSG) glutathione, the respective substances were added to erythrocyte suspensions, 10 min before the chloroform was introduced. Before adding the GSH or GSSG, the pH of the respective solutions was adjusted to 7.2.

Chloroform[2] was prepared in concentrations of 1.25, 2, 2.5, 5, and 10×10^{-2} *M* by pipetting the chloroform into the appropriate amount of saline and mixing for 15 min with an automatic shaker. This yielded a clear solution. Each concentration of chloroform shown represents the final calculated one. All experiments were conducted in a volume of 25 ml.

After the chloroform solution was added to the erythrocyte suspensions, the mixture was incubated at room temperature for periods varying from 7 to 45 min as indicated in the respective experiment. At the end of the period of incubation, cells and medium were separated by centrifugation for 10 min at 900*g*

[1] Former Fulbright scholar.

[2] USP Grade (Code 1566) obtained from Allied Chemical, General Chemical Division.

and the hemoglobin (Hb), lactic dehydrogenase (LDH), and malic dehydrogenase (MDH) content of unwashed cells and medium were determined. The LDH activity was determined by the method of Zimmerman and Weinstein (6), as adapted from that of Nielands (7), and the MDH, by the method of Ochoa (8). Enzyme activity was expressed as International Units (IU) per liter or per 10^{10} cells. The Hb was determined by the measurement of optical density at 540 m, after first demonstrating that the spectral curve of oxyhemoglobin was demonstrable in the presence of chloroform. All measurements were made in a Beckman DU-2 Spectrophotometer.

Results. Effects of chloroform. Hemoglobin loss. Exposure to $CHCl_3$ in a concentration of 5×10^{-2} *M* or greater, for 45 min led to almost complete hemolysis, as indicated by the disappearance of hemoglobin and enzymes from the cells and appearance of enzymes in the medium (Fig. 1). Erythrocytes exposed to 5×10^{-2} and 10×10^{-2} *M* chloroform had only 13 and 6%, respectively, of the hemoglobin content of the control preparation. Concentrations of 2.5×10^{-2} *M* and lower had no demonstrable effect on erythrocyte content of hemoglobin.

Enzyme leakage. Lactic dehydrogenase content of the cells was decreased significantly by exposure to $CHCl_3$ concentrations of 5×10^{-2} *M* or greater. At lower concentrations, the LDH content of the cells did not differ significantly from that of the control preparation (Fig. 1). The concentration of LDH in the medium also was increased significantly after exposure of cells to 5×10^{-2} *M* $CHCl_3$. At lesser concentrations, the effects were irregular.

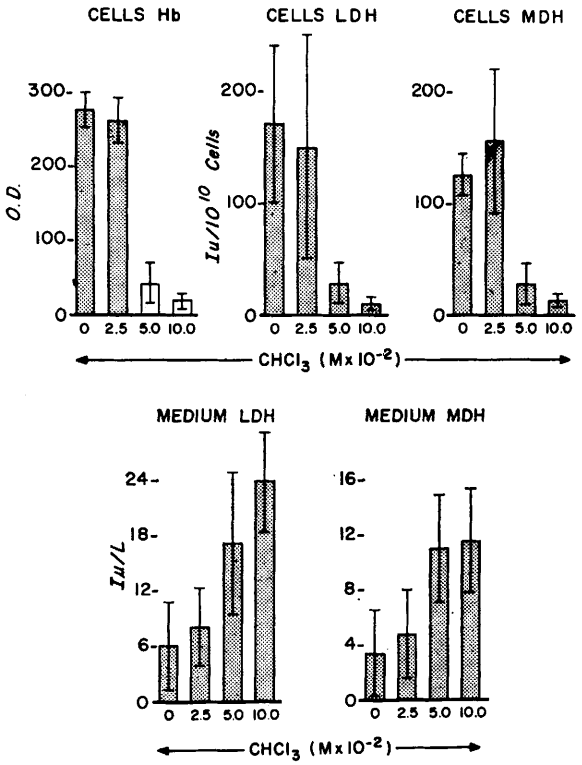

FIG. 1. Effects of $CHCl_3$ on RBC: Loss of hemoglobin (Hb) and of enzymes (LDH and MDH) from erythrocytes (upper panels) and appearance of enzymes in medium (lower panels) after exposure for 45 min to chloroform, in concentrations shown along abscissas. Enzyme values expressed in IU (μmoles of NADH formed/min) /liter or /10^{10} erythrocytes. Each bar shows mean $\pm$ SE of 14 experiments.

