ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 1989, p. 2092–2095
0066-4804/89/122092-04$02.00/0

Vol. 33, No. 12

Antibodies to Nystatin Demonstrate Polyene Sterol Specificity and Allow Immunolabeling of Sterols in *Saccharomyces cerevisiae*†

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Polyclonal antibodies elicited by injection into rabbits of a nystatin-bovine serum albumin conjugate were reactive with both nystatin and amphotericin B. Upon labeling of polyene-treated *Saccharomyces cerevisiae* sterol auxotrophs grown on various sterols, nystatin reacted specifically with ergosterol, while amphotericin B did not react preferentially with ergosterol, cholesterol, or cholestanol. Time course labeling experiments demonstrated the rate of ergosterol transport into cholesterol-grown cells.

Polyenes comprise a group of structurally related antifungal molecules which exert their effect by association with sterol moieties in fungal membranes and subsequent formation of cross-membrane channels which allow ion and metabolite leakage (1, 3, 10, 15). Several of these compounds have specificity for a particular subset of sterol molecular structures. Nystatin has been demonstrated to have a specific interaction with ergosterol, the predominant *Saccharomyces cerevisiae* sterol (1, 9, 10). Filipin has a slightly greater affinity for cholesterol (2–4, 6, 8, 14), while amphotericin B shows very little sterol specificity (1, 4). Also, if the C-18 carboxyl of amphotericin B is blocked, ergosterol-containing cells are permeabilized better than cholesterol cells, an alteration of the specificity of the antifungal molecule (6).

Ergosterol is the predominant sterol molecule found in fungi. Other sterols, when supplemented to yeast auxotrophs, can substitute for at least some of the native ergosterol (11, 12). We have defined several distinct levels of sterol function. Each of these levels has certain sterolic structural requirements as well as a minimal level needed to satisfy the particular function and allow abundant yeast growth. Our research has shown that the levels of ergosterol required to satisfy the most specific microrequirement are insufficient to allow nystatin to cause ion leakage and cell inviability (10). To define the different sterol functions more specifically, we are attempting to localize the sterols.

In this paper, we report the production of antibodies to nystatin and the use of these to demonstrate the ergosterol specificity of nystatin compared with that of amphotericin B or filipin and to detect ergosterol in yeast cells by immunocytochemistry and light microscopy. A previous report demonstrated the production of antibodies to amphotericin B (7).

MATERIALS AND METHODS

Conjugate formation. Twenty milligrams of nystatin dissolved in 2 ml of dimethylformamide-methanol (1:1) was added to 20 mg of bovine serum albumin in 96 ml of 0.1 M phosphate buffer, pH 7.0. To this mixture was added 20 mg of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide in 2 ml of buffer. The mixture was stirred at room temperature for 8 h and then dialyzed against water and 1.0% dimethylformamide in water for 24 h. The amount of nystatin dialyzed out was determined by the A_{306} of the dialysate ($E^{0.001\%} = 0.68$). The conjugate suspension was then frozen and lyophilized to a powder. Protein in the conjugate product was determined by a BCA protein assay (Pierce Chemical Co.) of an aqueous suspension. The UV spectrum of the suspension was also measured by using a DMS 100 spectrophotometer (Varian).

Antibody production and screening. Six-week-old New Zealand White rabbits were injected with 3.6 mg of conjugate in 4.5 ml of phosphate-buffered saline (PBS) homogenized with an equal volume of Freund complete adjuvant in two footpad and two neck sites. Four weeks later, rabbits were boosted with the same amount of antigen in Freund incomplete adjuvant. Blood was obtained from the ear vein for antibody titer analyses. Blood was allowed to clot, serum was decanted, and the immunoglobulins were precipitated with 40% saturated ammonium sulfate and suspended three times in PBS. Precipitated immunoglobulins were then screened by enzyme-linked immunosorbent assay (ELISA).

ELISA was performed by using a modification of the method of Swartz et al. (13) for water-insoluble antigens. Briefly, 100 ng of polyene in 10 μl of ethanol was pipetted into microdilution wells containing 90 μl of ethanol. Control wells contained 100 μl of ethanol. The ethanol was allowed to evaporate under a fume hood. Plates were stored foil wrapped under refrigeration overnight until used.

Wells were blocked with 100 μl of 1% ovalbumin in blocking PBS (pH 9.6) for 2 h at 37°C and then washed with PBS at pH 7.4. Immunoglobulins were diluted in 0.1% ovalbumin-PBS, placed in wells, and incubated 90 min at room temperature, and then the wells were washed 3 times with PBS. Anti-rabbit immunoglobulin G-alkaline phosphatase conjugate was diluted 1:500 in 0.1% ovalbumin-PBS, incubated in wells for 90 min at room temperature, and washed with PBS. Phosphatase substrate (*p*-nitrophenyl phosphate disodium, 1 mg · ml^{-1}) in diethanolamine buffer (9.7% diethanolamine, 0.5 mM magnesium chloride, 3 mM sodium azide [pH 9.8]) was added to the wells, and the A_{405} was read (Dynatech plate reader) after 20 to 30 min at room temperature.

