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Observations on the Cytolytic Activity of Lactoperoxidase Using a Continuous Assay[1] (42105)

STEVE J. McFAUL,[2] ELIZABETH L. STUYT, AND JOHANNES EVERSE

Department of Biochemistry, Texas Tech University Health Sciences Center, Lubbock, Texas 79430

Abstract. A turbidometric assay that allows continuous monitoring of the cytolytic activity of toxic agents toward various target cells has been developed. This assay monitors the change in absorbance at 600 nm (due to light scattering) of a suspension of human red blood cells as a function of time. The rate of cell lysis, $\Delta A_{600}/\Delta t$, can be expressed as the number of cells lysed per minute, which facilitates the determination of kinetic constants. Using this procedure we observed that the cytolytic activity exerted by various peroxidases in the presence of hydrogen peroxide and a halide ion proceeds in at least two stages. During the first stage no lysis occurs, but scanning electron microscopy showed that alterations in the target cell membrane take place. During the second stage the target cells lyse, resulting in a simultaneous release of metabolites and macromolecules. We conclude that the lytic action of peroxidases is directed toward the target cell membrane, which appears to acquire an increased rigidity and subsequently disintegrates.

Klebanoff and his colleagues have shown that the combination of myeloperoxidase, hydrogen peroxide, and a halide ion forms a cytotoxic mixture that is much more potent than the individual constituents (1, 2). This combination effectively kills bacteria, fungi, and viruses and lyses various mammalian cells (3–6). Similar cytotoxic and cytolytic properties are displayed by lactoperoxidase in the presence of hydrogen peroxide and a halide or thiocyanate ion (7–9).

An equally cytotoxic system is obtained when instead of hydrogen peroxide a peroxide-generating enzyme is used. Thus, lactoperoxidase in the presence of iodide yields a potent cytotoxic system upon the addition of glucose and glucose oxidase (10). The combination of hypoxanthine and xanthine oxidase was shown to be similarly effective in promoting the cytotoxic activity of myeloperoxidase (11).

A large body of evidence indicates that the biological function of various peroxidases may be that of a cytotoxic agent. Peroxidases are operative as cytotoxic agents in neutrophils, eosinophils, and other cell types with phagocytic properties (13–17). Lactoperoxidase is presumably responsible for maintaining the sterility of milk by exerting its cytotoxic activity against invading prokaryotes (7). The mechanism by which these peroxidases effect the killing of target cells, however, remains largely unknown. Various observations have led researchers to propose that the enzyme in the presence of hydrogen peroxide and a halide ion produces one or more highly reactive oxygen derivatives, that are lethal to the target cell. These oxygen derivatives may include superoxide (18, 19), hydroxyl free radicals (20), and singlet oxygen (21, 22). More recently, however, evidence has been presented suggesting that hypohalous acids may be responsible for the toxic activity of the peroxidases (23, 24).

We have recently developed a spectrophotometric assay that allows us to monitor cell lysis continuously. This assay has proved to be superior to the conventionally used endpoint assays (trypan blue uptake, ^{51}Cr release, etc.) in that more detail concerning the kinetic profile of cell lysis is readily obtained and that kinetic constants are obtainable with higher accuracy. In this communication we describe the assay as well as some initial observations made with lactoperoxidase concerning the kinetics of the cytolytic process.

Materials and Methods. *Materials.* Lactoperoxidase ($A_{412}/A_{280} \geq 0.90$), glucose oxi-

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[2] Present address: Department of Physiology, Texas Tech University Health Sciences Center, Lubbock, Texas 79430.

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dase (type V), glucose (grade III), and anhydrous monobasic potassium phosphate (grade III) were purchased from Sigma Chemical Company. Certified ACS grades of sodium chloride, potassium chloride, and potassium iodide were obtained from Fisher Chemical Company. Analytical grade dibasic sodium phosphate was purchased from Mallinckrodt, Incorporated. Reagent grade hydrogen peroxide was obtained from MCB Manufacturing Chemists, Incorporated.

Cytolytic assays. Fresh human erythrocytes and harvested mouse lymphoma L1210 cells were each diluted in phosphate buffered saline (PBS)[3] and the cells were counted using a hemocytometer. Appropriate dilutions in PBS were then made to yield cell suspensions of known concentration.

