Comparison of human red cell lysis by hypochlorous and hypobromous acids: insights into the mechanism of lysis

Margret C. M. VISSERS[1], Anitra C. CARR and Anna L. P. CHAPMAN

Free Radical Research Group, Department of Pathology, Christchurch School of Medicine, P.O. Box 4345, Christchurch, New Zealand

Human red blood cells are lysed by the neutrophil-derived oxidant hypochlorous acid (HOCl), although the mechanism of lysis is unknown. Hypobromous acid (HOBr), a similarly reactive oxidant, lysed red cells approx. 10-fold faster than HOCl. Therefore we compared the effects of these oxidants on thiols, membrane lipids and proteins to determine which reactions are associated with lysis. There was no difference in the loss of reduced glutathione or membrane thiols with either oxidant, but HOBr reacted more readily with membrane lipids and proteins. Bromohydrin derivatives of phospholipids and cholesterol were seen at approx. one-tenth the level of oxidant than chlorohydrins were. However, these products were detected only with high concentrations of HOCl or HOBr, which caused instant haemolysis. Membrane protein modification occurred at much lower doses of oxidant and was more closely correlated with lysis. SDS/PAGE analysis showed that band 3, the anion transport protein, was lost at the lowest dose of HOBr and at the higher concentrations of HOCl. Labelling the red cells with eosin 5-maleimide, a fluorescent label for band 3, suggested possible clustering of this protein in oxidant-exposed cells. There was also irreversible cross-linking of all the major membrane proteins; this reaction occurred more readily with HOBr. The results indicate that membrane protein modification is the reaction responsible for HOCl-mediated lysis. These effects, and particularly cross-link formation, might result in clustering of band 3 and other membrane and cytoskeletal proteins to form haemolytic pores.

INTRODUCTION

The hypohalous acids (HOX) are strong oxidants produced *in vivo* by phagocytic cells [1,2]. These cells (neutrophils, monocytes and eosinophils) contain peroxidases that catalyse the formation of HOX from H_2O_2 and a halide (X^-). Myeloperoxidase, the enzyme present in neutrophils and monocytes, will utilize Cl^-, Br^-, I^- and the pseudo-halide thiocyanate (SCN^-) [2,3]. However, Cl^- is the preferred substrate under physiological conditions, resulting in the production of hypochlorous acid (HOCl) [2,3]. Eosinophil peroxidase does not react readily with Cl^-; under physiological conditions it uses Br^- to form hypobromous acid (HOBr) and SCN^- to form hypothiocyanous acid (HOSCN) [4,5].

HOX are highly reactive and their toxicity has been well documented [2,6]. HOCl has been most frequently studied, and it is directly toxic to bacteria [6,7], endothelial cells [8], tumour cells [9] and red cells [10–12]. However, because HOCl reacts readily with a range of biological targets, it has been difficult to identify which reactions are critical for the cytotoxic effect. Although thiol groups are particularly reactive [13,14], many cells can repair oxidized thiols [14,15]. We have shown that regeneration of thiols did not prevent HOCl-mediated red cell lysis [14], and it is not clear whether thiol oxidation results in irreversible cell injury. Other reactive groups are Fe–S centres [7], thiolate bonds in zinc proteins [16] adenine nucleotides [17,18], alkenes [19,20] and amino groups, producing chloramines that are also strong oxidants [1,13]. The reaction with unsaturated fatty acids and cholesterol results in the formation of chlorohydrins [19,21], which are potentially destabilizing to the cell membrane and might cause permeability changes. The chemistry of HOBr is similar to that of HOCl [22,23] but little is known about its reactivity in biological systems. Bromamines are formed with amino groups [22], HOBr can react with H_2O_2 to give singlet oxygen ($O_2{}^1$) [24], and we have shown that HOBr adds to unsaturated fatty acids to form bromohydrins [25].

Red blood cells have been used extensively as a model system for investigating mechanisms of neutrophil-mediated cell injury [11,12,14,17,26]. Haemolysis is dependent on HOCl and is not readily mediated by chloramines [11,17]. However, the mechanism by which HOCl causes lysis is not known. We have shown previously that haemolysis occurs by a colloid osmotic mechanism that involves disruption of the membrane to cause K^+ leakage and swelling [14,27]. Although reduced glutathione (GSH) and membrane thiols were easily oxidized, this was not associated with lysis and the critical target remains unidentified.

The aim of the present study was to assess the contributions of lipid chlorohydrin formation and membrane protein oxidation to lysis. To do this we have compared the reactivity of HOCl and HOBr with red cells. Although these oxidants react similarly, HOBr is a weaker oxidant [22,25], and halogenation reactions might be favoured relative to oxidation reactions. Therefore we have compared the reaction of HOCl and HOBr with red cells to determine whether a difference in reactivity with particular cell targets might correlate with a change in the rate or extent of lysis. Initially we compared the rates of haemolysis initiated by both oxidants: the difference was related to oxidation of membrane thiols, membrane proteins and membrane lipids.

