Enzymatic Activation and Trapping of Luminol-Substituted Peptides and Proteins. A Possible Means of Amplifying the Cytotoxicity of Anti-Tumor Antibodies

(glucose oxidase/antibody-enzyme conjugates/sequential localization reactions)

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ABSTRACT Glutathione and glucose oxidase (EC 1.1.3.4) conjugates containing covalently bound luminol were prepared as prototypes for peptides and proteins with latent, enzyme-activatable chemical reactivity. In the presence of small quantities of activated horseradish peroxidase, conjugated luminol molecules were oxidized to unstable free radicals which reacted rapidly with soluble proteins and cells. These observations are of interest in regard to possible sequential localization reactions in which a few molecules of cell-bound antibody-horseradish peroxidase would be used to catalytically alter and trap many molecules of a second (luminol-substituted) enzyme, toxin, or hapten in the same area, as might be desirable in promoting selective cell destruction.

Recent studies have indicated that it is feasible to attach toxins to antitumor antibodies as a means of amplifying selective cytotoxicity (1-4), an idea first proposed by Paul Ehrlich in 1906 (5). We have suggested that it may be preferable to attach the antibody to an exogenous enzyme capable of converting a protoxin to a toxin, rather than directly to a toxin (1, 6, 7). This would permit the activity of the antibody to be catalytically amplified, providing for effective toxicity in situations in which only a limited number of conjugated antibody molecules can be localized on the cell surface. Particularly interesting results have been obtained with conjugates of antibody to glucose oxidase, a hydrogen peroxide generating enzyme with a very high turnover number. When glucose oxidase is attached through specific antibody to a target cell, and appropriate cofactors (glucose, O2, lactoperoxidase, and inorganic iodide) are added, the cell is iodinated and cell death occurs. In the complete enzyme system, with iodide or other protoxins, glucose oxidase conjugates of rabbit and goat antibodies to HeLa, HEp-2, L, human colon cancer cells (HT-29), and mouse myeloma (MOPC-315) cells have shown toxicity for their homologous cell lines in vitro (1, 7-9). Antibody-enzyme conjugates have also been shown to have marked cytotoxicity for bacteria (10) and parasites (11). In all of these cell systems immunologically specific cytotoxicity has been obtained and the antibody-enzyme conjugate has been more potent than the unconjugated antibody in the presence of excess complement.

In order to exploit the full potential of enzyme-mediated cytotoxicity in vivo, it will be necessary to localize as many enzyme molecules as possible in the tumor. One approach would be the utilization of a sequential localization reaction in which a few molecules of antibody-enzyme conjugate, specifically bound in the tumor, would be used to catalytically alter and trap many more molecules of a second enzyme in the same area. This might be accomplished by attaching a chemically unreactive organic substituent on the surface of the second enzyme and converting it to a highly reactive molecule at the level of the tumor surface. A theoretical scheme is shown in Fig. 1; E2-Ab is antibody-first enzyme conjugate; E2 is the second enzyme; X is chemically inert and attached to E2; X* is obtained from X in the presence of E2 and can react with the external cell membrane; and Y is a protoxin that is converted to Y*, a toxin, by E2.

Since the ultimate application of sequential localization would be in vivo, potentially useful types of trapping reactions are limited by the following factors: (i) The activation of X to X* should occur rapidly under physiologic conditions, even at low concentrations of E2. (ii) The reactivity of X* with the cell exterior should be high and its half-life in solution short, so that E2-X* will not diffuse away from the tumor area. (iii) In the absence of E2, X should remain stable so that the localization of E2-X* in normal tissues is avoided. This requires that X not be activated by enzymes normally present in tissue. As a candidate for X, we considered that 5-amino-2,3-hydro-1,4-phthalazinedione (luminol), which forms an unstable free radical when it is oxidized by horseradish peroxidase (12, 13), might be suitable, provided it could be conjugated to protein without losing its susceptibility to enzymatic activation. In the present report we describe a method for conjugating luminol to peptides and proteins and provide evidence that luminol conjugates of glutathione and glucose oxidase can be enzymatically activated to react rapidly with cells or soluble proteins.

