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IRON RELEASE AND ERYTHROCYTE DAMAGE IN ALLYL ALCOHOL INTOXICATION IN MICE

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Abstract—Allyl alcohol administration in a toxic dose (1.5 mmol/kg) to starved mice causes the development of hemolysis in nearly 50% of the animals. Malonic dialdehyde (MDA) appears in plasma of the animals showing hemolysis. The treatment of mice with desferrioxamine after allyl alcohol intoxication completely prevents lipid peroxidation and hemolysis, suggesting the involvement of iron in the allyl alcohol-induced erythrocyte damage. Erythrocytes obtained from intoxicated mice before the development of hemolysis show, upon incubation, release of iron, lipid peroxidation and lysis. Studies carried out with reconstituted systems of erythrocyte lysates, containing ghosts and different fractions of erythrocyte cytosol and incubated in the presence of acrolein (the major metabolite of allyl alcohol), strongly suggest that iron is released from hemoglobin. This iron appears to promote lipid peroxidation which is accompanied by erythrocyte lysis. Thus, the allyl alcohol-induced hemolysis appears to be a model for iron delocalization from iron stores.

Allyl alcohol is mainly metabolized in the liver by the cytosolic enzyme alcohol dehydrogenase to acrolein and other compounds [1, 2]. Acrolein, the most toxic member of the class of 2-alkenals [3, 4] may react spontaneously with nucleophiles, such as sulphydryl groups [5]. Cellular glutathione (GSH)§ is primarily involved in this reaction, and the result is a dramatic loss of GSH stores [3, 6–8], followed by hepatocellular necrosis. In addition, it has been suggested that allyl alcohol metabolites, released from the liver cell, cause damage to extrahepatic tissues [9].

We have previously observed [10] that allyl alcohol administration to mice produces, within a short period of time (15 min), GSH depletion in erythrocytes. This is followed by the appearance of malonic dialdehyde (MDA) in plasma and by a marked hemolysis.

In vitro studies [10] have shown that the addition of acrolein to mouse erythrocytes also produces a dramatic GSH depletion, which is followed by the development of lipid peroxidation and hemolysis. Inhibition of lipid peroxidation by an antioxidant (trolox C) or an iron chelator (desferrioxamine, DFO), prevents hemolysis despite a comparable GSH depletion. Studies on the mechanisms concerned with the induction of lipid peroxidation showed that a progressive increase in DFO-chelatable iron occurs in erythrocytes during the incubation with acrolein [10]. Thus an iron release from iron stores occurs in erythrocytes as a result of the interaction of acrolein with cellular molecules.

The aim of the present study was to investigate whether a similar release of iron also occurs in erythrocytes, after *in vivo* intoxication with allyl alcohol. In addition, experiments were designed to determine the iron store from which iron is released. The results indicate that the *in vitro* model previously studied reproduces the effects of the *in vivo* intoxication, and suggest that hemoglobin is the major source of iron release in erythrocytes.

MATERIALS AND METHODS

Desferrioxamine (Desferal,® DFO) was kindly supplied by Ciba-Geigy (Basel, Switzerland). The solvents used for high-pressure liquid chromatography (HPLC) analysis were of HPLC grade. All other chemicals were of analytical grade.

Male Swiss albino mice (Nossan, Correzzana, Milan, Italy) weighing 25–35 g and maintained on a pellet diet (Nossan) were used. The animals were starved for 16 hr before being used. Starvation decreases the GSH stores of liver cells and renders the animals more susceptible to the toxic effects of GSH-depleting agents.

In a first group of experiments (reported in Table 1), the animals received allyl alcohol (1.5 mmol/kg body wt; i.p.) dissolved in saline, or an equivalent volume of saline. Some of the intoxicated animals received DFO (50 μmol/kg body wt, dissolved in saline; i.p.) 10 and 40 min after allyl alcohol treatment. All animals were killed 1–2 hr after the intoxication. Blood was withdrawn from the abdominal aorta under ether anesthesia and heparinized. An aliquot was used for GSH determination according to Beutler *et al.* [11]. Another aliquot was centrifuged (800 *g* for 10 min) and the MDA content of plasma was determined after protein precipitation with trichloroacetic acid (final concentration 5%), by the addition of thiobarbituric acid [12]. The percentage of hemolysis was determined by measuring the optical density at 540 nm of the diluted plasma and comparing this value with the optical density of diluted blood completely hemolyzed by the addition of a hypotonic solution of EDTA (2.7 mM).

In a second group of experiments (reported in

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§ Abbreviations: GSH, glutathione; MDA, malonic dialdehyde; DFO, desferrioxamine; HPLC, high-pressure liquid chromatography.