EXPERIMENTAL

Materials

Eosin 5-maleimide (EMI) was from Molecular Probes Inc. Other chemicals were from Sigma Chemical Co, and were reagent grade or better. Sodium hypochlorite (NaOCl) was from Reckitt

Abbreviations used: DIDS, di-isothiocyano-2,2′-stilbene sulphonic acid; EMI, eosin 5-maleimide; GSH, reduced glutathione; HOX, hypohalous acid.

[1] To whom correspondence should be addressed.

and Coleman (NZ) (Auckland, New Zealand). The concentration of the stock solution was measured by reaction with thio-bis(nitrobenzoic acid) [28]. NaOCl was diluted with PBS (10 mM phosphate buffer, pH 7.4, containing 150 mM NaCl and 1 mM $MgCl_2$), and the pH of the solution was adjusted to 7.4 immediately before use. At this pH, the solution contains approximately equimolar amounts of HOCl and NaOCl, and is referred to hereafter as HOCl.

Solutions of OBr^- were generated by mixing equimolar amounts of OCl^- with Br^- at pH 9 [29]:

$$OCl^- + Br^- \rightarrow Cl^- + OBr^-$$

Spectral monitoring showed that under these conditions there was complete conversion into OBr^-, and the solutions contained no OCl^-. (HOBr λ_{max} at 260 nm, $\epsilon = 100\ M^{-1}\cdot cm^{-1}$: OBr^- λ_{max} at 329 nm, ϵ 332 $M^{-1}\cdot cm^{-1}$; OCl^- λ_{max} at 292 nm, ϵ 350 $M^{-1}\cdot cm^{-1}$ [29].) Because of the instability of $HOBr/OBr^-$, the solutions were generated within 30 min of use and adjusted to pH 7.4 immediately before addition to the red cells. Because the pK of HOBr is 8.7, solutions at pH 7.4 contain mostly HOBr.

Red cell lysis with HOCl and HOBr

Human blood was obtained on the day of use from healthy donors after obtaining informed consent. Red cells were separated by centrifugation, removal of the buffy coat and plasma, and washing four times with PBS. The cell suspensions were adjusted to a known concentration after measuring the haemoglobin concentration with Drabkins solution [30] and assuming that packed red cells (10^{10} cells/ml) contain 30 % (w/v) haemoglobin.

HOCl or HOBr solutions were added slowly with constant mixing to ensure uniform reaction with the red cells. Lysis was measured either by continuous monitoring of A_{700} or by measuring the haemoglobin concentration of the supernatant after centrifugation [27]. Oxidant solutions were added to cell suspensions at room temperature; any subsequent incubations were at 37 °C. We have previously shown that the rate of lysis is dependent on the dose of HOCl per cell rather than on the concentration of oxidant [27], and the results are therefore expressed as nmol of oxidant per 10^7 cells. In most experiments we used 2.5 % (w/v) cell suspensions with 100–2500 μM oxidant. When we measured chlorohydrins and bromohydrins, we used a 10 % (w/v) cell suspension with 40–160 mM oxidant. Because of the reactivity of HOCl and HOBr, it was important that the medium did not contain scavenging substances that could compete for reaction with the red cells. Therefore, when addition of dithiothreitol or 2-mercaptoethanol was required, these were added 3–5 min after exposure to oxidant.

Red cell GSH and membrane thiol estimates

GSH was measured by reaction with monobromobimane as described previously [14]. Red cell ghosts were prepared [31] after exposure to HOCl or HOBr, and membrane thiols were measured by reaction with 2,2-dithiobis-(5-nitrobenzoic acid) in the presence of 6 mg/ml SDS [27].

Preparation of EMI-labelled red cells

Red cells were suspended at 2 % haematocrit in 20 μM EMI in PBS, in the dark for 3 h at 37 °C, washed twice to remove unreacted EMI and resuspended in PBS [32]. After reaction with HOCl or HOBr the cells were left for 1 h, pelleted, mounted on glass slides under coverslips and immediately examined by fluorescence microscopy.

Detection of lipid halohydrins

Red blood cells treated with HOCl or HOBr were acidified and extracted with dichloromethane as previously described [21]. Normal-phase TLC was performed on silica-coated plates eluted with diethyl ether/light petroleum (b.p. 60–80 °C)/acetic acid (70:30:1, by vol.). Air-dried plates were sprayed with 40 % (v/v) sulphuric acid and complete charring was obtained by heating the plate to 180 °C for 5 min. Product identification was based on co-elution with synthesized standards and by MS analysis [21].