MATERIALS AND METHODS

Preparation of luminol-substituted polypeptides or proteins

Glutathione substituted with luminol (luminol-glutathione) was prepared as a model for a luminol-containing, small molecular weight polypeptide or hapten; glucose oxidase substituted with luminol-glutathione was prepared as a model for a luminol-substituted enzyme.

Synthesis of [3H]Luminol-glutathione. [3H]Luminol-glutathione was obtained by reacting 5-(2-chloracetamido)-luminol with reduced L-glutathione labeled with [3H] at the gly-
cine-2 position (New England Nuclear, Boston, Mass.). Powdered 5-chloracetamido-luminol (29 mg), prepared by the method of Cross and Drew (14), and a mixture of 'H-labeled (0.2 mCi) and unlabeled glutathione (31 mg) were suspended in 5 ml of deoxygenated water; 50 mg of solid NaHCO3 were added and the pH was adjusted to 8.5 with 1 M NaOH. Nitrogen gas was bubbled through the reaction mixture, the tube was stoppered, and incubation was carried out for 16 hr at room temperature in the dark. The reaction mixture was adjusted to pH 6.0 with 1 M HCl, lyophilized, and analyzed by ascending paper chromatography in a butanol-acetic acid-water (60:15:25) solvent system at room temperature. The results of the chromatographic studies indicated that the reaction of acetamido-luminol with glutathione was almost quantitative; >80% of the radioactivity was contained in a new, bright, white luminescent spot with an Rf of 0.25-0.35. Since the sulphydryl group in reduced glutathione reacts rapidly with alkylating agents under the reaction conditions used, it can be assumed that most or all of the acetamido-luminol groups were being introduced at this position (Fig. 2). In most of the experiments the [3H]luminol-glutathione product described above was used without further purification. Where specified, the [3H]luminol-glutathione was purified to homogeneity by preparative paper chromatography in the above chromatographic system.

**Conjugation of [3H]Luminol-glutathione to Glucose Oxidase.**

One milligram of *Aspergillus niger* glucose oxidase (EC 1.1.3.4; \( \beta \)-d-glucose:oxygen 1-oxidoreductase), at a specific activity of 166 IEC units/mg (Worthington Biochemicals, Freehold, N.J.), and 2 mg of [3H]luminol-glutathione (based on [3H]radioactivity) were dissolved in 1 ml of H2O2. The solution was adjusted to pH 5.5 with dilute acid and lyophilized. The lyophilized product was dissolved in 0.025 ml of water, and 1 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (Ott Chemical Co., Muskegan, Mich.) was added. The reaction was allowed to proceed for 16 hr in the dark at 22°. After dilution and dialysis against water and 0.15 M NaCl-0.01 M phosphate, pH 7.4 (phosphate-buffered saline), the conjugate was further purified by chromatography on Sephadex G-200; 2.5% of the original radioactivity remained associated with the protein (an average of about seven molecules of luminol-glutathione per molecule of protein). By spectrofluorometric analysis (15) the glucose oxidase was still catalytically active after the conjugation reaction although the estimated specific activity was reduced more than 90%. The activity was partially restored by the addition of FAD (the coenzyme for glucose oxidase), but only to about 20% of the original level.

**Reactions of luminol-glutathione or luminol-glucose oxidase with soluble proteins or cells**

[3H]Luminol-glutathione was reacted in phosphate-buffered saline with bovine gamma globulin or bovine serum albumin (Miles Laboratories, Kankakee, Ill.) in the presence and absence of horseradish peroxidase (Type II, Sigma Chemical Co., St. Louis, Mo.) and unconjugated glucose oxidase, for various time periods at room temperature (see Table 1, below). Glucose oxidase and n-glucose were included in the reaction mixture to provide a source of H2O2, which is needed to activate the horseradish peroxidase. Conjugation was determined by the level of protein-associated [3H]radioactivity after precipitation with trichloroacetic acid and washing. The molarities given below for bovine serum albumin, bovine gamma globulin, horseradish peroxidase, and glucose oxidase are calculated on the basis of molecular weights of 70,000, 150,000, 40,000, and 160,000, respectively.

