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Enzyme Immunoconjugates Utilizing Glucose Oxidase and Myeloperoxidase Are Cytotoxic to *Candida tropicalis*

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A dual-enzyme immunoconjugate system was evaluated for its cytotoxic effect on *Candida tropicalis*. Glucose oxidase, which generates hydrogen peroxide in the presence of glucose and oxygen, and myeloperoxidase, which catalyzes the oxidation of halides in the presence of hydrogen peroxide, were each conjugated to a *C. tropicalis*-specific monoclonal antibody. Neither the glucose oxidase nor the myeloperoxidase conjugates exhibited any significant cytotoxic effect by themselves. A combination of glucose oxidase conjugate (3.2 ng/ml) and myeloperoxidase conjugate (12.8 ng/ml) in the presence of 5 mg of glucose per ml, 150 mM chloride, and 50 μM iodide was cytotoxic to *C. tropicalis*, killing 99.9% of the treated sample. Flow cytometry was used to characterize the binding of the conjugates to yeast cells and demonstrated that the binding of both conjugates to the yeast cell surface is required for cytotoxicity. In addition, the concentrations of conjugates required for a cytotoxic effect were below the concentrations required to saturate all of the yeast cell surface antibody-binding sites.

Candida tropicalis infections are a significant cause of morbidity and mortality among immunocompromised patients (18, 24). The role of polymorphonuclear cells (PMNs) in protecting against fungal infections is illustrated by the observation that neutropenic mice are much more susceptible to fatal *C. tropicalis* infections than healthy animals (5).

One of the antimicrobial agents contained within the phagosomes of PMNs is myeloperoxidase (MPO). Hydrogen peroxide is generated by activated PMNs during a respiratory burst of metabolism. When hydrogen peroxide is present, MPO catalyzes the oxidation of halide ions which in turn causes oxidative damage and cell death. This hydrogen peroxide-peroxidase system has been shown to be effective against a variety of microorganisms, including fungi (11, 17). In addition, some MPO is released from the cell during phagocytosis (1), and since MPO has been shown to bind to *Candida* cell wall mannans, it has been implicated as a possible antifungal agent outside the PMN as well (25).

In this report, we describe the characterization and testing of anti-*C. tropicalis* enzyme immunoconjugates patterned after the oxidase-peroxidase system of PMNs. Okuda et al., using unpurified conjugates consisting of polyclonal anti-*C. albicans* antibodies coupled to xanthine oxidase and lactoperoxidase, first demonstrated that a peroxide-peroxidase immunoconjugate system could be cytotoxic to yeast cells (19). We further refined this approach by making anti-*C. tropicalis* enzyme immunoconjugates by using monoclonal antibodies of defined specificity which could be produced in large amounts and by purifying the conjugates to remove both unconjugated enzyme and antibody. The enzymes chosen for these conjugates, MPO and glucose oxidase (GO), have advantages over lactoperoxidase and xanthine oxidase for use as a potential in vivo therapy. MPO is an enzyme with established antifungal activity (17). In addition, it has been shown that the antimicrobial activities of MPO and hydrogen peroxide are potentiated by as little as 0.5 mM chloride, well below the physiological levels of chloride in vivo (11). In contrast, lactoperoxidase requires iodide, bromide, or thiocyanate (which is considered the physiological pseudohalide) to potentiate its cytotoxic effect but does not oxidize chloride (23). GO produces hydrogen peroxide from glucose and oxygen, substrates readily available in vivo, eliminating the need to supply the enzyme with a substrate, as in the case of xanthine oxidase.

MATERIALS AND METHODS

Yeast cultures. *C. tropicalis* (ATCC 28707) and *Saccharomyces cerevisiae* (ATCC 9763) were obtained from the American Type Culture Collection (Rockville, Md.). Stock cultures of yeast cells were grown at 37°C on Sabouraud dextrose agar plates (Difco Laboratories, Detroit, Mich.) and stored at 4°C. Before each experiment, a single colony was inoculated into 5 ml of Sabouraud dextrose broth (Difco) and grown for 16 h at 37°C without agitation. An A_{540} reading of the culture was determined and then diluted with Sabouraud dextrose broth to obtain 1.0 optical density unit (approximately 2×10^7 CFU/ml).

