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t-BUTYL HYDROPEROXIDE-INDUCED CHANGES IN THE PHYSICOCHEMICAL PROPERTIES OF HUMAN ERYTHROCYTES

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Summary

Treatment of human erythrocytes with micromolar concentrations of *t*-butyl hydroperoxide causes a variety of changes in the physical properties of the cells. Red cells exposed to concentrations of *t*-butyl hydroperoxide of less than 750 μM for 15 min exhibited significant decreases in cellular and membrane deformability, increases in membrane-associated protein cross-linking, osmotic fragility and the viscosity of the intracellular hemoglobin solution. No changes in the volume or density of the cells were observed. Changes in cellular deformability are probably attributable solely to changes in the mechanical properties of the cell membrane. Conversely, when red cells are exposed to *t*-butyl hydroperoxide concentrations in excess of 750 μM for 15 min they exhibited decreases in cellular deformability which may be related to increases in cell volume as well as membrane rigidity.

Introduction

In recent years a profusion of papers concerning the action of peroxides and peroxide-generating drugs have appeared. Interest in the area has been developing because of the large number of biological processes in which peroxides are produced [1,2] and because of the effects of certain families of peroxide-generating drugs on the lifespan and properties of cells. An area of particular

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Abbreviation: SDS, sodium dodecyl sulfate.

interest concerns the action of peroxides and peroxide-generating compounds on cells of the circulatory system; this family of chemical compounds has been implicated in altering the lifespan and physical properties of red cells [3—5]. Studies on the interaction of peroxides with molecules of biological interest have suggested a number of mechanisms by which cell damage may be related to the molecular architecture of the cell [6,7]. However, little information is available on the way in which alterations in the molecular architecture of cells are mirrored by changes in the physicochemical characteristics of the cell.

The present study has examined a number of physical and biochemical properties of red cells treated with an organic peroxide, *t*-butyl hydroperoxide, to determine the nature of its effect and locus of action. In particular, the effect of *t*-butyl hydroperoxide on cellular deformability was analyzed in terms of the physical properties of the cell which are thought to be determinants of cellular deformability. This required an investigation of the effect of *t*-butyl hydroperoxide on the volume and surface area of the cell, the viscosity of the intracellular hemoglobin solution and the mechanical properties of the cell membrane.

Materials and Methods

Reagents. All reagents were of analytical quality. Isotonic phosphate-buffered saline consisted of a 0.005 M NaH_2PO_4/0.16 M NaCl solution whose osmolality and pH were adjusted to 292 ± 3 mosM, 7.42 ± 0.02 at 25°C, respectively. When albumin-containing saline was required, human serum albumin (American Red Cross) was added to saline to a final concentration of 0.2%.

The hemoglobin standard for the establishment of a calibration curve was obtained from Hycel Inc. (Houston, TX). *t*-Butyl hydroperoxide (70%) was obtained from Aldrich Chemical Company (Milwaukee, WI).

Preparation of cells. Venous blood was taken from healthy donors into a heparinized syringe (0.05 mg heparin/ml blood). The blood was centrifuged, the plasma and buffy coat removed and the packed cells washed twice in albumin-containing saline. All centrifugations for washing the cells were at 17—21°C for 10 min at 2000 × *g*. After the second wash, the cells were suspended in saline to a 2% hematocrit. The cells were exposed to *t*-butyl hydroperoxide by mixing equal volumes of the aforementioned red cell suspension and a saline solution containing a known concentration of *t*-butyl hydroperoxide. When the red cell/peroxide suspension had been incubated at room temperature (22—24°C) for a given length of time, the cells were centrifuged (10 min, 900 × *g*) out of suspension and the supernatant removed. The cells were then diluted with an appropriate amount of peroxide-free, albumin-containing saline and measures of various cellular properties taken.

Cellular deformability. Cellular deformability was determined by a centrifugal deformation technique [8,9]. With this method, a dilute suspension of red cells (less than 10^7 cells/ml) is centrifuged at 15 000 × *g* so that the cells passed through their suspending medium into a saline solution containing 2% glutaraldehyde. The centrifugation process elongated the cells into a characteristic shape which was preserved by the glutaraldehyde. The ratio of the overall

deformed length of cells exposed, X, and not exposed, X_0, to t-butyl hydroperoxide, $(\bar{X}/\bar{X}_0)$, provides a measure of the change in extrinsic cellular deformability. For this assessment of extrinsic cellular deformability, all red cells were tested within 35 min of the time the t-butyl hydroperoxide-treated cells were resuspended in albumin-containing saline.