Detection of red cell fatty acyl halohydrins by ELISA was performed by using an anti-chlorohydrin monoclonal antibody by the method of Domigan et al. [33]. This antibody also recognized bromohydrins [33]. Briefly, 5×10^7 HOX-treated red cells were preincubated overnight at room temperature with an excess of monoclonal antibody. The samples were then added to wells in a 96-well plate coated with albumin-conjugated chlorohydrin, and the residual antibody that bound to the plate was measured. Detection was with a peroxidase-linked second antibody, with *o*-phenylenediamine dichlorohydride as substrate. The results are expressed as the residual amount of antibody detected in the presence of HOX-treated red cells. Control levels of antibody were detected in the presence of untreated cells.

Measurement of anion channel activity

Transport of $^{35}SO_4^{2-}$ after labelling the cells with $Na_2{}^{35}SO_4$ was measured in the presence and absence of di-isothiocyano-2-2′-stilbene sulphonic acid (DIDS) [34]. After reaction with HOCl or HOBr, the cell suspension was immediately cooled to 0 °C, centrifuged and resuspended in 20 mM Tris/HCl, pH 7.5, containing 40 mM NaCl, 20 mM Na_2SO_4 and 0.13 M sucrose, prewarmed to 37 °C. Samples were removed at stated times and spun for 1 min in a microfuge; $^{35}SO_4^{2-}$ in the supernatants was counted in scintillant. Total radioactive counts were determined in a water-lysed sample. Before counting, contaminating haemoglobin from lysed cells was bleached by incubation with 15 % (w/v) H_2O_2. Excess H_2O_2 was scavenged by adding a few grains of sodium ascorbate.

SDS/PAGE of red cell membrane proteins

Red cell ghosts [31] were washed in water, pelleted and solubilized by heating to 100 °C for 2 min in sample loading buffer containing 3 % (w/v) SDS and 2 % (v/v) 2-mercaptoethanol. SDS/PAGE [10 % (w/v) gel] was by the method of Laemmli [35], and staining was with Coomassie Blue.

HOCl- and HOBr-induced protein cross-link formation in purified globin

Haem-depleted human haemoglobin (globin) was prepared from a 30 % (w/v) red cell lysate by precipitation in ice-cold acid acetone, washed in cold acetone and air-dried [36]. Globin was dissolved in water and then dialysed into PBS. To initiate cross-linking, 1 vol. of HOCl or HOBr solution was added to 1 vol. of protein solution while vortex-mixing. The samples were left at room temperature for approx. 30 min, then analysed by SDS/PAGE [7–18 % (w/v) gradient gel] and stained with Coomassie Blue.

RESULTS

Effect of HOX on cell lysis

Exposure of red cells to increasing concentrations of HOCl or

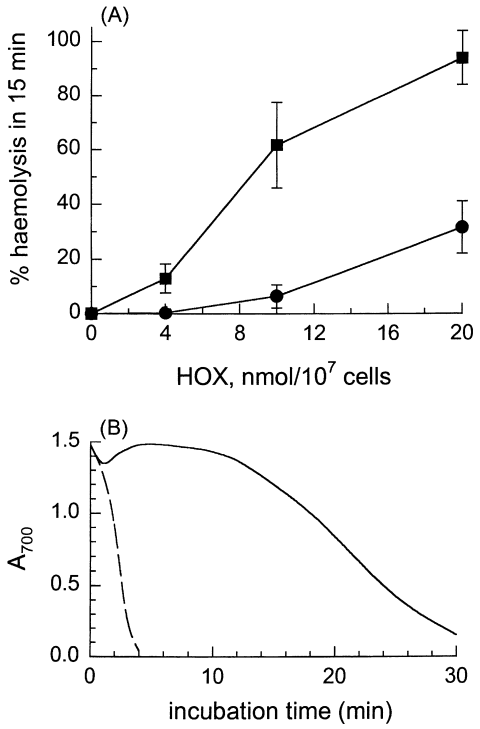

Figure 1 Red cell lysis after exposure to HOX

(**A**) A 2.5% (w/v) suspension of red cells was incubated with HOCl (●) or HOBr (■), and haemolysis was measured by the release of haemoglobin into the medium. Results are means ± S.D. for five experiments. (**B**) Haemolysis of red cells exposed to 20 nmol of HOX per 10^7 cells, measured continuously at 700 nm: solid line, HOCl; broken line, HOBr.