Anti-*C. tropicalis* monoclonal antibody purification and characterization. The monoclonal antibody used in this study was provided by Chemunex S.A., Maisons Alfort, France. The antibody, designated CTR-35, was generated by the immunization of a mouse with *C. tropicalis* followed by a standard hybridoma fusion protocol (28). CTR-35, a subclass 3 immunoglobulin G (IgG), was purified from ascites fluid by protein A affinity chromatography (4). The binding of the monoclonal antibody was analyzed by using a flow cytometer (FACStar; Becton Dickinson and Co., Paramus, N.J.). A 0.1-ml sample of a yeast culture (1.0 unit of optical density at 540 nm) was incubated for 1 h at 22°C with 1 μg of purified antibody in staining buffer (3% dialyzed fetal bovine serum in phosphate-buffered saline). After the samples were washed twice by centrifugation, the bound surface antibody was detected by staining with 5 μg of fluorescein isothiocyanate (FITC)-labeled goat anti-mouse immunoglobulins (Tago) per ml for 30 min at 4°C. The samples were washed, fixed with 0.5% paraformaldehyde in phosphate-buffered saline (16), and analyzed. To generate histograms, data were collected from 10,000 individual yeast cells.

GO. GO from *Aspergillus niger* was purchased from

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Sigma Chemical Co., St. Louis, Mo. (G-2133), and used without further purification.

MPO purification. MPO was purified from HL-60 cells (ATCC CCL 240), a human promyelocytic leukemia cell line, by using a modification of a previously described procedure (27). Briefly, 150 g of cells frozen at −70°C (about 1.5×10^{11} cells) was thawed, suspended in 480 ml of homogenizing buffer (6.7 mM sodium phosphate–3 mM sodium chloride–1 mM magnesium chloride–0.3% [vol/vol] Triton X-100 [pH 6.6]), and homogenized for 1 min by using an Ultra-Turrax homogenizer. The homogenate was centrifuged at 17,000 × *g* for 20 min, and the supernatant was discarded. The pellet was washed with homogenizing buffer and suspended in 480 ml of homogenizing buffer without Triton X-100. The cell suspension was stirred for 16 h at 4°C and centrifuged at 17,000 × *g* for 20 min. The supernatant was discarded, and the pellet was detergent extracted with 650 ml of 1% cetyltrimethylammonium bromide in 0.1 M potassium phosphate (pH 7.8) by homogenizing for 1 min with an Ultra-Turrax homogenizer and stirring for 2 h at 4°C. The extract was centrifuged at 17,000 × *g* for 20 min, and the supernatant was filtered with Whatman no. 1 filter paper. Solid ammonium sulfate was added to the supernatant to 70% saturation. After a 16-h incubation at 4°C, the precipitate was harvested by centrifugation at 17,000 × *g* for 20 min and the pellet was dissolved in 40 ml of 0.15 M sodium chloride–20 mM sodium citrate (pH 5.8). The crude MPO was further purified by size exclusion chromatography on a Sephacryl S-200 HR column (2.5 by 95 cm) equilibrated with 0.15 M sodium chloride–20 mM sodium citrate (pH 5.8). The peroxidase-containing fractions were determined by calculating the Rz value (optical density at 430 nm/optical density at 280 nm). Those fractions with Rz values of 0.7 or greater were pooled and concentrated to approximately 5 mg/ml on a YM-10 membrane (Amicon Corp., Lexington, Mass.). The sample was filter sterilized and stored at 4°C.

Enzyme-antibody conjugation. The enzyme-antibody conjugates were made by using a modification of a previously described method (14). GO and MPO were activated separately by incubating them with a 10-fold molar excess of succinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (Pierce Chemical Co., Rockford, Ill.) in 0.1 M sodium phosphate–0.5 mM EDTA (pH 7.0) for 30 min at 30°C. The unreacted reagent was removed by dialysis against 0.1 M sodium phosphate–0.5 mM EDTA (pH 7.0) for 16 h at 4°C.

Sulfhydryl groups were introduced into the purified monoclonal antibody by incubation for 3 h at 0°C with a 75-fold molar excess of 2-iminothiolane (Pierce) in 0.15 M sodium chloride–10 mM sodium phosphate–60 mM triethanolamine–1 mM EDTA (pH 8.0) under nitrogen. The unreacted reagent was removed by size exclusion chromatography on a P-6 (Bio-Rad Laboratories, Richmond, Calif.) column (1.6 by 16 cm) preequilibrated with 50 mM sodium chloride–5 mM bis-tris/acetate–1 mM EDTA (pH 5.8).