Each experimental cuvette contained 9.5 m*M* phosphate buffer, pH 7.2, 133 m*M* NaCl, 2.7 m*M* KCl, 25 μM KI, 10 m*M* glucose, 30 mUnit glucose oxidase, 1 μg (12 n*M*) lactoperoxidase, and either 2×10^6 red blood cells or 1×10^6 L1210 cells in a total volume of 1 ml. Control cuvettes contained all of the above except lactoperoxidase. Reactions were started with the addition of cells. Since the control cuvette also contained cells, a decrease in the absorbance of the sample cuvette would lead to a negative absorbance with respect to the reference cuvette. To avoid this problem the experimental cuvette was placed in the reference beam and the control cuvette was placed in the sample beam of a Beckman Model 34 double-beam recording spectrophotometer. The apparent increase in absorbance at 600 nm was then continuously monitored until no further change was detected. All assays were performed at 37°C.

The release of proteins and hemoglobin from peroxidase-treated erythrocytes was measured spectrophotometrically. At the indicated times an aliquot of the reaction mixture was centrifuged for 30 sec in a Beckman microfuge and the absorbance of the supernatant was determined at 280 nm for total protein content and at 415 nm for hemoglobin content.

[3] Abbreviations used: PBS, phosphate buffered saline, consisting of 9.5 m*M* Na-phosphate, pH 7.2, 133 m*M* NaCl, 2.7 m*M* KCl, 25 μM KI, and 10 m*M* glucose.

Assays for ATP were done as follows: at the indicated times, aliquots were removed from a reaction mixture containing erythrocytes, peroxidase, etc. as described above. The aliquots were immediately centrifuged in a Beckman microfuge for 30 sec. One hundred microliters of the supernatant was added to 500 μl Hepes buffer, followed by 100 μl of luciferin-luciferase reagent (Firelight kit, Analytical Luminescence Laboratory, Inc., San Diego, Calif.). Chemiluminescence was measured as counts/minute in a Lumitran Model 3000 integrating photometer.

Chromium release measurements were done using erythrocytes labeled with ^{51}Cr. About 4×10^7 erythrocytes in 2 ml PBS were incubated for 1 hr at 37° with 100 μCi $Na_2{}^{51}CrO_4$ (New England Nuclear Corp., Boston, Mass.). Unbound chromate was removed by several washings of the cells with PBS. About one-half (2×10^7) of the labeled cells were then added to a cytotoxic assay mixture of 10 ml total volume. Another 2×10^7 labeled cells were used for the control assays, lacking peroxidase. At the indicated times 1-ml aliquots were removed from the assay mixtures, centrifuged for 30 sec, and 500-μl aliquots of the supernatants were counted in a Beckman Gamma 5500 counter for 20 min.

Results. *Determination of monitoring wavelength.* Cell suspensions scatter both uv and visible light. To determine a wavelength that yields a significant difference in light scattering between whole cells and lysed cells, absorption spectra were generated for erythrocyte suspensions before and after peroxidase treatment. Figure 1 shows the results. The most significant difference in absorbance before and after lysis of the erythrocytes was found to be in the 600 to 800 nm region. Since none of the red blood cell constituents absorb in this region, the decrease in absorbance must be due to a decrease in light scattering, which in turn reflects a reduction in the number of intact cells. For these reasons, 600 nm was chosen as an appropriate wavelength to monitor cell lysis. Any of the higher wavelengths, however, could equally be used to monitor cell lysis.

Continuous cytolytic assay. Figure 2 shows the profiles of a typical continuous cytolytic assay, using either erythrocytes (A) or L1210

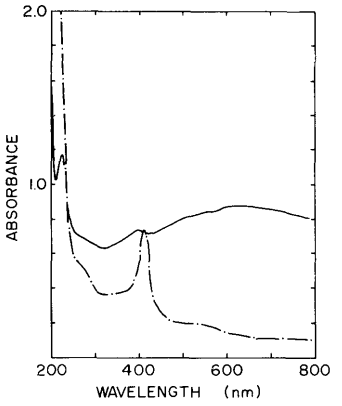

FIG. 1. Wavelength profile of a human erythrocyte suspension in phosphate buffered saline before (———) and after (- - - -) lysis of the cells with lactoperoxidase.

murine lymphoid leukemia cells (B). In both cases the cytolytic process involves a lag time of several minutes, followed by the actual lysis of the target cells. Both the duration of the lag time and the rate of cell lysis are dependent on the amount of peroxidase used in the assay. In fact, by using very small amounts of peroxidase we have observed lag times lasting as long as 30 min before lysis started. These results demonstrated that the peroxidase-promoted cell lysis does not follow Michaelis-Menten type kinetics, but involves several kinetically distinguishable steps.