Table 1. Hemolysis, erythrocyte GSH and plasma malonic dialdehyde (MDA) in mice intoxicated with allyl alcohol and administered either desferrioxamine (DFO) or saline

	No. of animals	Hemolysis (% relative to 100% hemolysis)	GSH (nmol/mL)	MDA (nmol/mL)
Controls	9	1.4 ± 0.5	1080.3 ± 77.1	—
Allyl alcohol (Group 1)	12	1.1 ± 0.2	273.9 ± 15.8	1.7 ± 0.3
Allyl alcohol (Group 2)	12	36.8 ± 4.0	214.7 ± 9.2*	19.0 ± 3.2
Allyl alcohol + DFO	18	1.6 ± 0.5	279.2 ± 15.3	1.1 ± 0.2

Allyl alcohol was given i.p. at a dose of 1.5 mmol/kg body wt. DFO was given i.p., 10 and 40 min after the intoxication, at a dose of 50 μmol/kg body wt. The animals were killed 1–2 hr after the intoxication. Group 1 is defined as having a level of hemolysis < 2% of complete hemolysis. Group 2 is defined as having a level of hemolysis > 2% of complete hemolysis. Results are given as means ± SE. Control values for MDA were subtracted from those of the intoxicated animals.

* Significantly different from both allyl alcohol (Group 1) and allyl alcohol + DFO, $P < 0.01$.

Table 2. Release of iron (DFO-chelatable iron), lipid peroxidation (malonic dialdehyde (MDA) formation) and hemolysis in erythrocytes obtained from allyl alcohol treated (AA-erythrocytes) or control (control erythrocytes) mice

	Incubation time (min)	DFO-chelatable iron (nmol/mL)	MDA (nmol/mL)	Hemolysis (%)
A. Control erythrocytes + DFO	0	2.6 ± 0.1	—	4.2 ± 0.4
	120	2.7 ± 0.3	0.4 ± 0.2	4.2 ± 0.4
B. AA-erythrocytes + DFO	0	2.7 ± 0.3	—	3.0 ± 0.4
	120	5.8 ± 0.5*	3.0 ± 1.3	11.4 ± 4.6
C. Control erythrocytes	0	2.6 ± 0.1	—	3.1 ± 0.7
(no DFO present during incubation)	120	2.4 ± 0.4	0.8 ± 0.2	3.8 ± 0.6
D. AA-erythrocytes	0	2.7 ± 0.3	—	4.7 ± 0.8
(no DFO present during incubation)	30	ND	25.1 ± 0.9	24.0 ± 4.7
	60	ND	38.4 ± 3.7	64.0 ± 3.4
	120	11.1 ± 1.4*	40.9 ± 4.0	101.0 ± 7.4

Erythrocytes were withdrawn from allyl alcohol poisoned mice, 15 min after the intoxication. DFO was added at 0 min to samples A and B. DFO was added at 120 min to samples C and D. Results represent the means ± SE of five experiments. Zero time values for MDA were subtracted from those of the incubated samples.

* Significantly different from the respective zero time value, $P < 0.001$.

ND: not determined.

Table 2), the animals were killed 15 min after receiving allyl alcohol as above. Washed erythrocytes were incubated as a 50% (v/v) suspension in 0.123 M NaCl, 28 mM K–Na phosphate buffer, pH 7.4. Iron contamination was removed from the buffer as previously described [10]. DFO was added (50 μM, final concentration) to the incubation system to measure the iron released from iron stores as a DFO–iron complex (see below). The addition of DFO was performed at the beginning of the incubation to chelate iron released from iron stores during the entire incubation period. Since DFO inhibits lipid peroxidation, in other experiments DFO was added at the end of the incubation to determine whether lipid peroxidation and hemolysis developed in these erythrocytes. Aliquots were withdrawn at the indicated times for the determination of DFO-chelatable iron, MDA and hemolysis. DFO-chelatable iron was determined as a DFO–iron complex. Briefly, the erythrocytes were hemolysed, the hemolysate was centrifuged and the supernatant was ultrafiltered as previously reported [10]. The determination of the DFO–iron complex was performed by HPLC according to Kruck *et al.* [13] with a number of modifications reported in detail in the previous paper [10].