Yeast culture and sterol shifts. *S. cerevisiae* X2180-1A(a *SUC2 mal mel gal2 CUP1*) was grown at 28°C on rich medium consisting of 0.5% yeast extract, 1.0% tryptone, and 2.0% glucose. *S. cerevisiae* RD5-R(a *hem1 erg3 erg7*) was cultured on complete medium consisting of 1.0% Casamino

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† Paper no. 12417 of the Journal Series of the North Carolina Agricultural Research Service, Raleigh.

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Acids (Difco Laboratories, Detroit, Mich.), 0.67% yeast nitrogen base, 2% glucose, and 30 mg each of adenine, methionine, and uracil ml^{-1} supplemented with 100 μg of unsaturated fatty acids (oleic-palmitoleic [4:1]) from a 1,000× stock solution in tyloxapol-ethanol (1:1) ml^{-1}. Sterols were added as indicated above from stock solutions in tyloxapol-ethanol (5 mg · ml^{-1}).

For shift experiments, sterol auxotrophic cultures were grown to late log phase, harvested by centrifugation, washed twice with sterile 0.5% tergitol in distilled water, and then suspended at low cell density in fresh medium containing the appropriate sterol. Samples were taken at time intervals, pelleted, washed with 0.5% tergitol, and then suspended in 4% formaldehyde-PBS to fix for peroxidase staining as described below.

Peroxidase immunolabeling procedure. Cells were harvested by centrifugation (500 × *g*, 5 min) and washed once with 0.5% tergitol. The resulting pellet was suspended in 4% (wt/vol) formaldehyde (freshly prepared from paraformaldehyde) in PBS (pH 7.4), incubated 2 h at 30°C, and washed twice with PBS. Cell walls were digested with 0.5 mg of Novozyme 234 ml^{-1} in PBS with 10 mM magnesium chloride for 30 min at 30°C with gentle shaking, and the spheroplasts were washed twice with PBS. After suspension in 4% formaldehyde, drops were applied to poly-L-lysine-coated slides which were then incubated in a moist chamber for 15 min at 30°C. Unless otherwise indicated, all further incubations were also done at 30°C in the moist chamber.

Slides were rinsed with a stream of PBS, allowed to stand with PBS for 5 min, and rinsed again. Cells attached to the slides were treated with polyene, as indicated in Results, at 40 U · ml^{-1} in PBS, incubated for 45 to 60 min, and then rinsed with PBS. Polyenes were diluted from freshly prepared 10,000 U · ml^{-1} dimethylformamide stock solution.

Nonspecific binding of antibodies was blocked by 1 h of incubation with a few drops of normal goat serum (NGS) on each slide. Excess serum was then shaken off, and a few drops (about 200 μl) of primary antibody, diluted in NGS to 100 μg of protein ml^{-1}, were incubated for 1 h on each slide. Unbound antibody was washed off by rinsing with PBS as described above. Nonspecific sites were again blocked with NGS for 30 min. Goat anti-rabbit immunoglobulin G diluted in NGS to 50 μg · ml^{-1} was incubated 1 h, and slides were rinsed in PBS. After another 30-min serum block, peroxidase-antiperoxidase diluted 1:50 in NGS was added and the mixture was incubated 1 h followed by rinsing with PBS.

Slides were reacted with 0.5 mg diaminobenzidine ml^{-1} and 0.2% H_2O_2 in 50 mM Tris hydrochloride, pH 7.6, for 15 to 20 min in the moist chamber at room temperature to develop color. Slides were rinsed with Tris buffer and dehydrated through a graded series of ethanol (30, 50, 70, 95, and 100%) followed by xylene in Coplin jars for 10 min each. Cover slips were mounted with Permount and allowed to set overnight before viewing.

Controls included cells not treated with polyene, primary antibody, or goat anti-rabbit immunoglobulin G.

Photographic documentation. Stained slides were viewed in a Zeiss microscope with a blue illuminator filter, and pictures were taken with Technical Pan 2415 film and identical exposure times and developed in D-19. Exposures of prints were adjusted for identical background densities.

Supplies. Formaldehyde, buffer chemicals, and solvents were purchased from Fisher Scientific Co. Novozyme 234 was from Novo Biolabs. Filipin was a generous gift from The Upjohn Co. Amphotericin B, tyloxapol, nystatin, adjuvants, serum, and other antibody reagents were from Sigma Chemical Co. The BCA protein reagents were obtained from Pierce.