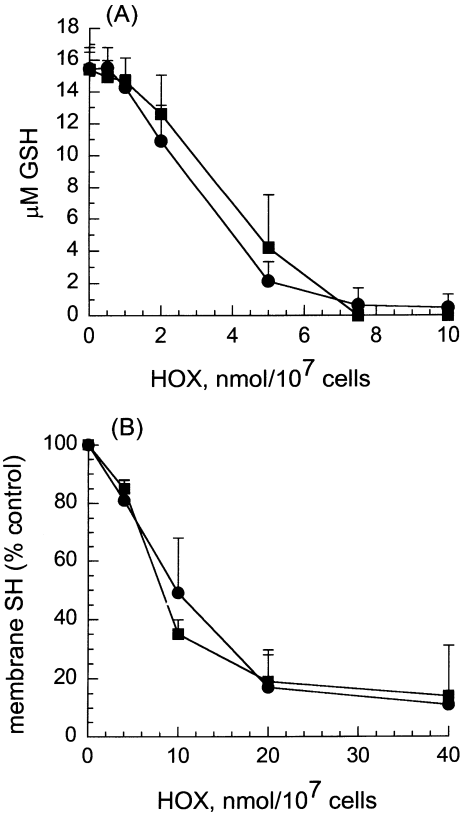

Figure 2 Thiol loss from red cells after exposure to HOCl and HOBr

(**A**) GSH content of a 1% (w/v) red cell suspension was measured with monobromobimane. Results are means ± S.D. for three experiments. (**B**) Membrane thiols were measured by reaction of red cell ghosts with 2,2-dithiobis-(5-nitrobenzoic acid). Results are means ± S.D. for three (HOCl) or two (HOBr) experiments. The control level of membrane thiols was 0.024 ± 0.011 μM SH/μg of protein. Symbols: ●, HOCl; ■, HOBr.

HOBr resulted in increased rates of haemolysis (Figure 1). At equivalent doses, HOBr caused significantly more lysis than HOCl. When measured continuously it was apparent that both the lag time and rate of lysis were affected (Figure 1B); on average, HOBr lysed red cells approx. 9–10-fold faster than did HOCl. To determine the reason for this difference we investigated the effects of both oxidants on red cell thiols, membrane proteins and lipids.

Oxidation of cell thiols

GSH was lost with low doses of oxidant, as observed previously with HOCl [14]. There was no significant difference between the extent of GSH loss with either HOBr or HOCl (Figure 2A) and membrane thiol loss occurred to a similar extent with each oxidant (Figure 2B). To determine whether a small difference in membrane thiol oxidation could account for faster lysis by HOBr, after oxidant exposure we reincubated the cells with 10 mM dithiothreitol or 10 mM 2-mercaptoethanol to regenerate membrane thiols. Neither reducing agent had any effect on haemolysis by either HOCl or HOBr (Figure 3).

Halohydrin formation

Cholesterol reacted with both HOCl and HOBr and the products of the reaction were analysed by TLC (Figure 4). We have previously identified the cholesterol chlorohydrin products [21] but this is the first time that the products of the reaction of HOBr with cholesterol have been described or have been detected in a cell system. Although they have not been fully characterized, the products of the reaction with HOBr co-elute with the cholesterol chlorohydrins 1, 2 and 3, and with the epoxides that result from base-catalysed degradation of cholesterol chlorohydrins [21]. This latter reaction occurs spontaneously with bromohydrins, and epoxide formation was increased with HOBr. The products detected with both oxidants were similar; it is apparent from a comparison of their intensities that the extent of halogenation was greater with HOBr than with HOCl at all doses (Figure 4). However, when compared with the amount of oxidant required to cause lysis (Figure 1), relatively high concentrations of HOCl and HOBr were required before a cholesterol halohydrin product was detectable (400 nmol of HOCl per 10^7 cells resulted in instant lysis).

The presence of phospholipid halohydrins was investigated directly by ELISA, with an antibody raised against oleic acid chlorohydrins, which also recognizes bromohydrins [33]. This allowed a more quantitative comparison between the two oxidant systems. Bromohydrin formation was detectable in red cells exposed to 50 nmol of HOBr per 10^7 cells, whereas ten times this amount of HOCl was required before chlorohydrins were detected (Figure 5). These results correlate with the levels of cholesterol oxidation seen by TLC, suggesting that similar levels of oxidant were required to produce either cholesterol or phospholipid halohydrins.

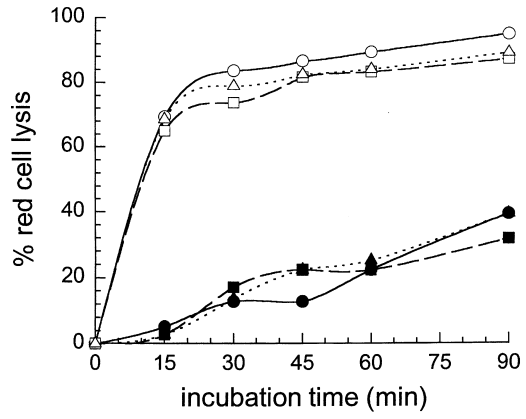

Figure 3 Effect of dithiothreitol and 2-mercaptoethanol on red cell lysis by HOCl and HOBr

At 2 min after the addition of 10 nmol of oxidant per 10^7 cells, 10 mM dithiothreitol (broken lines) or 2-mercaptoethanol (dotted lines) was added and lysis monitored; controls are shown by solid lines. Symbols: ●,■,▲, HOCl; ○,□,△, HOBr. The results of one of two representative experiments are shown, and similar results were obtained with 20 nmol of HOX per 10^7 cells.