In the experiments designed to investigate the possibility that iron was released from hemoglobin (reported in Table 3), washed erythrocytes from starved, non-intoxicated mice, were resuspended in the NaCl–K–Na phosphate buffer as above and hemolysed by freezing (−70°)–thawing. The ghosts were pelleted from the hemolysate at 60,000 *g* for 25 min. The supernatant fraction was recovered and the ghosts were washed twice with the same buffer. The supernatant fraction was ultrafiltered in an ultrafiltration cone (Centriflo CF 25, Amicon, molecular

Table 3. Acrolein-induced release of iron (DFO-chelatable iron) and lipid peroxidation (malonic dialdehyde (MDA) formation) in reconstituted systems of erythrocyte lysate containing ghosts, and different fractions (ultrafiltrate and protein moiety) of cytosol

Incubation system	Incubation time (min)	DFO-chelatable iron (nmol/mL)	MDA (nmol/mL)
A. Ghosts + aproteic ultrafiltrate + DFO	0	2.2 ± 0.5	—
	120	2.8 ± 0.4	0
B. Ghosts + aproteic ultrafiltrate + acrolein + DFO	120	2.9 ± 0.5	0
C. Ghosts + aproteic ultrafiltrate + protein moiety + DFO	0	2.5 ± 0.4	—
	120	3.6 ± 0.6	0
D. Ghosts + aproteic ultrafiltrate + protein moiety + acrolein + DFO	120	7.1 ± 1.0*	0
E. Ghosts + aproteic ultrafiltrate + protein moiety + acrolein (no DFO present during the incubation)	120	12.9 ± 2.6†	23.9 ± 5.0
F. Whole erythrocytes + acrolein + DFO	120	5.4 ± 0.5	0
G. Whole erythrocyte lysate + acrolein + DFO	120	6.1 ± 0.6	0

DFO was added at 0 min to samples A, B, C, D, F, G and at 120 min to sample E. Acrolein, where present, was added at a concentration of 6 mM. Results represent the means ± SE of four experiments. 0 min values for MDA were subtracted from those of the incubated samples.

* Significantly different from C, 120 min value, $P < 0.05$.

† Significantly different from D, 120 min, $P < 0.05$.

weight exclusion 25,000 daltons) by centrifugation at 700 *g* for 30 min. In this way the supernatant fraction was further fractionated into two subfractions: the hemoglobin free ultrafiltrate and the concentrate (on the filter). The supernatant fraction contained 100 ± 4 mg of hemoglobin/mL (assayed using the cyanmethemoglobin method [14]); the concentrate contained 212 ± 6 mg of hemoglobin/mL; the ultrafiltrate contained no hemoglobin nor proteins detectable by SDS–PAGE, according to Laemmli [15]. The amount of iron detectable as a DFO–iron complex in the ultrafiltrate was 2.02 ± 0.23 nmol/mL.

The ghosts equivalent to 1 mL of packed cells were resuspended with the ultrafiltrate equivalent to the same amount of packed cells. The mixture was incubated in the presence or absence of acrolein (final volume, 2 mL). In parallel experiments the ghosts (equivalent to 1 mL of packed cells) were resuspended with both the ultrafiltrate and the concentrate (each equivalent to 0.5 mL of packed cells) and incubated as above. Desferrioxamine was added at the beginning or the end of the incubation, as for the experiments with the whole erythrocytes (reported in Table 2). DFO-chelatable iron and MDA were determined as above.

RESULTS

In agreement with previous results [10], a severe hemolysis was observed in about 50% of the allyl alcohol intoxicated animals at 1–2 hr of intoxication (Table 1). A marked decrease in erythrocyte GSH was seen in all the intoxicated animals. Such a decrease was higher in the animals showing hemolysis (group 2) compared with the animals in which the hemolysis was virtually absent (<2%) (group 1). In the animals exhibiting hemolysis, a substantial amount of MDA was detected in plasma (Table 1). MDA appearance is indicative of the development of lipid peroxidation in erythrocyte membranes, as shown in the previous report [10]. Treatment of the intoxicated animals with DFO almost completely prevented both lipid peroxidation and hemolysis. In these animals the GSH content of erythrocytes was similar to that observed in the intoxicated animals not showing hemolysis and significantly higher than that observed in the intoxicated animals showing hemolysis (Table 1). This result suggests that the erythrocyte damage is in some way related to an iron release from iron stores.