The modified antibody (5 mg) was combined with a fivefold molar excess of activated GO or MPO and allowed to react at 4°C for 24 to 48 h under nitrogen. Unreacted free sulfhydryl groups were blocked by incubating with 2 mM iodoacetamide for 1 h at 22°C. Conjugates were purified from the unreacted enzymes by using protein A-Sepharose chromatography. After affinity chromatography, any remaining free antibody was separated from the conjugated antibodies by size exclusion chromatography on a column (1.6 by 97 cm) of Sephacryl S-300 HR equilibrated with 0.5 M sodium chloride–20 mM sodium citrate (pH 5.8). The conjugates eluted as a single peak well separated from the free antibody peak.

Immunoconjugates, evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 3 to 27% acrylamide gradient gel, were free of unconjugated antibody and enzymes (data not shown).

Enzyme assays. MPO activity was measured by a colorimetric assay with *o*-phenylenediamine (Sigma) as a substrate. Samples (100 μl) were diluted in phosphate-citrate buffer (0.103 M sodium phosphate, 0.0486 M citric acid [pH 5.0]) in 96-well enzyme-linked immunosorbent assay plates (Nunc, Roskilde, Denmark). The reaction was started by adding to each well 50 μl of phosphate-citrate buffer containing *o*-phenylenediamine (1 mg/ml) and 0.3% hydrogen peroxide. The rate of color development at 490 nm was measured for 2 min with a Molecular Devices V_{max} plate reader. This rate of color development, in milli-optical density units per minute, was directly proportional to the enzyme concentration over the range of 0.3 to 20 μg/ml. GO activity was measured by using essentially the same assay, except the substrate solution consisted of phosphate-citrate buffer containing 4% glucose, 5 U of horseradish peroxidase (Sigma) per ml, and 1 mg of *o*-phenylenediamine per ml. The GO assay was linear over the range of 0.03 to 1.0 μg/ml.

Enzymatic activity determinations showed that on the average, the immunoconjugates consisted of one enzyme molecule coupled to one antibody. The immunoconjugate concentrations used in this report were based on the enzymatic activity. Total protein concentrations were approximately twice the concentrations determined by the enzymatic assay.

Protein assays. Protein concentrations were determined by using the bicinchoninic acid protein assay (Pierce), with bovine serum albumin as a standard (21).

In vitro assay for *Candida* cytotoxicity. About 10^6 CFU of *C. tropicalis* was collected by centrifugation, suspended in 100 μl of staining buffer, and incubated with 25 μl of various concentrations of the antibody conjugates, free enzymes, antibody, or buffer control in sterile Eppendorf tubes for 1 h at 22°C. The samples were diluted 250-fold into 1 ml of substrate buffer (3% dialyzed fetal bovine serum, 5 mg of glucose per ml, 50 μM potassium iodide in phosphate-buffered saline) in sterile polypropylene-capped tubes (12 by 75 mm; Falcon; Becton Dickinson). The samples were incubated in a 37°C shaker incubator with the caps loosened to allow gas exchange. After being incubated for 4 h, the samples were chilled to 0°C to stop the enzymatic reaction. Several dilutions for each sample were made, and 75 μl was spread onto Sabouraud dextrose agar plates in triplicate. After incubating the plates for 24 to 48 h at 37°C, the number of visible colonies was tabulated and the number of CFU was determined.

Anti-enzyme fluorescence staining reagents. New Zealand White rabbits were immunized with either GO or MPO as described previously (3). IgG was purified from the immune sera by protein A affinity chromatography (4). Rabbit anti-GO antibodies were conjugated with biotin by using *N*-hydroxysuccinimidobiotin (8), and anti-MPO antibodies were labeled with FITC, yielding a fluorochrome/protein ratio of 1.4:1 (6).

Analysis of conjugate binding by flow cytometry. As described above, *C. tropicalis* cells were incubated for 1 h at 22°C with anti-*Candida* enzyme immunoconjugates, free enzymes, unconjugated antibody, or buffer control. The samples were washed twice and resuspended in 100 μl of staining buffer. The cells were incubated for 30 min at 4°C