[3H]Luminol-glutathione-glucose oxidase was reacted in phosphate-buffered saline with a single cell suspension of rat spleen cells in the presence and absence of horseradish peroxidase and glucose for various time periods at room temperature. To determine the extent of conjugation of [3H]luminol-glutathione-glucose oxidase to cells, the cells were washed three times with phosphate-buffered saline and studied for cell-bound, trichloroacetic acid-precipitable radioactivity or glucose oxidase activity.

**RESULTS**

The results of the reaction of [3H]luminol-glutathione with bovine gamma globulin are shown in Table 1. In the presence
acetic acid precipitation. Omission of horseradish peroxidase, glucose, or glucose oxidase almost completely eliminated the binding, indicating that the reaction requires both horseradish peroxidase and a source of hydrogen peroxide. Horseradish peroxidase could be replaced by lactoperoxidase, an oxidizing enzyme that is similar to horseradish peroxidase in that it has a broad substrate specificity and utilizes H₂O₂ (not shown). Very similar results were obtained when bovine gamma globulin was replaced by bovine serum albumin, indicating that the reaction takes place with other soluble proteins (not shown).

Additional data indicated that luminol was directly involved in the conjugation reaction: (i) When chromatographically purified luminol-glutathione was used under similar experimental conditions, up to 70% of the radioactivity was conjugated to protein. (ii) When unsubstituted [³H]glutathione (reduced or oxidized) was used instead of [³H]luminol-glutathione, protein binding was low (<5%) and no different after exposure to the complete enzyme system than in its absence.

The enzymatic activation of [³H]luminol-glutathione was efficient, as indicated by an ability to obtain conjugation at low concentrations of glucose oxidase or horseradish peroxidase. With a 60-min reaction time, 60 mM glucose, and 30 μM horseradish peroxidase, >20% coupling was obtained at glucose oxidase concentrations as low as 1 mM (not shown). With 3.6 μM glucose oxidase and the same glucose concentration, >20% coupling was obtained with horseradish peroxidase concentrations of 100 mM and above, and there was significant coupling (>8%) at a horseradish peroxidase concentration of 6 mM.

We next considered whether the protein to which [³H] luminol-glutathione was attached at the completion of the conjugation was serum protein (bovine gamma globulin or bovine serum albumin) or whether the enzymes themselves were involved. (Binding to horseradish peroxidase had to be particularly considered because it is directly involved in the activation of luminol.) To evaluate this question, reaction mixtures con-

![Graph](image_url)

**Fig. 3.** Chromatography of [³H]luminol-glutathione protein mixtures. A mixture of [³H]luminol-glutathione (36 μM), bovine serum albumin (BSA, 140 μM), horseradish peroxidase (HRP, 30 μM), glucose oxidase (GO, 3.6 μM), and glucose (60 mM) was incubated for 60 min at room temperature. After dialysis of the reaction mixture against phosphate-buffered saline, an aliquot containing 3.3 absorbance units (at 280 nm) was chromatographed on a 1.3 × 34-cm column of Sephadex G-100 in phosphate-buffered saline at 4°C. Effluent fractions were monitored for enzyme activity, absorbance at 280 nm (●), and radioactivity (○); 79% of the radioactivity and 92% of the 280 nm absorbance applied to the column were recovered. The column was calibrated separately with the individual proteins (elution volumes containing the highest concentrations of the respective proteins are indicated by the arrows).