It is obviously impossible to measure iron release from erythrocytes *in vivo*, since the DFO–iron complex could be formed in hepatic and extrahepatic cells and is rapidly excreted in urine and bile [16, 17]; therefore we examined the ability of erythrocytes from allyl alcohol-intoxicated mice to release iron and to undergo lipid peroxidation and hemolysis upon incubation. Erythrocytes were withdrawn from mice as early as 15 min after allyl alcohol intoxication, i.e. at a time when GSH depletion was already at a maximum, but virtually no hemolysis nor lipid peroxidation were evident. These erythrocytes were incubated, for 2 hr at 37°, under physiological conditions (Table 2). As can be seen (Table 2; B),

the amount of DFO-chelatable iron increased during the incubation (as documented by the increase in the DFO–iron complex measured by HPLC). Lipid peroxidation was minimal in these samples because of the addition of DFO (at time 0) necessary to chelate free iron. Likewise, hemolysis was relatively low (nearly 10%) and was probably due to an increased fragility of the erythrocytes of the intoxicated animals. When DFO was added at the end of the incubation (Table 2; D), the amount of iron detectable as a DFO–iron complex was markedly higher than in the samples (Table 2; B) in which lipid peroxidation did not occur. Lipid peroxidation and hemolysis rapidly developed in the absence of DFO (Table 2; D). It seems, therefore, that the interaction of allyl alcohol with erythrocytes *in vivo* makes them more likely to release iron from iron complexes. Such iron appears to promote lipid peroxidation which, in turn, leads to a further release of chelatable iron.

The finding of an iron release raised the obvious question of where the iron was released from, and therefore, the possibility that iron was released from hemoglobin was investigated.

To this end, erythrocytes from normal mice were hemolysed, and the ghosts and the cytosol separately recovered. In a first set of experiments, erythrocyte ghosts were incubated with deproteinized cytosol and purified mouse hemoglobin, either in the presence or absence of acrolein. These experiments gave no helpful indication, because the prepared hemoglobin itself was found to contain significant amounts of DFO-chelatable iron. Similar results were obtained with the use of commercial bovine hemoglobin.

In further experiments the supernatant fraction of erythrocytes, after removal of ghosts, was subfractionated by ultrafiltration (see Materials and Methods) into an ultrafiltrate and a concentrate (hereafter referred to as "protein moiety"; proteins of mol. wt > 25 kDa). The ghosts were incubated either with the ultrafiltrate alone, or the ultrafiltrate and the protein moiety. As shown in Table 3, when the ghosts were incubated with the ultrafiltrate alone in the presence of acrolein (Table 3, B), no release of iron was seen. When, on the other hand, the ghosts were incubated with the ultrafiltrate and the protein moiety, in the presence of acrolein (Table 3; D), a release of iron comparable to that obtained with the whole erythrocytes (Table 3; F) or with the whole erythrocyte lysate (Table 3; G) was observed. Lipid peroxidation was again inhibited (Table 3; D), because of the addition of DFO (necessary to chelate iron) at the beginning of incubation. However, when DFO was added at the end of the incubation (Table 3; E), lipid peroxidation markedly developed in the reconstituted system, and the amount of released iron was higher as compared to the samples in which lipid peroxidation was inhibited by the presence of DFO.

These results clearly indicate that during the interaction of acrolein with erythrocyte molecules, iron is released from the protein moiety. The basal content of DFO-chelatable iron in the ultrafiltrate does not promote lipid peroxidation in ghosts (not shown in Table 3). Acrolein cannot cause a release of iron and promotion of lipid peroxidation when the protein moiety is absent. Since hemoglobin constitutes nearly 99% of non-membrane protein [18], it is reasonable to assume that iron is released from hemoglobin. Of course, the direct demonstration for this should come from experiments carried out with reconstituted systems in which purified mouse hemoglobin is present or absent. As previously stated, these experiments were unsuccessful. However, the hypothesis that hemoglobin is the source of released iron is also supported by the results of experiments (Table 4) in which a progressive increase of DFO-chelatable iron was seen by incubating erythrocyte lysates with increasing amounts of acrolein. In these experiments the amounts of released iron (up to 26 μM) are compatible with a quantitatively important iron store. Such an iron store in the erythrocytes can only be hemoglobin, since the other iron containing proteins (catalase, diaforase) could not, by themselves, account for such a high release of iron.

Table 4. Release of iron (DFO-chelatable iron) in erythrocyte lysates incubated with different concentrations of acrolein

Incubation system	Acrolein concentration (mM)	DFO-chelatable iron (nmol/mL)
Erythrocyte lysate + acrolein	3	3.3 ± 0.2
	6	8.5 ± 0.6
	15	9.0 ± 0.4
	30	17.6 ± 0.8
	60	26.5 ± 0.4

The incubation time was 120 min. DFO was added at 0 min. Results are given as means ± SE of three experiments.