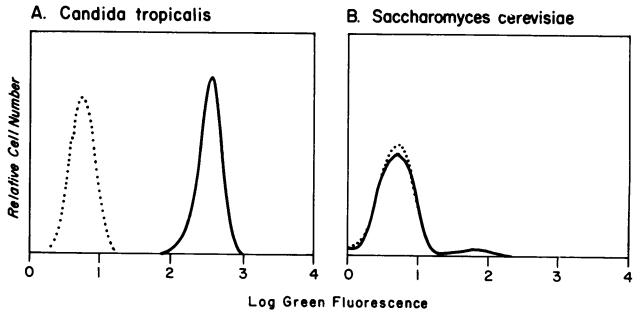

FIG. 1. Fluorescence histograms of yeast cells incubated with monoclonal antibody CTR-35 and stained for murine IgG binding. (A) *C. tropicalis* ATCC 28707; mean, 379; SD, ±137. (B) *S. cerevisiae* ATCC 9763; mean, 5.8; SD, ±3.1. Mean background fluorescence with FITC-labeled goat anti-mouse IgG staining alone was 6.0 (SD, ±3.0) for both samples. Dashed lines indicate background staining of yeast cells with FITC-labeled goat anti-mouse IgG alone. Solid lines indicate yeast cells incubated with monoclonal antibody CTR-35 followed by FITC-labeled goat anti-mouse IgG.

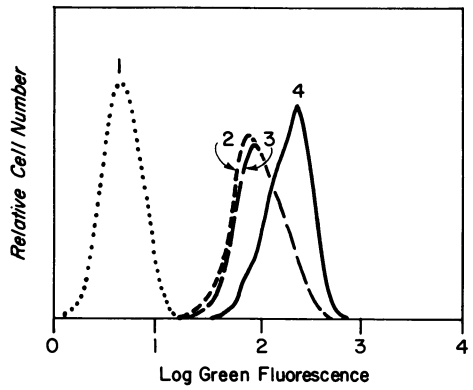

FIG. 2. Fluorescence histograms of *C. tropicalis* ATCC 28707 incubated with monoclonal antibody CTR-35 or GO or MPO enzyme immunoconjugates and stained for murine IgG binding. Cells were incubated with FITC-labeled goat anti-mouse IgG only (mean, 6; SD, ±5.8) (peak 1), GO conjugate (mean, 118; SD, ±74.3) (peak 2), MPO conjugate (mean, 127; SD, ±74.9) (peak 3), or unconjugated CTR-35 antibody (mean, 223; SD, ±107) (peak 4).

with a 25-μl mixture of biotinylated rabbit anti-GO antibody and FITC-labeled rabbit anti-MPO antibody, at concentrations of approximately 15 μg/ml for both staining antibodies. The yeast cells were washed again and incubated for 30 min at 4°C with 25 μl of a solution (10 μg/ml) of phycoerythrin-avidin (Fisher Scientific Co., Pittsburgh, Pa.). After a final wash, the yeast cells were analyzed on a FACStar flow cytometer. To generate contour plots, data on 10,000 yeast cells per sample were collected.

Statistical analysis. Statistical significances between the untreated sample and the test samples were determined by the Student's *t* test.

RESULTS

Monoclonal antibody and enzyme immunoconjugate-binding characterization. The binding specificity of CTR-35 was determined by flow cytometry. Yeast cells were incubated with the anti-*Candida* antibody and then stained with FITC-labeled goat anti-mouse immunoglobulins. The monoclonal antibody CTR-35 reacted strongly with *C. tropicalis*, resulting in a relative mean fluorescence signal ± a standard deviation (SD) of 379 ± 137 (Fig. 1). When the antibody was incubated with an irrelevant yeast, *S. cerevisiae*, the relative mean signal was only 5.8 ± 3.1, which was equivalent to the background fluorescence (Fig. 1). Approximately 7% of the *S. cerevisiae* population exhibited a fluorescence signal above the background fluorescence. This signal was due to nonspecific binding of FITC-labeled goat anti-mouse reagent alone and not to CTR-35 binding. Yeast cells treated with FITC-labeled goat anti-mouse reagent alone or with irrelevant murine IgG and then stained had identical histogram profiles with mean fluorescence values of 6.0 ± 3.0 (Fig. 1).

Histograms showing a comparison of the binding activity of the enzyme immunoconjugates with unconjugated antibody are shown in Fig. 2. *C. tropicalis* treated with the GO or MPO conjugates resulted in relative mean fluorescence signals of 118 ± 74.3 and 127 ± 74.9, respectively. A slightly higher mean fluorescence signal of 223 ± 107 was seen with yeast cells treated with the unconjugated antibody. A mean fluorescence signal of 6.0 ± 3.0 was seen with yeast cells treated with FITC-labeled goat anti-mouse reagent alone.