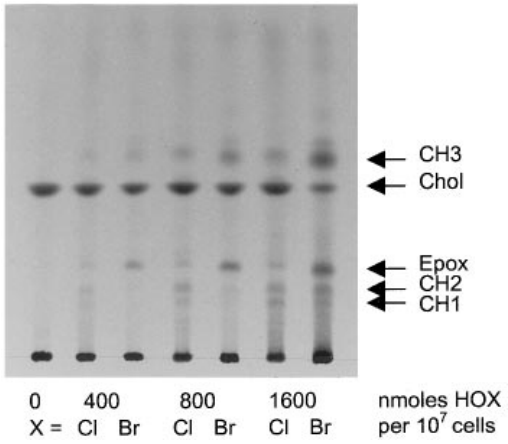

Figure 4 TLC of red cell membrane lipids after exposure to HOCl or HOBr

Red cells (10^9/ml) were treated with the stated amounts of oxidant and the lipids were extracted with dichloromethane as described in Experimental section. The products of the reaction with HOCl have been characterized previously [21]; their positions are marked. Abbreviations: chol, cholesterol; CH, chlorohydrins; epox, epoxide.

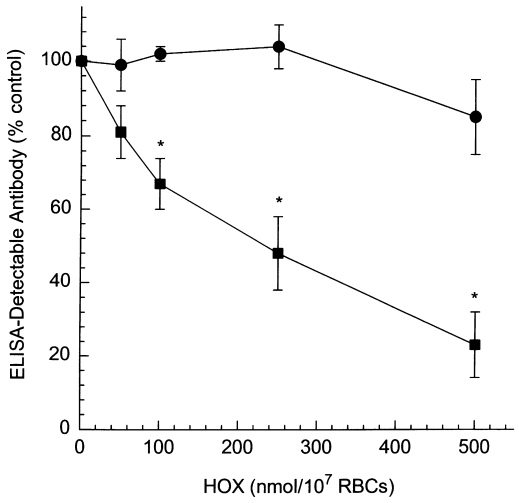

Figure 5 Detection of fatty acid halohydrin derivatives by ELISA

Red cells (RBCs) that had been exposed to HOX were preincubated with an excess of monoclonal antibody to chlorohydrins and added to microtitre wells coated with albumin-conjugated oleic acid chlorohydrin. The residual amount of antibody that was not removed by oxidant-treated cells was measured. The control represents the amount of antibody binding in the presence of untreated red cells. Results are means ± S.E.M. for four experiments, each carried out in duplicate. Symbols: ●, HOCl; ■, HOBr. Values that are signficantly different from control levels ($P < 0.05$ by Student's paired t test) are indicated with an asterisk. At 1000 nmol of HOCl per 10^7 cells, antibody levels were 31% of control ($n = 1$, in duplicate).

Membrane protein modification

As shown previously with HOCl [27], there were major changes to the SDS/PAGE membrane protein profile with increasing doses of oxidants (Figure 6). Some of these changes were reversed by 2-mercaptoethanol, but there was an accumulation of material at the top of the gel that was not reducible [27] (Figure 6). HOBr also caused irreversible cross-linking (Figure 6) and at equivalent levels of oxidant exposure there were more extensive changes to the membrane proteins with HOBr than with HOCl. At the lowest dose of oxidant used (4 nmol of HOBr per 10^7 cells) there was complete loss of band 3, and at 20 nmol of oxidant per 10^7 red cells most bands were decreased with accumulation of material at the top of the gel. By comparison, approx. 40–100 nmol of HOCl per 10^7 cells was required to cause a similar degree of modification. Band 3 did not show the same susceptibility to HOCl as to HOBr, but was altered at higher levels. Spectrin (bands 1 and 2) was also readily modified.

To confirm the greater effect of HOBr than HOCl on proteins, we used a simpler, pure protein solution and compared the reaction of HOCl and HOBr with haem-depleted globin. SDS/PAGE analysis showed that both oxidants caused cross-linking, as seen by the formation of dimers, trimers and extensively cross-linked material at the top of the gel (Figure 7). Even with a 1:1 molar ratio of oxidant to globin, the formation of dimers and trimers was considerable. HOBr was more reactive: at a 10:1 molar ratio of HOBr to globin, most of the material was at the top of the gel and none of the globin monomer or dimers remained (Figure 7).