DISCUSSION

A great deal of experimental evidence suggest that in a number of pathological conditions in which an oxidative stress is involved, iron is released from iron stores and that such "iron delocalization" induces cellular damage both by generating hydroxyl radicals through the Fenton reaction and by promoting lipid peroxidation [19–24]. Normally, iron is transported and stored in specific proteins (transferrin, ferritin, lactoferrin and haem proteins) which prevent its reaction with reduced oxygen metabolites [25, 26]. However, in spite of these safeguards, increasing evidence suggests that reactive iron becomes available during some disease states. A number of studies have shown that the specific iron chelator DFO can modify tissue changes observed in inflammatory, degenerative and ischemic damage [27–31]. In model systems *in vitro* it has been shown [32] that the superoxide anion ($O_2^{\cdot-}$) generated by xanthine oxidase promotes release of iron from ferritin, and that this released iron can promote lipid peroxidation in phospholipid liposomes. Also, it has been shown that liver microsomes contain ferritin [33], and that $O_2^{\cdot-}$ generated by paraquat via redox-cycling releases

iron from ferritin added to ferritin-free microsomes [34]; this iron then promotes peroxidation in microsomal lipids. Release of iron from ferritin [35, 36] or from other non-heme, non-ferritin iron sources [37–39] has also been described under various experimental conditions.

Here, and in a previous report [10], we have shown the possibility that an iron release from iron stores occurs under conditions in which an oxidative stress is imposed on intact cells, and that such free iron induces lipid peroxidation and cellular damage. The model cell used in these studies was the erythrocyte, which is a unique biological structure containing high concentrations of iron, oxygen and unsaturated fatty acids in the ligand state. The experimental approach was an *in vitro* system in which mouse erythrocytes were incubated with acrolein, which reacts immediately with erythrocyte GSH producing a dramatic GSH depletion. The use of acrolein, the major metabolite of allyl alcohol, derived from the observation that allyl alcohol administration to mice produces a high incidence of hemolysis. This was accompanied by a dramatic depletion of erythrocyte GSH by lipid peroxidation. Here it is shown that the administration of DFO to the intoxicated animals prevents both lipid peroxidation and hemolysis, indicating that the allyl alcohol-induced hemolysis is mediated by lipid peroxidation. The possibility that an iron release from iron stores is in some way related to the erythrocyte damage is also indirectly inferred.

Our reports indicate that in intact erythrocytes, or in an erythrocyte lysate, or in a reconstituted erythrocyte system acrolein can induce a release of iron in a form that is readily chelatable by added DFO. When DFO is added at the beginning of the incubation, lipid peroxidation does not occur and virtually no hemolysis is observed. On the other hand, when DFO is added at the end of the incubation (i.e. the incubation is carried out in the absence of DFO), lipid peroxidation and hemolysis rapidly develop probably because iron is now available to initiate lipid peroxidation through an iron oxygen complex as postulated by Aust [40, 41]. Under these conditions the release of iron is higher than in the samples in which lipid peroxidation does not occur. Thus lipid peroxidation leads to a further release of iron from complexes. In liver microsomes incubated in the NADPH–Fe dependent system, it has been shown that lipid peroxidation leads to a rapid degradation of heme and hemoprotein cytochrome P-450 [42–44]. If some heme degradation occurs as a consequence of lipid peroxidation in erythrocytes, then some iron may be released from degraded heme and may amplify the extent of lipid peroxidation.

An interesting feature of the present report is the indication that iron is released from hemoglobin during the incubation of erythrocytes with acrolein. It has been suggested that heme released from hemoglobin is capable of initiating a number of oxidative reactions in the erythrocyte [45, 46]. It is not certain, however, whether these effects are due to heme itself or iron released from the molecule. Further studies in our laboratory will investigate this point.

Another point which needs to be clarified is whether the acrolein-induced iron release is the result of GSH depletion, or whether GSH simply acts to protect hemoglobin from the interaction with acrolein, so that such interaction can occur only when erythrocyte GSH is almost completely consumed. Even if the latter occurrence seems more likely, it must be considered that H_2O_2, which can easily be formed in a GSH-depleted cell, can release iron from hemoglobin as shown by Gutteridge [47].

Another interesting feature of the present report is the demonstration that a release of iron similar to that observed during the interaction of erythrocytes with acrolein *in vitro*, also occurs in red blood cells after the *in vivo* intoxication with allyl alcohol. Again, lipid peroxidation developed in these cells and this process led to a further release of DFO chelatable iron. Thus, on the whole, these studies indicate that allyl alcohol-induced hemolysis represents a model of iron delocalization and iron-induced cell damage. Very early results from our laboratory seem to indicate that a release of iron from iron complexes also occurs in red blood cells during aging.

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