Intrinsic membrane deformability. The parallel-plate flow channel technique of Hochmuth and Mohandas [10] was used to assess intrinsic membrane deformability. Red cells were allowed to attach to the glass surfaces of the channel which had been previously exposed to saline containing 5% (v/v) autologous plasma and 0.2% albumin (v/v). The cells were then deformed by causing a saline/autologous plasma mixture (7% (v/v) plasma) to flow over the red cells at preselected volumetric flow rates. The fluid shear stress acting on the cells was calculated from the known volumetric flow rate and the measured geometry of the flow channel. Erythrocyte deformation was observed microscopically and recorded via a video system. Post-experimental measurements of the non-deformed, L_0, and deformed, L, lengths of the cells at different shear stresses were made using a video micrometer. Only 'point-attached' cells were selected for these length measurements [10]. All measurements of L_0 and L were completed within 3 h of the time the cells were first exposed to t-butyl hydroperoxide.

Using the known fluid shear stress and the measured ratio, L/L_0, it is possible to calculate the shear modulus of elasticity of the cell membrane, μ. This is a parameter which is proportional to the resistance of the cell membrane to stretching. At a given fluid shear stress, the shear modulus is inversely related to the ratio L/L_0 [10]. Thus, for the purposes of this work, the ratio L/L_0 at various levels of shear stress was used as an index of membrane elasticity rather than calculating μ.

Determination of red cell density. Red cell densities were determined by the phthalate oil method of Danon and Marikovsky [11] using an IEC Model MB microhematocrit centrifuge. Heat-induced changes in the density distributions were minimized by keeping the centrifuge lid open during the 10 min centrifugation.

Osmotic fragility. The effect of t-butyl hydroperoxide on red cell osmotic fragility was determined by mixing a 2% red cell suspension with an equal volume of saline solution containing t-butyl hydroperoxide. After a 15 min incubation period the cells were centrifuged for 10 min at $2000 \times g$, decanted and resuspended to a 2% hematocrit in albumin-containing saline. The osmotic fragility of this cell suspension with respect to hypotonic (138 mosM) pH 7.4 saline was then assessed as a function of time. The extent of hemolysis was determined by the technique of Gear [12]. This required the use of an Electrozone-Celloscope (Particle Data, Inc., Elmhurst, IL) fitted with a 30 μm orifice and interfaced with a PDP-8A minicomputer.

Cell volume. Mean cell volumes were determined electronically by the technique of Waterman and coworkers [13] with the aforementioned Electrozone-Celloscope equipped with a 76 μm orifice. The Electrozone-Celloscope was calibrated with polystyrene latices (Coulter Diagnostic Products, Hialeah, FL) of known diameter. A shape factor of 1.5 was used in calibrating the particle-sizing apparatus with the polystyrene latices, and a 20% rejection level

was employed to minimize artifacts associated with non-axial transit of particles through the orifice. Replicate measures of cell volumes indicated a coefficient of variation on the order of 2%.

Determination of hemoglobin viscosity. After treatment with *t*-butyl hydroperoxide, the red cells were collected by centrifugation and resuspended in saline to a hematocrit in excess of 50%. The suspension was then centrifuged for 5 min at 12 000 × *g* and hemoglobin solutions for viscosity measurements were prepared from the packed cells by a modification of the method of Cokelet and Meiselman [14]. In this modified procedure the hemoglobin solution was washed with CCl_4 twice rather than once. The concentration of hemoglobin in the final solution was determined by the cyanomethemoglobin procedure [29]. The viscosities of the hemoglobin solutions were determined at 25.0 ± 0.1°C with a Cannon-Fenske viscometer. These determinations were carried out approx. 6—8 h after the initial exposure of the cells to *t*-butyl hydroperoxide.

SDS-polyacrylamide gel electrophoresis. The preparation of red cell ghosts and SDS-polyacrylamide gel electrophoresis of the ghost membrane proteins was done according to the technique of Fairbanks et al. [15].