Band 3 anion transport and fluorescence

Because band 3 was very sensitive to HOBr, we measured the anion transport activity with $^{35}SO_4^{2-}$ in the presence and in the absence of the inhibitor DIDS. Anion transport was not affected by HOCl or HOBr (Table 1), although at levels of oxidant that caused haemolysis there was passive leakage of label from the cells that reflected the extent of lysis. However, active transport was measured even at 4 nmol of HOBr per 10^7 cells (Table 1), when the SDS/PAGE profile showed almost complete loss of band 3 (Figure 6). The red cells were also labelled with EMI, which forms a covalent bond with band 3, and analysed by fluorescence microscopy after exposure to HOCl and HOBr but before lysis (Figure 8). With low doses of HOCl there was an obvious shape change (echinocyte formation) and a partly increased fluorescence at the periphery of the cell membrane. With higher levels of oxidant, many intensely fluorescent spots were seen on the cell surfaces, which might indicate clustering of band 3. A similar effect was seen with both HOCl and HOBr: the changes corresponded to the dose of oxidant. At equivalent

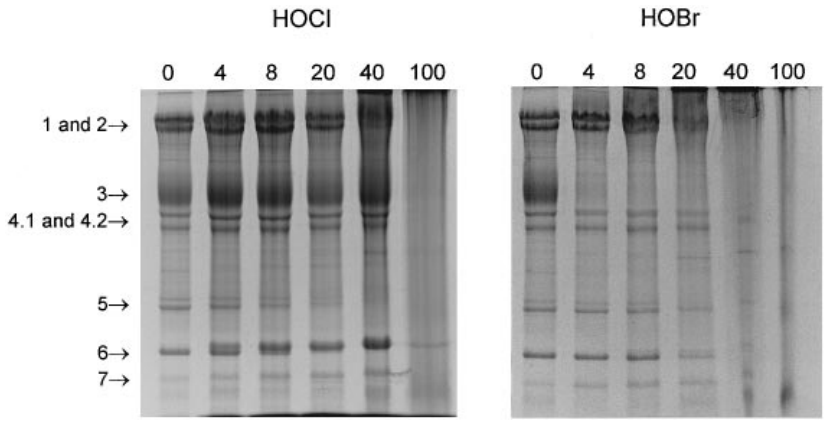

Figure 6 SDS/PAGE of red cell membrane proteins after exposure to HOCl or HOBr

Samples were prepared under reducing conditions. The dose of oxidant (nmol per 10^7 cells) is given above each lane, and the positions of the major bands are indicated.

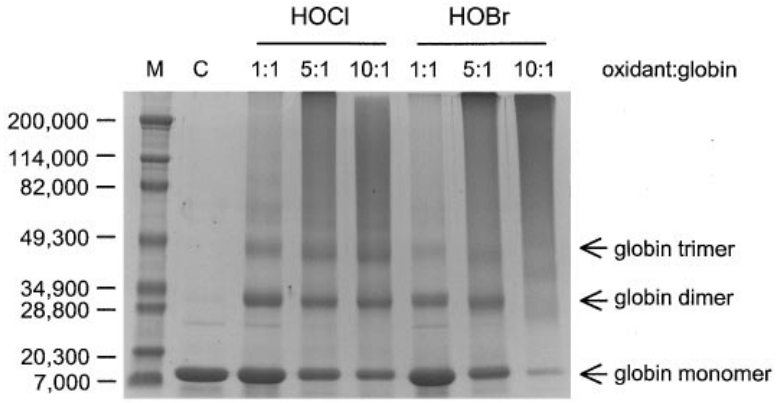

Figure 7 HOX-induced cross-linking of purified globin

Globin was reacted with increasing doses of either HOCl or HOBr, and analysed after 30 min by SDS/PAGE under reducing conditions. The molar ratio of oxidant to globin is given and the positions of dimers and trimers are indicated. Lane C, control; lane M, molecular mass markers (molecular masses in Da are indicated at the left). Cross-linking increased with the amount of oxidant, and HOBr treatment resulted in a greater loss of globin and formation of high-molecular-mass material.

Table 1 DIDS-inhibitable transport of $^{35}SO_4^{2-}$ after exposure to HOCl and HOBr

Under control conditions, normal $^{35}SO_4^{2-}$ transport from the cells was completely inhibited by DIDS. Similarly, after exposure to oxidants, $^{35}SO_4^{2-}$ transport was inhibited by DIDS except with 4 nmol of HOBr per 10^7 cells, when there was some leakage of $^{35}SO_4^{2-}$ that paralleled the rate of haemolysis. The results shown are from one of two experiments, which gave similar results.

	Relative transport (% of total d.p.m.)							
Time (min) ...	0		20		30		45	
Oxidant (nmol per 10^7 cells)	−DIDS	+DIDS	−DIDS	+DIDS	−DIDS	+DIDS	+DIDS	−DIDS
Control	4	1	59	3	81	3	98	3
HOCl (4)	2	2	47	2	68	3	98	3
HOCl (10)	4	1	55	3	83	5	100	7
HOBr (2)	2	2	42	3	69	4	105	6
HOBr (4)	6	4	57	15	83	27	94	53