**Table 1.** Conjugation of [³H]luminol-glutathione to bovine gamma globulin in the presence and absence of horseradish peroxidase and a source of hydrogen peroxide

<table>
<thead>
<tr>
<th>Reaction condition</th>
<th>[³H]Luminol-glutathione, μM</th>
<th>Bovine γ-globulin, μM</th>
<th>Horseradish peroxidase, μM</th>
<th>Glucose oxidase, μM</th>
<th>Glucose, mM</th>
<th>Protein conjugated with protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>36</td>
<td>72</td>
<td>30</td>
<td>3.6</td>
<td>60</td>
<td>41</td>
</tr>
<tr>
<td>2</td>
<td>36</td>
<td>18</td>
<td>30</td>
<td>3.6</td>
<td>60</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>36</td>
<td>3</td>
<td>30</td>
<td>3.6</td>
<td>60</td>
<td>9</td>
</tr>
<tr>
<td>4</td>
<td>36</td>
<td>—</td>
<td>30</td>
<td>3.6</td>
<td>60</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>36</td>
<td>72</td>
<td>—</td>
<td>3.6</td>
<td>60</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>36</td>
<td>72</td>
<td>30</td>
<td>—</td>
<td>60</td>
<td>4</td>
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<tr>
<td>7</td>
<td>36</td>
<td>72</td>
<td>—</td>
<td>3.6</td>
<td>60</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>36</td>
<td>72</td>
<td>30</td>
<td>3.6</td>
<td>—</td>
<td>4</td>
</tr>
</tbody>
</table>

The reaction was carried out in phosphate-buffered saline in the dark at room temperature for 60 min in a final volume of 0.085 ml. The protein in the reaction mixture was precipitated by the addition of an equal volume of 20% trichloroacetic acid at 4°C. The precipitate was washed three times with cold 10% trichloroacetic acid, dried at 70°C, dissolved in liquid scintillation mixture, and counted for 10 min in a Nuclear Chicago liquid scintillation counter.

**Table 2.** Effect of time on the conjugation of enzyme activated luminol-glutathione with bovine gamma globulin

<table>
<thead>
<tr>
<th>Total reaction time, min</th>
<th>Time of addition of bovine γ-globulin, min</th>
<th>% Radioactivity conjugated to protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>6.2</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>7.4</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>7.4</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>28</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>6.6</td>
</tr>
<tr>
<td>60</td>
<td>0</td>
<td>37</td>
</tr>
<tr>
<td>60</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>60</td>
<td>5</td>
<td>8</td>
</tr>
</tbody>
</table>

Each reaction mixture contained 60 mM glucose, 30 μM horseradish peroxidase, 3.6 μM glucose oxidase, and 36 μM [³H]-luminol-glutathione in phosphate-buffered saline at room temperature. At various times (0 min, 1 min, or 5 min) bovine gamma globulin was added to a final concentration of 72 μM. The reaction was stopped by the addition of an equal volume of cold 20% trichloroacetic acid, and the radioactivity in the protein precipitate was determined (see legend to Table 1).
taining bovine serum albumin and the other reactants were dialyzed without prior trichloroacetic acid precipitation and chromatographed on Sephadex G-100 (Fig. 3). Under the conditions used, a satisfactory separation of horseradish peroxidase from bovine serum albumin and glucose oxidase was obtained. More than 90% of the radioactivity was in the bovine serum albumin and glucose oxidase fraction, indicating that when bovine serum albumin is present in the medium, horseradish peroxidase does not react to a major extent with luminol-glutathione. Additional studies on Sephadex G-200 indicated that >90% of the radioactivity in the higher-molecular-weight protein fraction migrated with bovine serum albumin rather than glucose oxidase. As further evidence that the reaction of luminol-glutathione with the two enzymes is limited, there was markedly reduced binding of radioactivity to trichloroacetic acid-precipitable protein when bovine serum albumin and bovine gamma globulin were omitted from the reaction mixture (Table 1). Finally, oxidation of up to 500 nmol of luminol-glutathione in the presence of 5 nmol each of horseradish peroxidase and glucose oxidase did not result in detectable inactivation of either enzyme.