Cytotoxic effects of the enzyme conjugates in vitro. Three essential components of the peroxidase-mediated antimicrobial system are hydrogen peroxide, the peroxidase enzyme, and an oxidizable halide or pseudohalide, such as thiocyanate. It has been reported that iodide is more effective than chloride on a molar basis as the halide component of the MPO-mediated antimicrobial system (11). As a result of this observation and in order to compare our results with those of other peroxidase immunoconjugate systems utilizing lactoperoxidase (which does not oxidize chloride), we used a buffer containing 150 mM chloride and 50 μM iodide to evaluate the cytotoxic effects of MPO. In our system, buffers that contained both iodide and chloride gave results equivalent to those given by buffers containing 150 mM chloride as the only halide source (data not shown).

It is known that hydrogen peroxide at high concentrations is toxic to microorganisms (10). For the strain of *C. tropicalis* tested (ATCC 28707), 1 mM hydrogen peroxide was toxic, but concentrations lower than 100 μM had no measurable effect on cell viability.

To compare the cytotoxic effect of the MPO immunoconjugate with free MPO on *C. tropicalis*, free or conjugated MPO (8 μg/ml) was first allowed to bind to the yeast cells. The samples were then diluted 250-fold into buffer, with or without 100 μM hydrogen peroxide. After the incubation period, serial dilutions of each sample were made and a sample was removed and grown on Sabouraud dextrose agar plates to quantitate viable yeast cells. A 32-ng/ml dose of the MPO conjugate (enzyme concentration) plus hydrogen peroxide resulted in a reduction of viable yeast cells from 5×10^5 to 1.25×10^4 CFU (Fig. 3). This corresponds to 97.5% killing relative to the untreated culture. Unconjugated MPO (32 ng/ml) plus hydrogen peroxide resulted in 20% killing. Ten percent or less of the yeast cells were killed when the culture was treated with free MPO, MPO conjugate, or hydrogen peroxide alone (Fig. 3).

Instead of supplying hydrogen peroxide to the MPO conjugate exogenously, we tested a hydrogen peroxide-generating immunoconjugate composed of monoclonal antibody CTR-35 coupled to GO. The dual-enzyme immunoconjugate system was tested by incubating *C. tropicalis* with the conjugates, singly or in combination, and then diluting the mixture 250-fold into a substrate solution containing glucose. The samples were incubated, with agitation, for various times at 37°C. Initial experiments showed that a 4-h incuba-

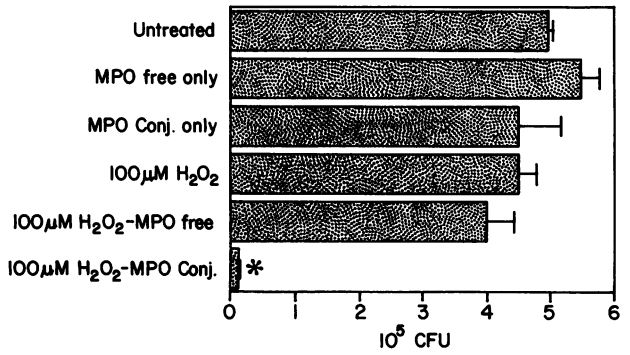

FIG. 3. Effects of MPO conjugate (Conj.) or free MPO and hydrogen peroxide on *C. tropicalis* ATCC 28707. About 5×10^5 CFU of yeast cells was incubated with 8 μg of MPO conjugate or free MPO per ml for 1 h at 22°C. Samples were diluted in substrate-containing buffer with or without 100 μM H_2O_2 and treated as described in the text. Values are means ± the standard error of the mean (n = 3). *, Significantly different from value for untreated sample ($P < 0.005$).

tion with substrate produced maximal killing. We also observed that it was necessary to agitate the samples to achieve maximal killing. Neither enzyme immunoconjugate alone showed any statistically significant killing at a final concentration of 32 ng/ml (enzyme concentration) (Table 1). The maximum cytotoxic effect was seen with a combination of GO conjugate (3.2 ng/ml) and MPO conjugate (12.8 ng/ml) which resulted in a 99.9% reduction in *C. tropicalis* viability. If the GO conjugate was reduced to 1.6 ng/ml, the cytotoxic effect was slightly less, resulting in 98.0% killing. However, the cytotoxic effect of 1.6 ng of GO conjugate per ml could be augmented if the MPO conjugate was increased twofold from 12.8 to 25.6 ng/ml. This combination resulted in 99.9% killing of the yeast cells. The cytotoxicity of a suboptimal combination of 6.4 ng of MPO conjugate per ml and 1.6 ng of GO conjugate per ml, which resulted in 96.6% of the yeast cells being killed, could likewise be augmented by increasing the GO conjugate 16-fold to 25.6 ng/ml, resulting in a 99.9% reduction in yeast viability.