Phosphorus assay. The red cells were washed twice and resuspended in a NaCl solution buffered with 0.005 M Tris (pH 7.42 ± 0.02, 292 ± 3 mosM) to a concentration of $2.3 \pm 0.1 \cdot 10^8$ cells/ml. The suspension was then mixed with an equal volume of NaCl/Tris buffer containing *t*-butyl hydroperoxide. After 15 min incubation period, the cells were centrifuged from suspension and the phosphorus released into the supernatant determined. Direct assessments of the amount of phosphorus-containing material released into the supernatant by treated and non-treated cells were made with an automatic phosphate analyzer [16]. The coefficient of variation of replicate determination of phosphorus was approx. 25%. This large variation was a result of the low levels of phosphorus in the supernatant fractions, since the coefficient of variation for replicate measurements in more concentrated calibration solutions was on the order of 5%.

Results

Fig. 1 shows the effect of varying concentrations of *t*-butyl hydroperoxide on the deformability of red blood cells. It is apparent that after 15 min exposure to concentrations of *t*-butyl hydroperoxide which exceed 300 μM, the deformability of the cells decreases significantly ($P < 0.05$). Conversely, red cells incubated with 3.0 mM *t*-butyl alcohol, for 90 min, exhibited no changes in cellular deformability, volume or density (data not shown). Although no changes in red cell deformability were seen when the cells were exposed to *t*-butyl hydroperoxide concentrations less than 300 μM for 15 min, significant ($P < 0.05$) changes occurred with longer incubation periods. When cells were exposed to 150 μM *t*-butyl hydroperoxide (Fig. 2) for 75 min or more, significant decreases in cell deformability were observed.

In order to elucidate the factors causing the observed decrease in cellular deformability, we first examined the effect of *t*-butyl hydroperoxide on the red cell surface vs. volume relationship. Fig. 3 shows that apparent increases

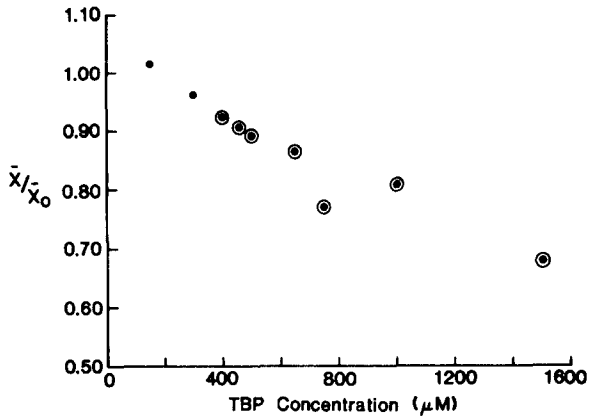

Fig. 1. Erythrocyte cellular deformability after exposure to various *t*-butyl hydroperoxide (TBP) concentrations for 15 min. Erythrocyte deformability was measured via a centrifugal technique and at least 60 cells were measued for each point. Values of X/X_0 which are significantly ($P < 0.05$) less than the control (i.e., $X/X_0 = 1.00$) value are circled.

in cell volume are observed only when erythrocytes are exposed to *t*-butyl hydroperoxide concentrations in excess of 750 μM for 15 min. Incubating red cells for up to 120 min in 150 μM *t*-butyl hydroperoxide, as previously described (e.g., Fig. 2), produces no change in cell volume (data not shown).

To determine if decreases in membrane surface area were causing the aforementioned alterations in cellular deformability, the osmotic fragility and extent of phosphorus loss of the *t*-butyl hydroperoxide-treated erythrocytes were examined. Fig. 4 indicates that cells exposed for 15 min to *t*-butyl hydroperoxide concentrations up to 400 μM exhibited essentially no change in osmotic fragility. However, exposure to 750 μM or higher concentrations of *t*-butyl