Comparison of the turbidometric assay with other cytolytic assays. To assure that the sigmoidal profiles obtained by following the decreases in absorption at 600 nm were not an artifact of the assay method we measured the cytolytic activity of lactoperoxidase by several other well-established methods. These include hemoglobin release, total protein release, ATP release, and ^{51}Cr release. The obtained results are shown in Fig. 3. These results clearly indicate that the sigmoidal profile is not an artifact of the method used, but demonstrates the actual progress of the reaction. A microscopic examination of the reaction in progress further confirmed that cell lysis does not take place until about 5 min after the addition of peroxidase and H_2O_2.

It is noteworthy that the profile obtained by measuring ATP release is not significantly different from the profiles obtained from hemoglobin and total protein measurements. These results, which were repeatedly obtained, suggest that the total cell content may be released simultaneously rather than small molecules being released before large molecules. These results are therefore not consistent with the concept that the damage to the erythrocyte membrane involves a progressive increase in porosity of the membrane, but rather with the concept that at a given time

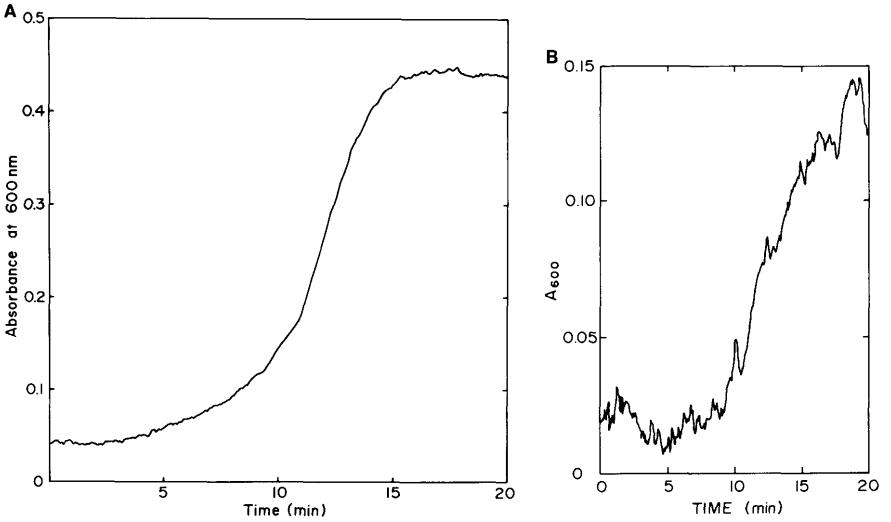

FIG. 2. Turbidometric profiles of peroxidase-mediated cytolysis using erythrocytes (A) or L1210 cells (B) as target cells. Assay cuvettes contained PBS, 30 mUnit glucose oxidase, 1 μg lactoperoxidase and 2 $\times$ 10^6 erythrocytes or 1 $\times$ 10^6 L1210 cells in 1 ml total volume.

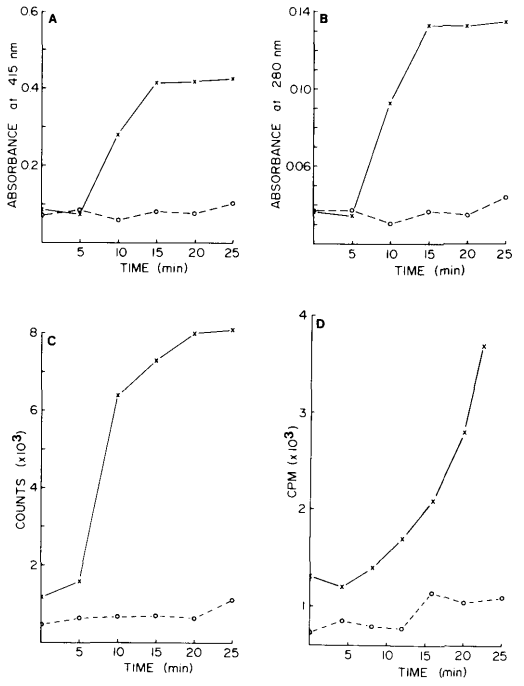

FIG. 3. Release of cellular components from human erythrocytes upon treatment with the glucose oxidase/lactoperoxidase system. The reaction mixtures were as described in Fig. 2A. At the specified time intervals the cells were removed by centrifugation and the supernatant was analyzed for its hemoglobin content (A), protein content (B), and ATP content using the luciferase reagent (C). The release of ^{51}Cr from cells previously incubated with this isotope is shown in D. (×) Complete reaction resulting in cell lysis; (○) lactoperoxidase omitted from reaction mixture.

the membrane "cracks" open like an eggshell. The ^{51}Cr release profile also supports this concept in that no appreciable amount of ^{51}Cr is released prior to the release of macromolecules.