Free enzymes were also tested to determine their cytotoxic effects on *C. tropicalis*. No statistically significant cytotoxicity was seen with 32 ng of MPO or GO per ml alone or with any of the combinations found to be cytotoxic for the conjugates.

TABLE 1. Effects of enzyme immunoconjugates on *C. tropicalis* viability in vitro

Treatment	CFU (10^5)[a]
None	7.90 ± 0.53
Antibody alone (32 ng/ml)	6.30 ± 0.22
Enzyme immunoconjugates (GO/MPO)[b]	
32.0/0	5.50 ± 0.05
0/32.0	6.20 ± 0.15
19.2/12.8	<0.01[c]
6.4/12.8	<0.01[c]
3.2/12.8	<0.01[c]
1.6/12.8	0.16 ± 0.02[c]
1.6/6.4	1.90 ± 0.14[c]
1.6/25.6	<0.01[c]
25.6/6.4	0.27 ± 0.02[c]

[a] Mean ± standard error of the mean (n = 3).
[b] Values expressed as nanograms per milliliter.
[c] $P < 0.005$ versus value for untreated sample.

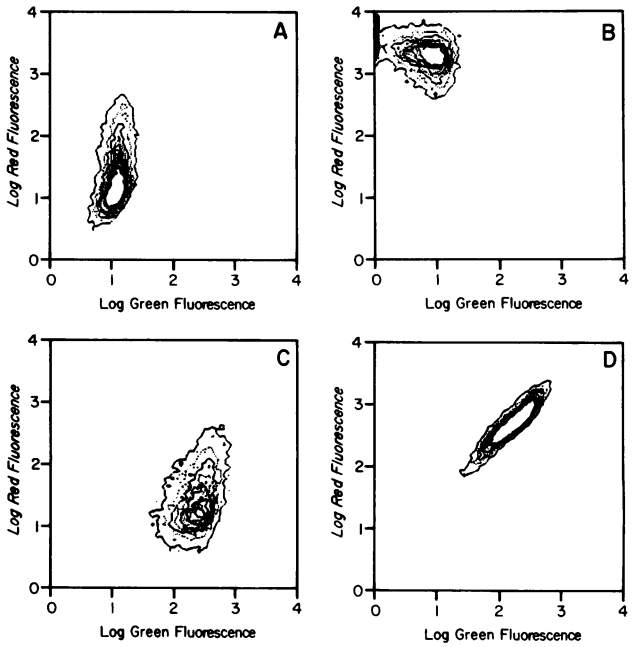

FIG. 4. Contour density plots of *C. tropicalis* ATCC 28707 incubated with GO or MPO enzyme immunoconjugates alone or in tandem and then stained with FITC-labeled anti-MPO (green fluorescence) and biotin-labeled anti-GO plus avidin-phycoerythrin (red fluorescence). Log green fluorescence, representing MPO binding, is plotted on the x axis, and log red fluorescence, representing GO binding, is plotted on the y axis. (A) Cells incubated with CTR-35 (8 μg/ml; mean x, 13.3; mean y, 46.5); (B) cells incubated with GO conjugate (8 μg/ml; mean x, 7.1; mean y, 2,370.0); (C) cells incubated with MPO conjugate (8 μg/ml; mean x, 282.2; mean y, 48.9); (D) cells incubated with GO conjugate (0.8 μg/ml) and MPO conjugate (3.2 μg/ml) (mean x, 196.2; mean y, 651.9).

Flow cytometry analysis of dual-immunoconjugate binding. We measured the simultaneous binding of the GO and MPO conjugates by flow cytometry by using a dual-color fluorescence staining technique. The yeast cells were incubated with various combinations of GO and MPO conjugates and then stained with an FITC-labeled anti-MPO antibody (green fluorescence) and a biotin-labeled anti-GO antibody plus a phycoerythrin-avidin conjugate (red fluorescence).

Contour density plots showing the simultaneous staining for both enzymes are shown in Fig. 4. The fluorescence signal due to MPO conjugate binding is plotted along the x axis, while the signal due to GO conjugate binding is plotted along the y axis. Panel A shows the background fluorescence of yeast cells incubated with unconjugated CTR-35 antibody and stained with both anti-enzyme reagents. The mean relative fluorescence signals due to nonspecific binding of the anti-MPO and anti-GO reagents were 13.3 and 46.5, respectively.