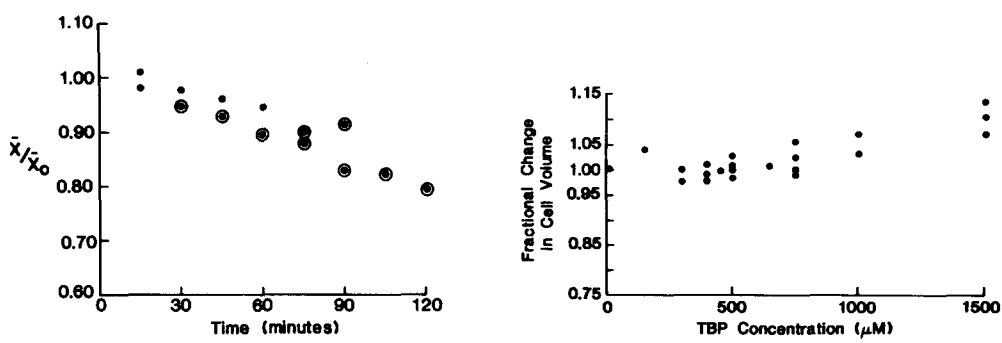

Fig. 2. Erythrocyte cellular deformability on exposure to 150 μM *t*-butyl hydroperoxide for various periods of time. The results from several experiments are shown, with values of X/X_0 which differ significantly ($P < 0.05$) from unity indicated by the circled data points.

Fig. 3. Fractional change in cell volume after exposure to various *t*-butyl hydroperoxide (TBP) concentrations for 15 min. Each point represents a single experimental measurement. Note that cell volume increases are only observed at *t*-butyl hydroperoxide concentrations in excess of 750 μM.

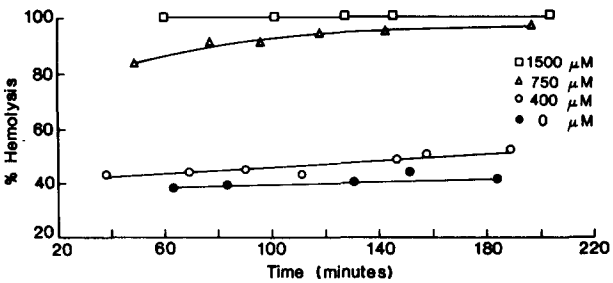

Fig. 4. Osmotic fragility of red cells exposed to *t*-butyl hydroperoxide for 15 min. The ordinate represents the percent of cells which hemolyzed when placed in a hypotonic (138 mosM) saline solution. The abscissa indicates the length of time which had elapsed since the cells were removed from the various *t*-butyl hydroperoxide solutions.

hydroperoxide caused large increases in the osmotic fragility of the cells.

Since increases in the osmotic fragility of red cells suggest but do not prove the loss of membrane, a more direct measure of membranes loss was examined by determining the loss of phosphorus-containing molecules from erythrocytes treated with various concentrations of *t*-butyl hydroperoxide. Over the range of *t*-butyl hydroperoxide concentrations investigated, 0—1500 μM, the amount of phosphorus released was approx. 5 nmol/ml supernatant (mean = 5.0, range = 4.5—5.3). No trend with respect to *t*-butyl hydroperoxide concentration was observed nor did the amount released differ significantly from the control, peroxide-free suspension. Attempts to extract and quantitate the amount of phospholipid released into the supernatant were inconclusive.

Investigations of the role of hemoglobin viscosity in decreasing cellular deformability showed that the viscosity of a 30 g% hemoglobin solution from cells exposed to 500 μM *t*-butyl hydroperoxide was slightly (10 ± 7%) greater, but not by a statistically significant amount, than a solution of equal concentration prepared from untreated cells. When the cells were treated with 1500 μM *t*-butyl hydroperoxide, the viscosity of a 20 g% hemoglobin solution was approx. 90% greater than the hemoglobin from untreated cells. These increases in viscosity are only approximate since the viscosity of hemoglobin solutions from *t*-butyl hydroperoxide-treated cells increased slowly with time. This increase was accompanied by the formation of a flocculent precipitate which was visible under phase microscopy. The amount of precipitate and the speed with which it formed increased with the concentration of *t*-butyl hydroperoxide to which the cells were exposed.