It is also apparent that the release of ^{51}Cr is significantly slower than the release of ATP and the proteins. The reason for this is not clear. It is possible that at least some of the chromate ions are not freely dispersed in the cytoplasm, but are bound to the membrane, from which they may be slowly released after the cell has lysed.

Electron microscope studies. In an attempt to obtain further information concerning the fate of the erythrocyte membrane as a result of its exposure to the cytolytic action of lactoperoxidase we took a series of electron micrographs of the erythrocytes at various points during the lytic reaction.

Within 30 sec after the addition of lactoperoxidase to a suspension of erythrocytes the cells swelled to a round shape and the surface showed numerous "blebs" (Fig. 4B). Normal erythrocytes could no longer be found in these preparations. In the absence of glucose oxidase the erythrocytes maintained this appearance for at least 30 min, displaying no additional observable changes. In the presence of the complete cytolytic system the same initial changes in appearance were observed. After 5 to 10 min, however, the cells started to show indentations in the membrane, as if the cells were being deflated. At about the same time cracks developed in the membranes and a number of pictures showed what appears to be the release of intracellular material (Figs. 4C–E). After 20 to 25 min, when lysis appeared to be complete, all cells had the appearance of deflated cells with one or more "cracks" in their membranes. It was obvious from the large number of such membranes that were present in each of the various preparations that these membranes do not spontaneously reseal any longer.

When glucose oxidase in sufficient amounts is added to erythrocytes in the absence of lactoperoxidase, the erythrocytes change to a crenated form (Fig. 4F).

We conclude from these observations that lactoperoxidase interacts rapidly with the erythrocyte membrane, causing dramatic changes in its appearance within seconds after contact. The presence of H_2O_2 is required, however, to lead to lysis of the erythrocytes, in agreement with our kinetic observations.

Discussion. Cytolysis is generally measured by trypan blue exclusion, ^{51}Cr release, ATP release, or a similar technique. Each of these techniques represents an endpoint analysis. To establish the rate of the cytolytic reaction as well as other kinetic parameters a large number of intermediate points need to be taken. In many cases this is not possible since no means are available to stop the lytic reaction instantly. Measuring the amount of ^{51}Cr, ATP, etc. that has been released at a given time point during the cytolytic reaction requires a separation of the remaining intact