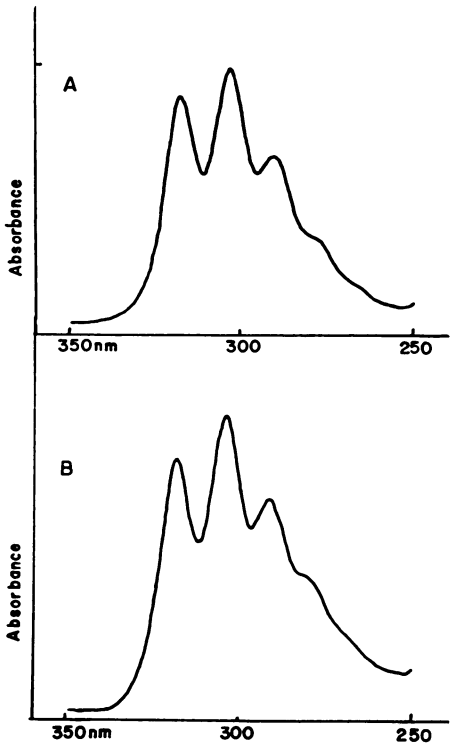

FIG. 1. UV absorbance spectra for aqueous suspension of nystatin (6 μg · ml^{-1}) (A) and nystatin-bovine serum albumin conjugate (100 μg · ml^{-1}) (B).

RESULTS AND DISCUSSION

Polyenes have very characteristic structures which would presumably be quite antigenic if presented in a suitably large molecular form. We have conjugated nystatin to bovine serum albumin in order to produce an immunogenic compound. To demonstrate conjugation of nystatin to bovine serum albumin, the UV absorbance spectrum of a suspension of the conjugated, dialyzed material was measured (Fig. 1). The conjugate demonstrated peaks at 320, 306, and 289 nm, which is characteristic of the nystatin suspension. The retention of the UV absorbancy indicated that the nystatin was still in its native configuration. The conjugate contained 0.7 mg of protein per mg of solid.

Upon analysis of ammonium sulfate-precipitated rabbit immunoglobulins by ELISA, good reactivity was seen with both amphotericin B (0.828) and nystatin (0.781) but not with filipin (0.046). This indicated that the antibodies were mainly recognizing part of the polyene structure common to both amphotericin B and nystatin. The most notable structural difference was the mycosamine moiety, present in both amphotericin and nystatin but absent in filipin. This carbohydrate group would provide a suitable antigenic determinant. We were able to detect nystatin and amphotericin B to nanogram levels with the ELISA.

Immunostaining of *S. cerevisiae* by using the peroxidase-antiperoxidase second antibody indirect method confirmed the nystatin reactivity of the antibodies. Only nystatin-treated cells showed labeling, while control cells, which had no polyene treatment, were unstained (Fig. 2). Additionally,