The effect of *t*-butyl hydroperoxide treatment on the cross-linking of proteins is shown by the SDS-polyacrylamide gels pictured in Fig. 5. 30 μg of ghost protein were placed on each of the three gels pictured. The ghosts were prepared from red cells exposed for 15 min to either 0, 500 or 1000 μM *t*-butyl hydroperoxide. The protein banding patterns for all three gels appears to be typical for human erythrocytes, with the exception of the high molecular weight material at the top of the gel. The amount of this material increased with the concentration of *t*-butyl hydroperoxide used to treat the red cells. Scans of the distribution of absorbances of the gels (not shown) did not reveal

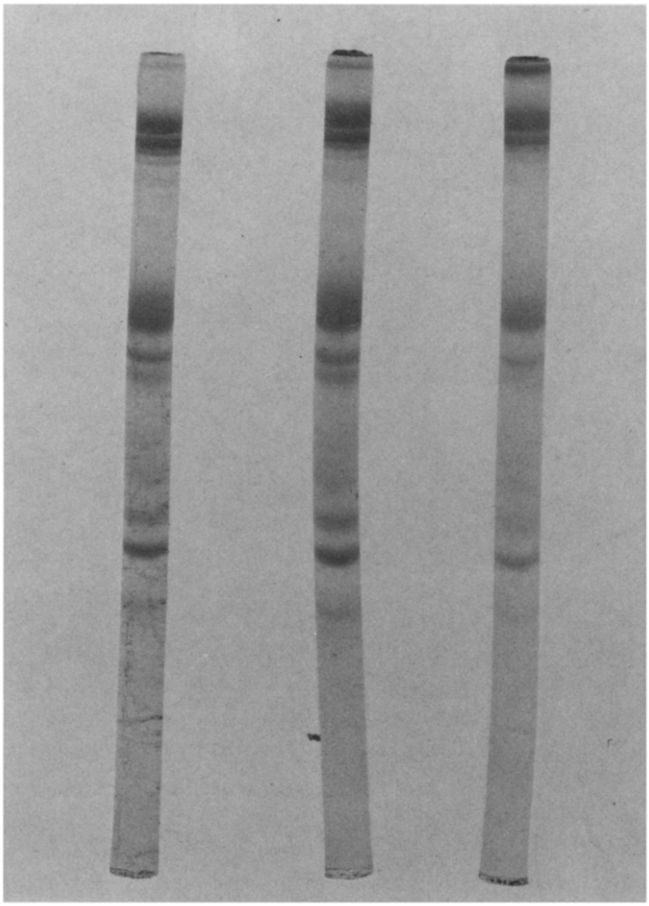

Fig. 5. Effect of *t*-butyl hydroperoxide treatment on membrane proteins. 30 μg of ghost protein were placed on each of the SDS-polyacrylamide gels pictured. The ghosts were prepared from erythrocytes exposed for 15 min to either 0, 500 or 1500 μM *t*-butyl hydroperoxide (left to right). Note the concentration-dependent high molecular weight material at the top of the gel.

a decrease in any particular membrane protein comparable to the amount of high molecular weight aggregate at the top of the gel.

The effects of *t*-butyl hydroperoxide treatment on the mechanical properties of the red cell membrane are shown in Fig. 6 where the average L/L_0 of red cells exposed to different concentrations of *t*-butyl hydroperoxide is plotted against the fluid shear stress to which the cells were exposed. Analysis of these data indicates that at all shear stresses, the values of L/L_0 obtained for cells treated with 500 and 750 μM *t*-butyl hydroperoxide were significantly ($P < 0.05$) smaller than those for untreated cells (with the sole exception of the 500 μM *t*-butyl hydroperoxide, 1.5 dynes/cm^2 data point). Conversely none of the data points for cells treated with 250 μM *t*-butyl hydroperoxide differed significantly ($P > 0.05$) from control cells. It should be noted that although measurement of the L/L_0 values often took up to 3 h, there was no evidence that membrane deformability (as L/L_0) changed with time within this period.