The kinetics of the enzyme-mediated reaction of luminol-glutathione with bovine gamma globulin were studied (Table 2). Under the conditions used, the reaction of luminol-glutathione with protein was more than 60% complete within 60 sec. When bovine gamma globulin was added 1 min after the initiation of luminol-glutathione oxidation, binding of luminol-glutathione to trichloroacetic acid-precipitable protein was reduced more than 5-fold. Evidently, then, the reactive form of luminol-glutathione decays rapidly if a suitable target protein is not available.

The properties of the reactive intermediate of luminol-glutathione (rapid generation, formation in the presence of two different peroxidases, a high rate of reactivity, and rapid decay) are consistent with the free radical mechanism outlined in the introduction. This mechanism is further supported by blocking studies in which compounds containing functional groups present on proteins were used in an attempt to inhibit the reaction. The reaction with bovine gamma globulin was carried out in the presence of 100 mM of tyramine, acetate, ethanol, e-aminocaproate, α-N-acetylimidine, and 2-mercaptoethanol under the reaction conditions given in the legend to Table 1. The order of inhibition was 2-mercaptoethanol (98%) > tyramine (92%) > e-aminocaproate (30%), with no blocking by other agents. This is the order of reactivity that would be expected with a free radical scavenger. The reaction mechanism can, therefore, be formulated as follows, assuming the 2-electron transfer mechanism shown for unconjugated luminol (16) holds for conjugated

\[
\begin{align*}
\text{glucose oxidase} & \\
\text{O}_2 + \text{glucose} & \rightarrow \text{H}_2\text{O}_2 + \text{gluconic acid} \quad [1] \\
\text{HRP} + \text{H}_2\text{O}_2 & \rightarrow \text{HRP-complex I} \quad [2] \\
\text{HRP-complex I and 2 LG} & \rightarrow \text{HRP + 2 - LG} \quad [3] \\
\cdot\text{LG}^+ + \text{protein (SH, PheOH, NH}_2) & \rightarrow \text{protein-LG} \quad [4]
\end{align*}
\]

where HRP-complex I is the initial complex formed between horseradish peroxidase and H\textsubscript{2}O\textsubscript{2}, and LG is luminol-glutathione.

From the experiments with [\textsuperscript{3}H]luminol-glutathione it was clear that small-molecular-weight peptides containing luminol could be efficiently attached to soluble proteins. To study the ability of enzymes containing luminol to react in a similar fashion, we next conjugated [\textsuperscript{3}H]luminol-glutathione to glucose oxidase by means of a water-soluble carbodiimide, which promotes peptide bond formation between the protein and free amino or carboxyl groups on the peptide. This provides a means of attaching luminol-glutathione to the enzyme without oxidizing the luminol portion of the molecule. The reactivity of the conjugate with rat spleen cells was then studied. In the presence of soluble horseradish peroxidase and glucose, as many as 39% of the luminol-glutathione oxidase molecules could be conjugated to cells (based both on \textsuperscript{3}H radioactivity and enzyme activity). With nonderivatized glucose oxidase, cells, and the same cofactors, 2% or less binding was obtained (Table 3).

**DISCUSSION**

This study indicates that peroxidases will activate luminol-substituted peptides or proteins, resulting in their attachment to soluble proteins and cells. The kinetics of the reaction of conjugated luminol with horseradish peroxidase have not been analyzed in detail, although it is clear that the reaction is rapid. This is not surprising since unconjugated luminol has an estimated second order rate constant for its reaction with the horseradish peroxidase-H\textsubscript{2}O\textsubscript{2} complex of 2.3 × 10\textsuperscript{3} mol\textsuperscript{-1}·sec\textsuperscript{-1} (16). As discussed in the introduction and directly demonstrated with glucose oxidase, luminol can be attached to an enzyme and oxidized, creating the possibility of stacking enzyme molecules on the cell surface and using their catalytic activity to produce cytotoxicity. At a more speculative level, once a luminol-substituted enzyme had been localized, the principle could be extended to still another enzyme, creating a man-made enzymatic cascade. The amplification inherent in this system is of particular interest for human tumors in which the density of tumor-specific antigen on the cell surface is too low to permit useful levels of antibody- or lymphocyte-mediated cytotoxicity directly.