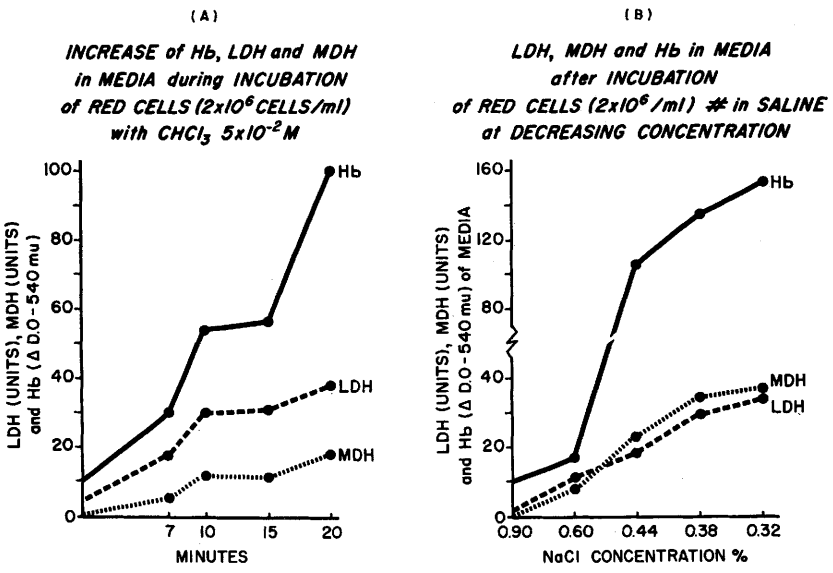

FIG. 2. Rate of leakage of hemoglobin and enzymes (LDH and MDH) from erythrocytes exposed to $CHCl_3$ (left). The Hb, LDH, and MDH in the medium by 20 min, may be compared with amounts released from erythrocytes on exposure to maximally hemolyzing hypoisomolarity (right). Enzyme levels expressed in IU/liter (see Fig. 1 for meaning). Each value represents the mean of four experiments.

Malic dehydrogenase changes in the cells and medium (Fig. 1) were similar to those of LDH.

Effects of duration of exposure on hemolysis. The effects of exposing erythrocytes, for periods ranging from 7 to 20 min, to $CHCl_3$ in a concentration of 5×10^{-2} *M* was studied in four experiments (Fig. 2). Exposure of cells for the briefest period (7 min), measurable under the conditions of the experiment, led to appreciable leakage of Hb, LDH, and MDH. A progressive increase in the amounts of Hb in the medium was found at 10, 15, and 20 min; but by 10 min, most of the LDH and MDH activity, that was to be demonstrated at 20 min, was found in the medium.

Comparison of $CHCl_3$ effects with those of hypotoxicity. The effects of suspending erythrocytes in varying concentration of saline also are illustrated in Fig. 2. In 0.6% saline, there was slight leakage of LDH and MDH but no apparent Hb leakage. Decreasing concentrations of saline led to increasing degrees of enzyme leakage into the medium, that reached a peak at the concentration (0.32%) of total hemolysis. At this concentration, the activity of LDH and MDH was somewhat greater than that observed after hemolysis induced by $CHCl_3$.

Effects of GSSG and other agents on chloroform-induced hemolysis. The effects of incubating erythrocytes with GSSG and GSH, respectively, before exposing the cells to $CHCl_3$ are shown in Figs. 3 and 4. The concentrations of GSSG tested, were 2.5, 1.5, 0.75 and 0.37×10^{-4} *M*. The molarity of the GSH tested was twice that of GSSG, to provide equal numbers of GS group in the two preparations. The data in Fig. 3 indicate that chloroform-induced hemolysis was inhibited by GSSG. The degree of inhibition seemed proportional to the concentration of the GSSG, as measured by loss of Hb from the erythrocytes. At the highest molarity (25×10^{-4} *M*) of GSSG tested, hemolysis was only 14% that of the cells exposed to $CHCl_3$ without GSSG. This proportionality could not be deduced by the leakage of LDH or MDH.