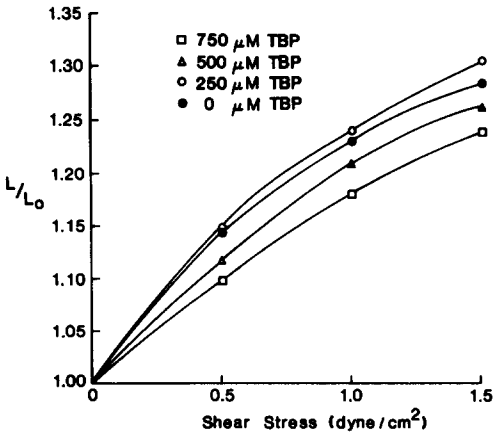

Fig. 6. Erythrocyte membrane rigidity after exposure to various *t*-butyl hydroperoxide (TBP) concentrations for 15 min. Cells were deformed in a parallel-plate flow chamber at several levels of fluid shear stress. At a given shear stress, the reduced length of the cell (L/L_0) is inversely related to the shear modulus of elasticity of the cell membrane. Each point represents the mean of at least 15 different cells.

Discussion

Exposure of red cells to *t*-butyl hydroperoxide produces time and concentration-dependent decreases in cellular deformability (Figs. 1 and 2). These alterations could have been caused by a change in the cellular surface area to volume (S/V) ratio, the viscosity of the intracellular hemoglobin or in the mechanical properties of the membrane [17]. To elucidate the mechanism by which *t*-butyl hydroperoxide decreased the deformability of the cells, a systematic evaluation of these parameters was undertaken.

Analysis of the effect of *t*-butyl hydroperoxide on cellular S/V ratios required an examination of cell volume and membrane surface area after exposure to *t*-butyl hydroperoxide. Measurements of erythrocyte volume (Fig. 3) following a 15 min exposure to low or moderate *t*-butyl hydroperoxide concentrations (less than 750 μM) indicated a negligible (less than 2%) increase in cell volume. Note, however, the approx. 15% decrease in cellular deformability, as X/X_0, when cells are exposed to 600—700 μM *t*-butyl hydroperoxide (Fig. 1). Since modest (less than 15%) increases in cell volume cause X/X_0 to decrease at a rate of about 1%/percent increase in cell volume [8], increases in cell volume cannot account for the observed decrease in cell deformability (*t*-butyl hydroperoxide concentration less than 750 μM). When red cells are exposed to high (greater than 750 μM) concentrations of *t*-butyl hydroperoxide, decreases in cell deformability may be partially attributable to increases in red cell volume. However, this apparent increase in volume produced by high concentrations of *t*-butyl hydroperoxide may be partially spurious inasmuch as glutaraldehyde, a protein cross-linking agent which also decreases the deformability of red cells, can cause artifactual increases in erythrocyte volumes as measured by an orifice-type electronic system [18].

To determine whether *t*-butyl hydroperoxide might decrease cell deform-

ability by causing membrane loss, assessments of cellular osmotic fragility and phosphorus loss were made. Measurements of osmotic fragility were made on the assumption that any losses in cell membrane would be reflected by an increased osmotic fragility. Cells exposed to low *t*-butyl hydroperoxide concentrations (400 μM) did not show a significant increase in osmotic fragility (Fig. 4) while exposure to high *t*-butyl hydroperoxide concentrations (greater than 750 μM) caused a dramatic increase in osmotic fragility. These data are consistent with the hypothesis that the exposure of red cells to high *t*-butyl hydroperoxide concentrations causes a loss of membrane. However, a more direct evaluation of membrane loss (see below) did not support this hypothesis.

Measurement of the amount of phosphorus-containing material released into the suspending medium by *t*-butyl hydroperoxide-treated cells indicated that cells (1% hematocrit) exposed to *t*-butyl hydroperoxide concentrations up to 1500 μM released approx. 5 nM of phosphorus/ml of medium. Assuming a total red cell phosphorus content of 719 mg/l of red cells [19], this suggests that only approx. 2% of the total cellular phosphorus was released. If all of the phosphorus-containing material in the supernatant was phospholipid, approx. 13% [20] of the lipid in the red cell membrane would have been lost. However, it is unlikely that much of the material released was of a phospholipid nature, as the amount of phosphorus released was independent of the concentration of *t*-butyl hydroperoxide used to treat the cells. Further, cells exposed to low or moderate *t*-butyl hydroperoxide concentrations did not undergo any of the morphological transformations that would be expected of cells which had lost membrane, but rather, maintained the biconcave shape. Thus it is improbable that *t*-butyl hydroperoxide-induced decreases in cell deformability are related to a loss of cell membrane.