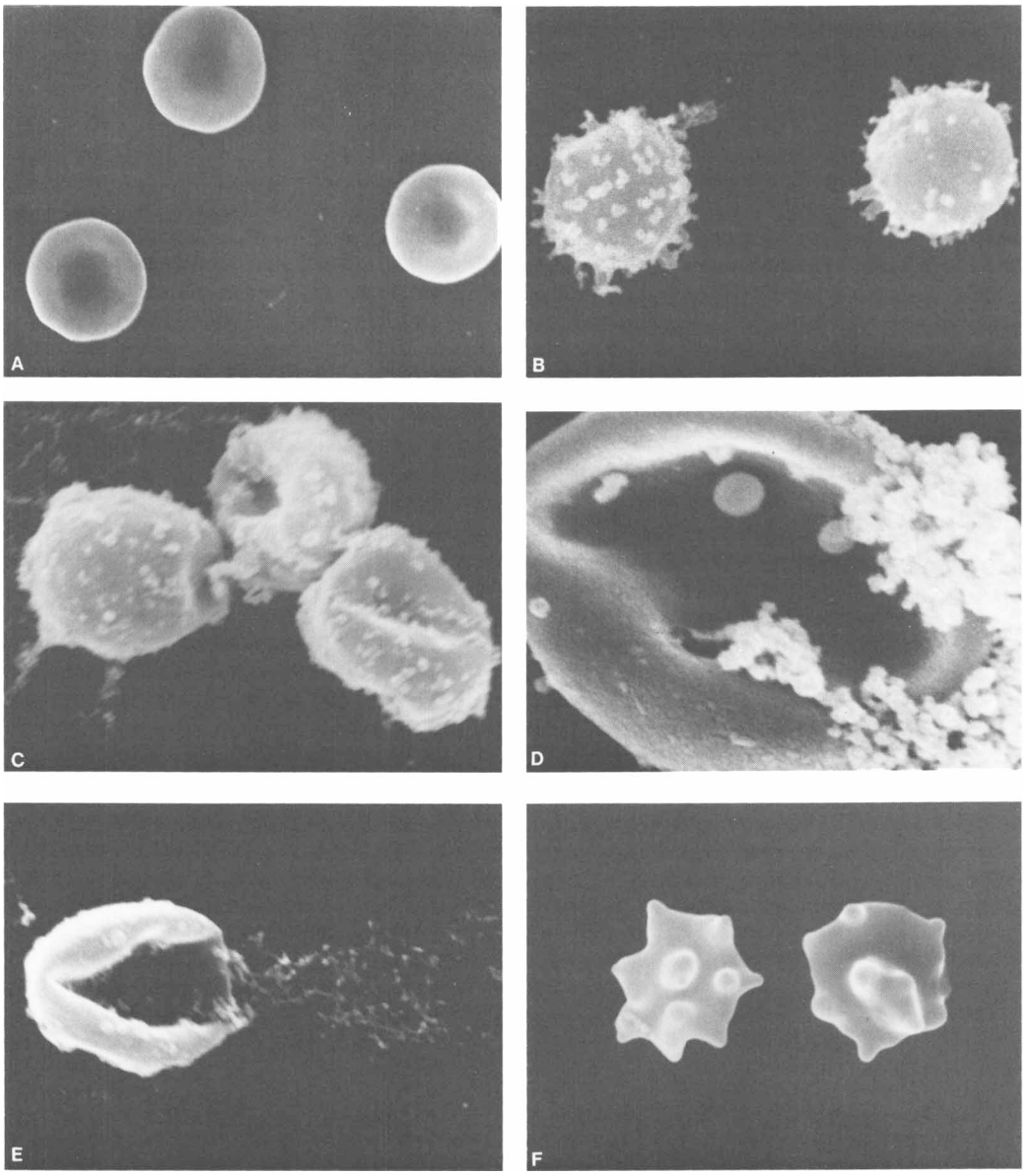

FIG. 4. The effect of lactoperoxidase-mediated cytolytic activity on fresh erythrocytes. (A) Erythrocytes suspended in PBS; (B) lactoperoxidase (1 μg/ml) added to suspension 30 sec prior to fixation; (C, D, E) lactoperoxidase (1 μg/ml) and glucose oxidase (15 mU/ml) were added to the suspension 20 min prior to fixation; (F) glucose oxidase without lactoperoxidase added 20 min prior to fixation.

cells from the released material. During this separation (usually by centrifugation) the reaction proceeds, which prevents one from establishing the exact time parameter. As a result, few reports have appeared dealing with the kinetics of a cytolytic reaction.

The turbidometric method presented here overcomes the difficulties just indicated. The kinetic behavior of the lytic process is instantly obtained, and valid data concerning the rate of cell lysis can readily be calculated.

The results presented in this paper clearly demonstrate that the lysis of erythrocytes by the lactoperoxidase–H_2O_2–halide system in-

volves a complex mechanism. This is evidenced by the considerable amount of time that elapses before any cell lysis occurs. Scanning electron microscopy has shown, however, that during this time the enzyme system is damaging the cell membrane. Surprisingly, this damage is not of a nature that results in the leakage of small molecules from the cytosol, followed by larger molecules when the damage increases. Instead, it appears that the total cell content is released at once. The swelling of the erythrocytes that occurs soon after contact with the peroxidase could indicate that the cell can no longer maintain a balanced interaction with its environment. The cytolytic system apparently continues to further damage the membrane to the point that it loses its fluidity. The development of cracks in the membrane associated with or closely following a deflation of the swollen cell, as well as the apparent inability of the cell membrane to reseal itself, are consistent with the concept that the membrane loses its fluidity and acquires a more rigid structure as a result of the action of the peroxidase.

In this context the observations of Foerder *et al.* (25, 26) are noteworthy. These authors found that the outer membrane of a sea urchin egg, which contains a significant amount of ovoperoxidase, changes its entire structure and becomes more rigid within seconds after being penetrated by a sperm cell. This prevents a second sperm cell from entering the egg. Since the event is most likely triggered by the sperm cell penetrating at a single point on the membrane, the ensuing reaction must be autocatalytic in that it rapidly spreads through the whole egg membrane. This decrease in membrane fluidity occurring in the sea urchin egg resembles the apparent increase in rigidity found with the lactoperoxidase-treated cells. The nature of the chemical changes that take place in a membrane after exposure to the action of a peroxidase are still unknown. Crosslinking of tyrosine residues to form dityrosines has been shown to occur during this reaction and this may at least in part be responsible for the increase in rigidity (25).

Information concerning the mechanism of action of the peroxidase may also be obtained by measuring the kinetic parameters of the lytic reaction under various conditions. The dependence of the initial lag time and of the rate of lysis on the concentration of peroxidase, H_2O_2, erythrocytes, and halide ion as well as on pH, ionic strength, etc. can be accurately determined with the turbidometric method and the results obtained with different peroxidases can be quantitatively compared. The results of such studies will be presented in several forthcoming publications.

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