Preincubation of cells with GSH also inhibited hemolysis; but the inhibition as measured by Hb loss, did not increase as the concentration of GSH increased. At the

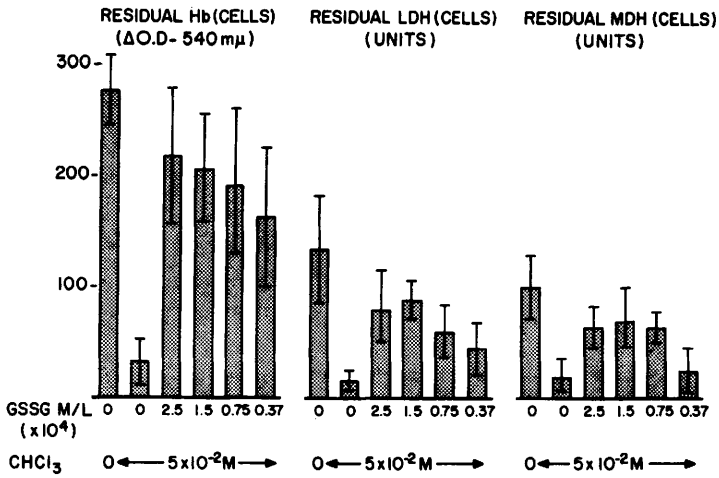

FIG. 3. Inhibition of $CHCl_3$ hemolysis by GSSG: Effect of incubation of erythrocytes with varying concentrates of oxidized glutathione prior to exposure to chloroform on loss of hemoglobin and enzymes (expressed as IU/10^{10} cells). Note that inhibition of chloroform-induced leakage of hemoglobin and of enzymes is maximal at the highest concentrations of glutathione. Each value represents the mean ± SE of 10 experiments.

lowest molarity (0.75×10^{-4} *M*) of GSH employed, only 28% of the erythrocyte Hb was lost; at the highest concentration, the loss was 32%; and in the "unprotected" cells exposed to $CHCl_3$, the Hb loss was 87% (Fig. 4).

Discussion. The present results indicate that chloroform induces lysis of red cells *in vitro*. The concentration of chloroform at which hemolysis was noted ($2.5–5.0 \times 10^{-2}$ *M*) is similar to that at which another chlorinated hydrocarbon, carbon tetrachloride, is able *in vitro* to induce a leakage of enzymes from suspensions of cells from a tissue culture strain of laryngeal carcinoma (1). This concentration is higher than that observed in the liver of the intact animal after toxic doses of CCl_4. In the latter, the concentration was approximately 10 mmoles/kg, that is equivalent to one of 10^{-2} *M* (9). However, in the present study, a concentration of 5×10^{-2} *M* was required to produce complete hemolysis. The concentration of $CHCl_3$ at which *some* degrees of hemolysis was observed, (2.5×10^{-2} *M*), however, *in vitro* approaches that of CCl_4 demonstrated in the liver *in vivo*. The rapidity of the hemolytic effect of $CHCl_3$ (less than 7 min), resembles that of the adverse effect of CCl_4 in *in vitro* systems (1, 10) and in the whole animal (4, 14).

The erythrocytes are very simplified cells which lack nucleus, mitrochondria, and endoplasmic reticulum (3). Since the enzymatic machinery for metabolizing chlorinated hydrocarbons is associated with the endoplasmic reticulum (4, 5), it is unlikely that erythrocytes can metabolize the chloroform to a toxic derivative. The ability of chloroform to injure these cells is not consistent with the hypothesis that the chlorinated hydrocarbons injure cells and their membranes *only* after their conversion to a toxic metabolite. Indeed, the data suggest that the adverse effect on the red cell is induced by the unmodified chloroform molecule. These observations support the deduction from *in vitro* studies with CCl_4 (1) that chlorinated hydrocarbons can be cytotoxic even without conversion to a free radical or other toxic metabolite; although there is ample evidence that metabolism of CCl_4 enhances its hepatotoxic effects (4, 5).

Studies of the hepatotoxicity of CCl_4 have focused on the injury to mitochondria, endoplasmic reticulum, or lysosomes (4, 12–17). The ability of chloroform to injure the red cells, which lack these subcellular particles, suggests that it also can exert adverse effects

on other components of the cell namely the plasma membrane. It seems reasonable to infer that the cytotoxicity of other chlorinated hydrocarbons may be the expression of injury of the plasma membranes as well of intracellular organelles.

The precise mechanism by which the chloroform induces the injury, is unclear. Injury of membranes might result from interference with metabolic pathways needed to maintain their integrity or from direct alteration of their lipid or protein components. The *promptness* of *erythrocyte* damage after brief exposure to $CHCl_3$, suggests that the cellular injury does not depend on interference with active metabolic pathways but rather on an effect on the cell membrane. The possibility that $CHCl_3$ acts directly to produce general membrane damage, is consistent with some current views (2, 4, 5, 18, 19), but remains to be proved. Damage to hepatocytes in carbon tetrachloride poisoning has been attributed to peroxidation of lipids of the membranes, induced by a metabolite of CCl_4, perhaps a free radical (4). Chloroform itself, is not an oxidizing agent and metabolic conversion to one by the erythrocyte is unlikely. Like other solvents, however, $CHCl_3$ might be able to lead to non-oxidative denaturation of membrane protein. This suggestion is supported by the studies of Dianzani *et al.* (19), who reported that chlorinated hydrocarbons alter serum lipoproteins by increasing the density of the protein fraction.