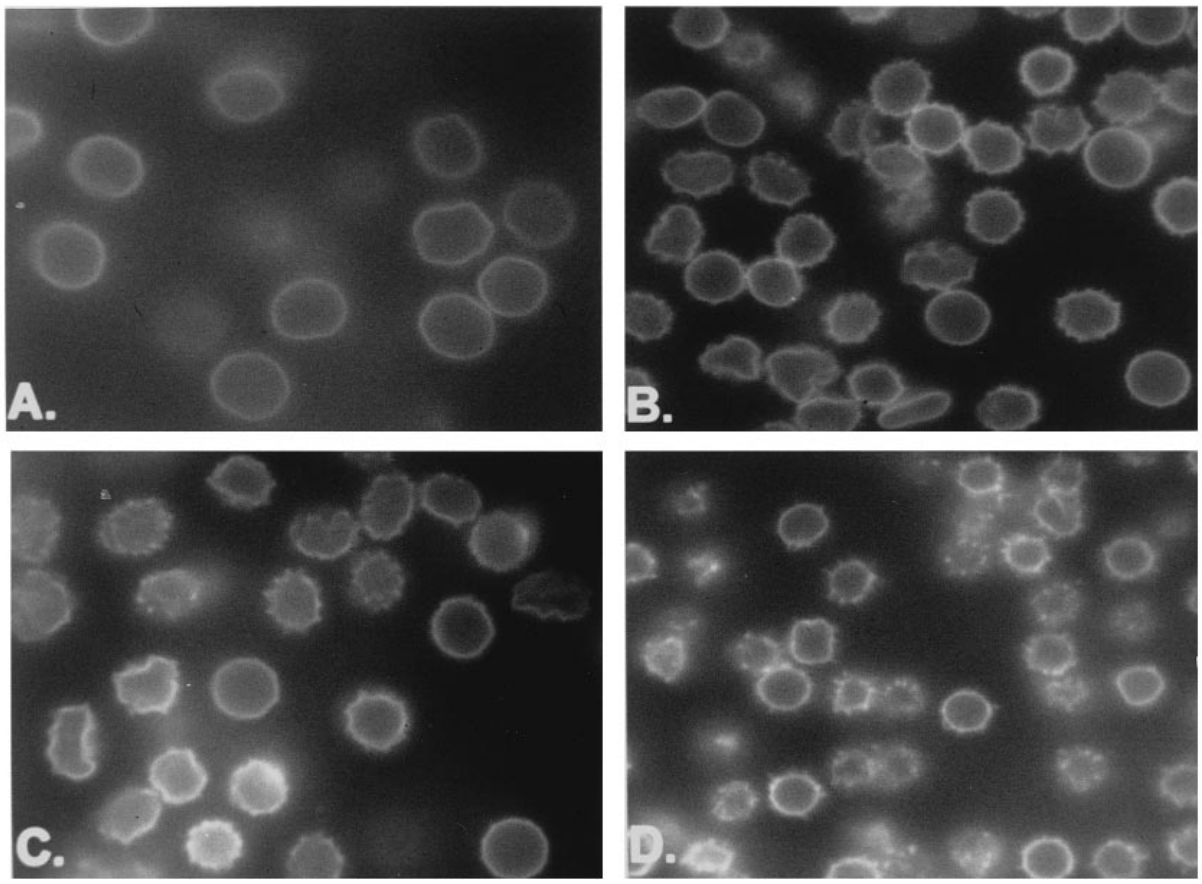

Figure 8 Fluorescence microscopy of EMI-labelled red cells

(**A**) Control cells. (**B**) After exposure to 4 nmol of HOCl per 10^7 cells there is an apparent shape change (echinocytes), and some bright spots of enhanced fluorescence appear on the cell periphery. (**C**, **D**) Cells treated with 4 nmol of HOBr per 10^7 cells (**C**) or 20 nmol of HOCl per 10^7 cells (**D**) show intensely fluorescent regions that indicates clustering of band 3.

doses, HOBr caused more extensive modification than HOCl (Figure 8).

DISCUSSION

We have shown that both HOCl and HOBr cause lysis of red blood cells, and that, on an equimolar basis, HOBr is 10-fold more efficient as a lytic agent. To relate this difference to a reaction that could account for lysis we compared the effects of these oxidants on specific cell targets. Reduced thiols are the most susceptible cell targets, and our finding that there was no significant difference in the loss of GSH and membrane thiols suggests that they react with HOCl and HOBr at similar rates. It is unlikely that even small changes in the extent of thiol oxidation could account for the increased lysis by HOBr because dithiothreitol and 2-mercaptoethanol, which reverse membrane thiol loss, could not prevent lysis. These results confirm our previous finding that thiol oxidation is not associated with haemolysis [14,27], and others have also noted that neutrophil-mediated lysis of red cells is not related to the GSH content [37].