Panel B of Fig. 4 depicts yeast cells incubated with GO conjugate alone and shows an increase in the fluorescence signal due to anti-GO binding (mean, 2,370) with no significant shift in the signal due to anti-MPO binding (mean, 7.1). Panel C represents yeast cells incubated with MPO conjugate alone showing an increase in the mean fluorescence signal due to anti-MPO binding (mean, 282.2) with no significant change in the fluorescence signal due to anti-GO binding (mean, 48.9). These results demonstrate that the anti-enzyme reagents were specific and did not cross-react.

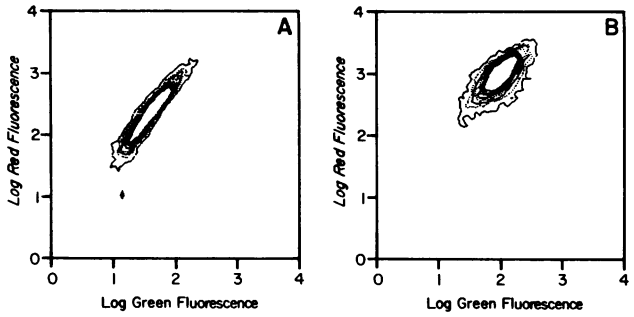

FIG. 5. Qualitative determination of saturating levels of enzyme immunoconjugates bound to *C. tropicalis* by flow cytometry analysis. Yeast cells were incubated with GO and MPO conjugates followed by fluorescent anti-enzyme reagents. Contour density plots represent the MPO and GO conjugate binding on the *x* and *y* axes, respectively. (A) MPO conjugate (4.8 μg/ml) and GO conjugate (3.2 μg/ml) (mean *x*, 46.4; mean *y*, 298.4); (B) MPO conjugate (48 μg/ml) and GO conjugate (32 μg/ml) (mean *x*, 107.0; mean *y*, 1,037.2).

Yeast cells were incubated with 0.8 μg of GO conjugate per ml and 3.2 μg of MPO conjugate per ml (equivalent to a cytotoxic combination of 3.2 ng of GO conjugate per ml and 12.8 ng of MPO conjugate per ml after a 250-fold dilution in the killing assay) and then stained with both anti-enzyme reagents. A contour plot of cells treated with both conjugates is shown in Fig. 4, panel D, with mean fluorescence values of 651.9 for GO conjugate binding and 196.2 for MPO conjugate binding.

Concentrations of the dual immunoconjugates which elicited effective killing did not saturate all the available conjugate-binding sites. A contour plot of yeast cells incubated with a cytotoxic combination of GO conjugate (3.2 μg/ml) and MPO conjugate (4.8 μg/ml) (equivalent to 12.8 ng of GO per ml and 19.2 ng of MPO per ml after a 250-fold dilution in the killing assay) is shown in Fig. 5, panel A. Yeast cells incubated with a 10-fold-higher concentration of both conjugates showed an increase in both mean fluorescence signals (GO binding, 298.4 to 1,037.2; MPO binding, 46.4 to 107.0) (Fig. 5, panel B).

DISCUSSION

In this study, we have demonstrated an effective cytotoxic system against *C. tropicalis* by using MPO and GO immunoconjugates. Other investigators have used similar targeting approaches by using polyclonal antibody conjugates against bacteria as well as yeast cells. Knowles et al. observed a cytotoxic effect on various bacteria when a GO antibody was used in combination with lactoperoxidase, glucose, and potassium iodide (12). A polyclonal anti-*C. albicans* antibody carrying both xanthine oxidase and lactoperoxidase was shown to be cytotoxic (19), and immunoconjugates have been used to target amphotericin B-containing liposomes to *C. albicans* (7).

The enzyme immunoconjugates used in this study showed only a slight reduction in antigen binding relative to that of native antibody. From this result, we concluded that the coupling did not significantly alter the binding properties of the enzyme immunoconjugates.