The possibility that the cytotoxicity of $CHCl_3$ is mediated, at least in part through the effects on glutathione metabolism is suggested by the protective effects of adding glutathione to the system. The inhibition of chloroform-induced hemolysis by both reduced and oxidized glutathione, however, cannot be attributed to the known ability of GSH to prevent oxidative denaturation of proteins. The effectiveness of GSSG and the improbability of reduction by unaltered $CHCl_3$ requires another interpretation for

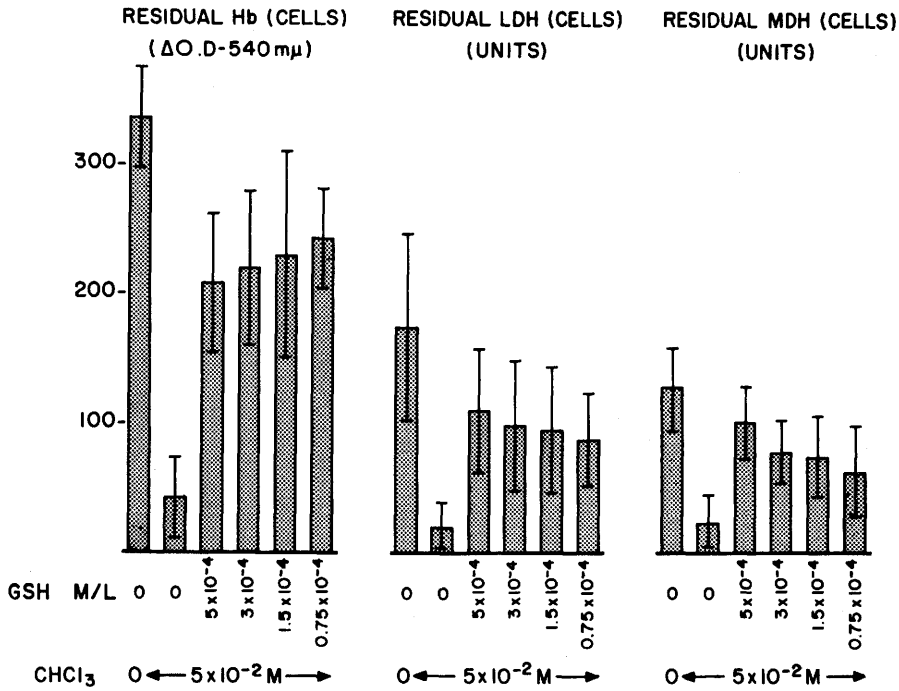

FIG. 4. Inhibition of $CHCl_3$ hemolysis by GSH: Effect of incubation of erythrocytes with varying concentrations of reduced glutathione, prior to exposure to chloroform, on loss of hemoglobin and enzymes (expressed as IU $\times$ 10^{10} cells). Inhibition of leakage of hemoglobin and enzymes was approximately equal at all concentrations of glutathione. Each value represents the mean $\pm$ SE of 10 experiments.

the role of glutathione in this system. It is possible that both forms of glutathione interfere with nonoxidative denaturation by providing a physicochemical, rather than metabolic effect.

The presently reported observations of an *in vitro* hemolysis by $CHCl_3$ and the demonstration of *in vitro* cytotoxicity by CCl_4 for other nonhepatic cells (1) are consistent with the view that the hepatotoxicity of chlorinated hydrocarbons is a product of the concentration in the liver. It appears that these agents can damage a variety of cells, effecting the damage through alteration of membranes at the surface and in the interior of the cells. The evidence that hepatic conversion of the chlorinated hydrocarbon to a metabolite enhances the hepatotoxicity (2, 4, 5) provides additional explanation for the particular vulnerability of the liver to agents that are also general cytotoxins.

Summary. The effect of chloroform on a suspension of erythrocytes was studied, in order to test the hypothesis that a hepatotoxic chlorinated hydrocarbon could exert a cytotoxic effect without first being metabolized. Chloroform in a concentration of 5×10^{-2} *M* was found to induce hemolysis and leakage of lactic dehydrogenase and malic dehydrogenase from the red cells, effects that could be partially inhibited by reduced or oxidized glutathione. These data support the view that $CHCl_3$ can have a directly adverse effect on a cell membrane, although they do not preclude a role of a $CHCl_3$ metabolite in hepatotoxicity in the intact animal.

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