In contrast with these results, HOBr gave much higher yields of phospholipid and cholesterol halogenation products and resulted in greater oxidative modification of membrane proteins. Phospholipid bromohydrins were seen by ELISA with 50 nmol per 10^7 cells, but ten times this amount of HOCl was required before chlorohydrins were detected. This correlates well with the detection of cholesterol chlorohydrins (400 nmol of HOCl per 10^7 cells). These doses of HOCl and HOBr cause complete haemolysis within seconds, suggesting that lipid modification does not occur to a significant extent except at very high doses of oxidant. Although the extent of lipid modification that would be required to disrupt the cell is unknown, 2–3 % lipid peroxidation has been shown to cause slow K^+ leakage [38]. In a separate study we have shown that fatty acid and cholesterol chlorohydrins are disruptive to micelle and liposome structure and that their incorporation into red cell membranes results in rapid lysis (A. C. Carr, N. M. Domigan, M. C. M. Vissers and C. C. Winterbourn, unpublished work). However, only a small fraction of the cells were lysed and when lipid chlorohydrins represented approx. 2 % of the total cell membrane fatty acids there was only 2–3 % haemolysis. The ELISA results suggest that only a small percentage of the phospholipid is halogenated with levels of oxidant that result in complete and immediate haemolysis (50 nmol of HOBr per 10^7 cells or 500 nmol of HOCl per 10^7 cells). Taken together, these results indicate that lipid modification is not correlated with haemolysis by HOCl.

Protein modifications, however, were detected at much lower doses of oxidant. Extreme changes to the SDS/PAGE profile were seen with 20 nmol of HOBr per 10^7 cells and 100 nmol of HOCl per 10^7 cells. Because halohydrins are undetectable at

these levels, it seems likely that modification of membrane and cytoskeletal proteins is responsible for haemolysis. It is interesting that studies with different oxidative systems have also found that protein modification, rather than lipid peroxidation, is associated with haemolysis [32,39,40]. There were two major differences in the effects of HOBr and HOCl on membrane proteins: (1) with HOBr there was selective loss of band 3 that was almost complete at the lowest dose of oxidant (4 nmol per 10^7 cells) and (2) irreversible protein cross-linking of most of the proteins occurred more readily with HOBr.

The loss of band 3 was particularly notable and might be related to lysis. Fluorescence microscopy with EMI-labelled cells indicated that there might be clustering of band 3 in the membrane: this was observed with both HOBr- and HOCl-treated cells, although higher concentrations of HOCl were required. Oxidation of band 3 has been shown to result in clustering to form haemolytic pores [32], but this has never been demonstrated with HOCl. Band 3 has separate and independent domains; the transmembrane domain is the anion transport channel and the cytoplasmic domain has structural and regulatory roles in interactions with the cytoskeleton and glycolytic enzymes [41]. We were able to measure active anion transport at a level of HOBr exposure that showed an almost complete loss of band 3 on SDS/PAGE, which suggests that the membrane-spanning region of the molecule was not inactivated and that HOBr might react more readily with the cytoplasmic region. This reaction could include cross-linking with the cytoskeletal proteins, particularly spectrin, or with other band 3 molecules, resulting in clustering and pore formation.

Other membrane proteins are also affected by HOCl and HOBr. Our results show that the formation of non-reducible cross-links with most of the membrane proteins is a major consequence of exposure to both HOCl and HOBr and is correlated closely with haemolysis. This mechanism has similarities to other oxidant systems that cause red cell lysis: photo-oxidation of protoporphyrin also causes protein cross-linking and clustering of the membrane proteins that is closely associated with lysis [42,43]. Protein cross-linking by HOCl has been reported previously [44–46] and our results with globin demonstrate that a substantial amount of dimerization occurred even at a 1:1 ratio of oxidant to protein. This is surprising in view of the number of potentially reactive amino acids [13], and indicates that this is a major reaction of HOCl and HOBr. Together with the prevalence of this reaction in the red cell, these results suggest that irreversible protein cross-linking might contribute significantly to the cytotoxicity of these oxidants. However, the mechanism of cross-link formation has not been characterized. Although generation of Schiff's bases by the condensation of lysine-derived chloramines and reactive carbonyl groups has been proposed [45], our experiments indicate that the reaction occurs within 30 s and precedes chloramine decay (A. L. P. Chapman and A. J. Kettle, unpublished work).

In summary, a comparison of the effect of HOCl and HOBr on red cells has proved useful to determine the reactions responsible for lysis. This has enabled us to distinguish between the relative importances of protein and lipid oxidation, and to correlate specific protein changes, namely band 3 clustering and protein cross-linking, with lysis. Although we have used HOBr simply to gain this comparison, these results are also of interest for the information they give on the general cytotoxicity of HOCl and HOBr. HOBr is formed in significant amounts by eosinophils [4,5] but the mechanism of its toxicity is not well understood. The demonstration that HOBr causes more protein cross-linking and lipid oxidation provides an interesting insight into the relative cytotoxicities of these oxidants.

We thank Professor Christine Winterbourn for helpful discussion and critical reading of the manuscript, and Dr. Christine Morris (Cytogenetics laboratory, Christchurch School of Medicine, Christchurch, New Zealand) for help with the fluorescence microscopy. This study was supported by the Health Research Council of New Zealand.

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Received 4 August 1997/22 September 1997; accepted 6 October 1997