Neutrophils utilize a system composed of MPO, hydrogen peroxide, and an oxidizable halogen to kill pathogenic bacteria and yeast cells inside the phagocytic vacuole of the cell (11). Previous studies have shown that the toxicity of neutrophil MPO occurs only when the MPO is in close proximity to the organism (9, 25). We showed that as little as 32 ng of MPO immunoconjugate per ml could kill *C. tropicalis* with the addition of 100 μM exogenous H_2O_2. Although neutrophil MPO has a fairly high affinity for *C. albicans* cell wall mannans (K_d, 1.2×10^{-5} M) (26), we were not able to demonstrate any binding of free MPO (32 ng/ml) to *C. tropicalis* by either cytotoxicity or immunological tests. These results demonstrate that the conjugated MPO has an increased affinity for *C. tropicalis* relative to that of free MPO while retaining its cytotoxic activity.

In our study, an enzyme immunoconjugate composed of GO was used to generate hydrogen peroxide at the yeast cell surface in close proximity to the MPO immunoconjugate. This system would be expected to be more efficient than adding hydrogen peroxide exogenously where it may decompose before it can interact with the MPO. Similar hydrogen peroxide-generating immunoconjugates by themselves have been shown to be cytotoxic to tumor cells (22). Even though we could detect bound GO conjugate on the yeast cell surface by using flow cytometry techniques, GO conjugates alone (32 ng/ml) had no effect on *C. tropicalis* viability. On the basis of this result and the finding that this strain of *C. tropicalis* was sensitive to 1 mM hydrogen peroxide but resistant to 100 μM hydrogen peroxide, we concluded that the amount of hydrogen peroxide generated by the GO conjugate was not sufficient to have a cytotoxic effect.

When we tested for the cytotoxic effects of adding both MPO and GO conjugates in tandem, we found that a combination of MPO and GO, 12.8 and 3.2 ng/ml, respectively, was very cytotoxic and resulted in a 99.9% reduction in viable yeast cells. We found that this combination was not the only effective cytotoxic dose in this dual-immunoconjugate system (Table 1). A range of concentrations, 1.6 to 25.6 ng/ml for the GO conjugate and 6.4 to 25.6 ng/ml for the MPO conjugate, could be effectively used.

When we treated yeast cells with a combination of enzyme immunoconjugates that were found to be cytotoxic, we observed by flow cytometry analysis that both enzyme immunoconjugates were bound to the cell surface. We concluded from this observation that the cytotoxic effect of the dual-immunoconjugate system was due to the combined action of both enzymes bound at the cell surface via the antibody. Both immunoconjugates had similar binding affinities, and neither conjugate bound preferentially to the yeast cells while displacing the other. In addition, flow cytometry analysis showed that yeast cells treated with minimal amounts of each conjugate required for a cytotoxic effect were not saturating all the available antigen-binding sites.

These results have important implications in the use of this dual-enzyme immunoconjugate system in vivo. When a suitable ratio of MPO conjugate and GO conjugate is determined, an excess amount of both conjugates could be administered and the optimal ratio of these conjugates would be maintained on the yeast cell surface.

The MPO-GO enzyme immunoconjugate system offers several advantages over other enzyme immunoconjugate systems as a potential in vivo therapeutic agent. MPO is a natural human enzyme and would be expected to be nonimmunogenic and nontoxic to humans. Although human lactoperoxidase has been isolated in small quantities from the colostrum (15), the only practical source of large quantities of the enzyme is bovine milk. Lactoperoxidase does not readily oxidize chloride, in contrast to MPO, and must be supplied with iodide, bromide, or thiocyanate to exert its cytotoxic effect (23). In addition, when we compared the enzymatic activity of MPO with that of lactoperoxidase, our

preparations of MPO were at least four times more active than equimolar amounts of commercially available lactoperoxidase.

GO is a readily available enzyme, and there are advantages to its use for immunoconjugates because of its enzymatic properties. It can generate hydrogen peroxide from glucose and oxygen, substances readily available in the blood. In other systems that use xanthine oxidase immunoconjugates (19, 22), xanthine, at concentrations that have yet to be determined, must be supplied for the conjugates to exert a cytotoxic effect. In addition, GO has been shown to have low toxicity in vivo (20).

The current treatment for candidiasis is amphotericin B therapy. This approach is often ineffective in treating fungal infections in neutropenic and immunodeficient patients (2). In addition, amphotericin B has been shown to be highly toxic to vital organs (13). We have demonstrated in an in vitro model system that enzyme immunoconjugates patterned after the hydrogen peroxide-MPO system of neutrophils are effective in killing *C. tropicalis*. With further development and testing, the enzyme immunoconjugate approach has the potential for becoming an adjunct to current drug treatment, leading to a safe and effective therapy for *Candida* infections in vivo.

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