A specific scheme by which the luminol reaction might be exploited to obtain immunologically directed target cell damage is shown in Fig. 4. This would involve the action of cell-bound antitumor IgG or Fab fragments conjugated to

**Table 3. Conjugation of [\textsuperscript{3}H]luminol-glutathione-glucose oxidase to rat spleen cells**

<table>
<thead>
<tr>
<th>Type glucose oxidase</th>
<th>% Radioactivity bound to cells</th>
<th>% Total enzyme activity bound to cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conjugated to [\textsuperscript{3}H]luminol-glutathione</td>
<td>+</td>
<td>30</td>
</tr>
<tr>
<td>Unconjugated</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Rat spleen cells (2 × 10\textsuperscript{6}) were incubated in phosphate-buffered saline with 12 μM horseradish peroxidase, 10 mM or 0 mM glucose, and 6 μM glucose oxidase (either conjugated to [\textsuperscript{3}H]luminol-glutathione or unconjugated). The final reaction volume was 0.05 ml. The reaction was at room temperature (24°) for 120 min. The cells were washed, and cell-associated radioactivity ([\textsuperscript{3}H]luminol-glutathione-glucose oxidase only) and glucose oxidase activity were determined.
Peroxidase

ing the cytotoxic oxidized, supplied. The LGO bound oxidase. The galactose) cell inorganic which from this selective peroxidase molecules, oxidase would turn to react with the surface of the cell. Unbound L-GO is then cleared. Lactoperoxidase and iodide are supplied. The lactoperoxidase utilizes H₂O₂ produced by cell-bound L-GO to promote cell iodination and death.

horseradish peroxidase on luminol-substituted glucose (or galactose) oxidase. The luminol-glucose oxidase would generate H₂O₂ from endogenous glucose, activating the horseradish peroxidase, which would in turn promote the reaction of the luminol moiety with the cell, locking the glucose oxidase on the cell exterior. After clearing unreacted luminol-glucose oxidase (by exchange transfusion), excess lactoperoxidase and inorganic iodide would be supplied. As shown in previous studies from this laboratory, H₂O₂ generated by glucose oxidase bound to the cell surface will activate nearby lactoperoxidase molecules, resulting in cell iodination and death (7–9). The reaction is sufficiently well localized to produce highly selective cell damage in heterogeneous cell mixtures (17).

While we have not yet attempted to obtain target cell damage with luminol-substituted enzymes, a major gain in cytotoxicity is potentially obtainable, particularly if inactivation of the enzyme can be avoided both during the conjugation of luminol to the enzyme and at the time the luminol portion of the conjugate is activated by horseradish peroxidase. With the luminol-glutathione-glucose oxidase conjugate, used in the present study, inactivation in the presence of horseradish peroxidase has not appeared to be a problem. However, at the time of the attachment of luminol-glutathione to the enzyme, there is a considerable reduction in catalytic activity, indicating a need for systematic studies with other enzymes and other conjugation procedures to insure that conjugates with maximal specific activities are obtained. Potential problems are presented by peroxidases, catalases, soluble proteins, and amino acids present in tissue which might inhibit or misdirect the reaction. It may be necessary to explore other enzyme-activatable systems to avoid problems of nonspecific tissue localization.

Antibodies, haptenes, coenzymes, metals (18), growth inhibitory glycolipids (19), or other proteins might also be fixed to cells through luminol and used to inhibit cell growth. For example, by attaching large numbers of haptenic groups to cells, the cells would be primed to undergo complement-mediated damage in the presence of excess antihapten antibody.

The possible application of enzymatically mediated conjugation reactions is not limited to the production of selective cytotoxicity. In the presence of activated horseradish peroxidase, luminol-substituted proteins and polypeptides with affinity for cells should combine covalently with cellular receptors, providing an alternative to photoaffinity labeling for obtaining binding that is both selective and irreversible. In addition, luminol derivatives containing heavy metals are alternative substrates for horseradish peroxidase in immunoperoxidase studies of antigen or antibody localization at the ultrastructural level.

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