The effect of the viscosity of the intracellular hemoglobin on controlling cell deformability in certain situations has been addressed by Hochmuth et al. [21]. In their studies, they have presented calculations relevant to the amount of energy required for an erythrocyte to undergo shape transformations similar to those involved in making cell deformability measurements. Their work has shown that even if the viscosity of the intracellular hemoglobin solution were increased 2-fold, the energy dissipated by that solution is only a minor fraction (less than 1%) of that dissipated by the membrane for cellular deformations of the type employed in this study. Thus, the 10% increase in the viscosity of 30 g% hemoglobin solutions from red cells treated with 500 μM *t*-butyl hydroperoxide is strong evidence that moderate concentrations of *t*-butyl hydroperoxide do not decrease cell deformability by increasing the viscosity of the intracellular hemoglobin solution.

Changes in the mechanical behavior of the cell membrane consequent to exposure to *t*-butyl hydroperoxide were evaluated via a flow channel technique (Fig. 6). As cells were treated with increasing concentrations of *t*-butyl hydroperoxide (greater than 250 μM), the rigidity of the membrane, as indexed by L/L_0, exhibited significant increases similar to decreases observed with measurements of cell deformability (see Fig. 1). This correlation suggests [30,31] that alterations in membrane rigidity represent the mechanism by which *t*-butyl hydroperoxide reduces cell deformability.

In an attempt to relate the *t*-butyl hydroperoxide-induced decreases in cell

and membrane deformability to changes in the molecular architecture of the cell, polyacrylamide gel electrophoresis studies of the membrane proteins were undertaken. These studies indicated that exposing the red cells to higher concentrations of *t*-butyl hydroxperoxide increased the amount of high molecular weight material near the origin of the gel. Gel scanning analysis of the protein staining patterns indicated that the amount of this high molecular weight material appeared to increase without a decrease in any other membrane proteins. These observations partially corroborate the findings of other workers [22,23] who have shown that protein cross-linking agents can decrease the deformability of red cells. In particular, Fischer and coworkers [23] have shown that treatment of red cells with sulfhydryl cross-linking agents causes the formation of a high molecular weight material, probably cross-linked spectrin molecules, whose presence coincides with a decrease in cellular deformability. The nature of their material probably differs from the present situation in that the bulk of the high molecular weight material near the origin of the gels from *t*-butyl hydroperoxide-treated cells most likely consists of hemoglobin cross-linked to various membrane components. This hypothesis is supported by the following observations: the treatment of cells with peroxides and peroxide-generating drugs produces agents capable of cross-linking proteins [7,24]; *t*-butyl hydroperoxide-induced cross-linking of intracellular hemoglobin would account for the increased viscosity of hemoglobin solutions from *t*-butyl hydroperoxide-treated red cells; the brownish color (i.e., methemoglobin) of ghosts prepared from *t*-butyl hydroperoxide-treated red cells increased with the concentration of *t*-butyl hydroperoxide used to treat the cells. However, the specific role of membrane-bound hemoglobin in decreasing cell deformability is unknown, since it may be possible that the observed decreases in cellular deformability are attributable solely to cross-linking of membrane components.

In summary, our results indicate that *t*-butyl hydroperoxide can cause a number of changes in the physicochemical properties of red cells, with the nature and extent of these changes being dependent on the concentration of *t*-butyl hydroperoxide and the exposure time. Although the concentrations of *t*-butyl hydroperoxide employed herein are higher than peroxide levels expected in vivo, it is apparent from Fig. 2 that red cell properties can be altered by low concentrations of *t*-butyl hydroperoxide if extended exposure times are involved. It should be noted that erythrocyte deformability has been shown to be an important determinant of capillary blood flow [25] and appears to be related to the in vivo lifespan of the red cell [26,27]. Thus, a speculation which can be formulated from our data is that by cross-linking cell membrane components with each other or with hemoglobin, peroxides or peroxide-generating drugs cause decreased red cell deformability, thereby decreasing the lifespan of the cell. Needless to say, this speculation gives no consideration to the role of the immune system [28] in eliminating effete red cells, but does provide an interesting possibility which warrants further consideration.

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