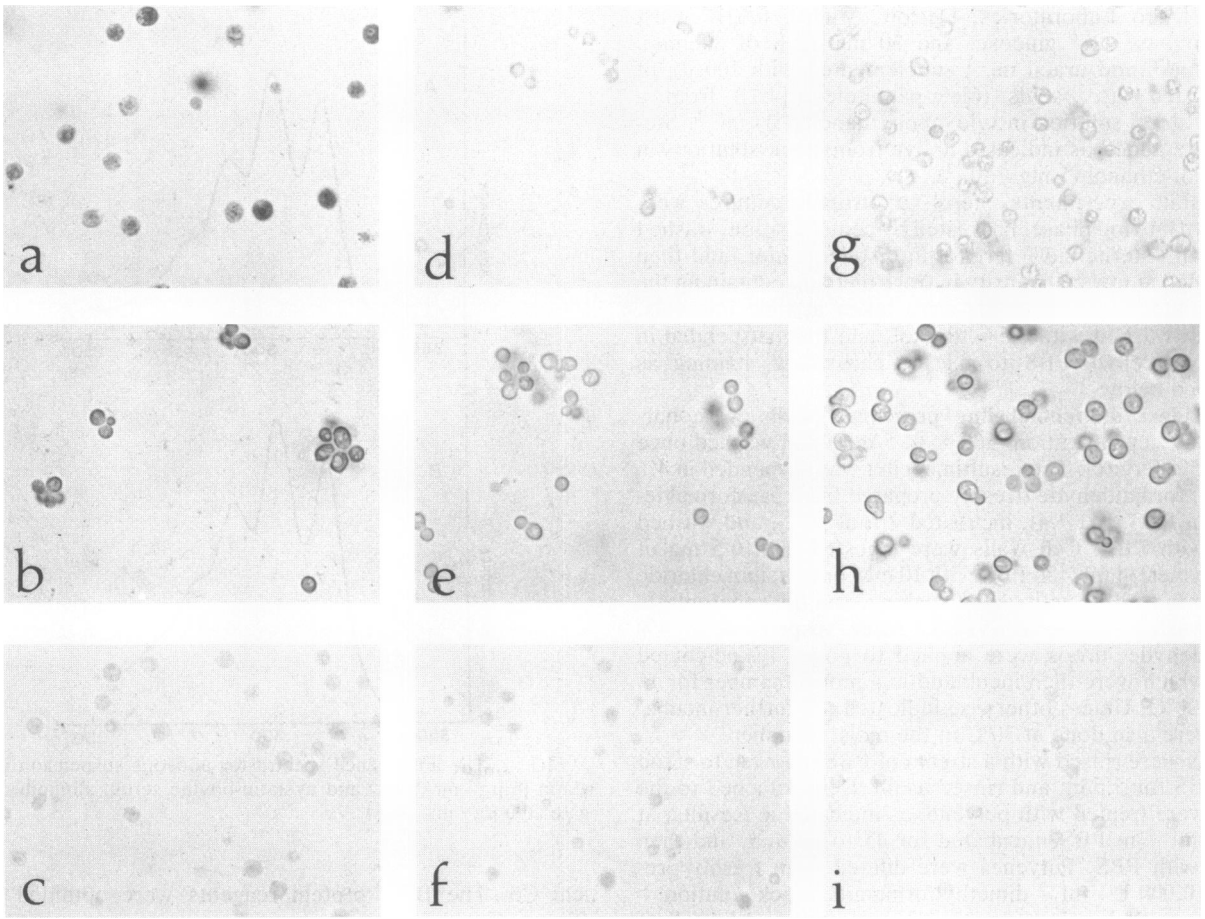

FIG. 2. Yeast sterol auxotrophic cells treated with polyene and stained with diaminobenzidine-peroxidase after growth in ergosterol (a through c and i), cholesterol (d through f) or cholestanol (g and h). Cells were treated with nystatin (a, d, and g), amphotericin B (b, e, and h), filipin (c and f), or no polyene (i).

amphotericin treatment allowed good cell staining but filipin-treated yeast cells showed little or no staining. The reactivity of antibody with both nystatin and amphotericin B in combination with our yeast sterol auxotroph, which can be grown to have various membrane sterol compositions, provides a useful tool for the study of polyene sterol specificity.

Yeast sterol auxotrophs grown on a $\Delta^{5,7}$-sterol and then cycled on cholestanol grow very poorly once endogenous $\Delta^{5,7}$-sterol is sufficiently diluted. We consider cells cycled in this manner ergosterol depleted. The Δ^5-unsaturated cholesterol molecule can satisfy yeast sterol functions in an auxotroph when supplied at a medium concentration of 5 μg of cholesterol ml^{-1} (11, 12). Cells grown on this sterol have little or no ergosterol because it has been replaced by cholesterol. Ergosterol-grown, cholesterol-grown, and cholestanol-cycled cells were polyene treated and immunostained (Fig. 2). All cultures treated with amphotericin B stained equally well, demonstrating that amphotericin B lacks sterol specificity. Other reports have also shown amphotericin B to be effective against many different sterols and even to interact with membrane phospholipids (3, 4). Among the nystatin-treated cultures, only ergosterol-grown cells, and not cholesterol- or cholestanol-grown cultures (ergosterol depleted) showed peroxidase staining. These data confirm other reports of nystatin having a specificity for ergosterol in yeast membranes (3, 9, 10, 14).

This ergosterol-specific "tagging" procedure was then applied to the study of ergosterol dilution during culture growth after sterol shifts. When an auxotrophic yeast culture cycled on cholesterol was washed and shifted to medium containing ergosterol, nystatin-treated sterol labeling was not readily detectable until 2 h or more of growth in ergosterol medium (Fig. 3). In the opposite sterol shift (from ergosterol to cholesterol), labeling was seen at all time points up to 12 h (data not shown), indicating that 12 h is insufficient for appreciable dilution of ergosterol in the cells. These results indicate that ergosterol got into cells quickly. When cells grown on cholesterol with or without sparking levels ($10\ ng \cdot ml^{-1}$) of ergosterol were immunolabeled with peroxidase, no staining was visible. Presumably, this is because the amount of ergosterol per cell is low and thus not detectable. One set of calculations suggests that there are only 14.5×10^4 molecules of ergosterol per cell grown under these conditions (R. J. Rodriguez, Ph.D. thesis, Oregon State University, Corvallis, 1983). Previous studies of these ergosterol-stimulated cells found that they do not demonstrate nystatin-induced potassium ion leakage as ergosterol-grown cells do (5, 6, 10).

This antibody immunostaining method provides a useful tool for the detection of ergosterol in yeast cells, provided a sufficient level of ergosterol per cell is present. It will be applicable to further studies of ergosterol in yeast auxotrophs.

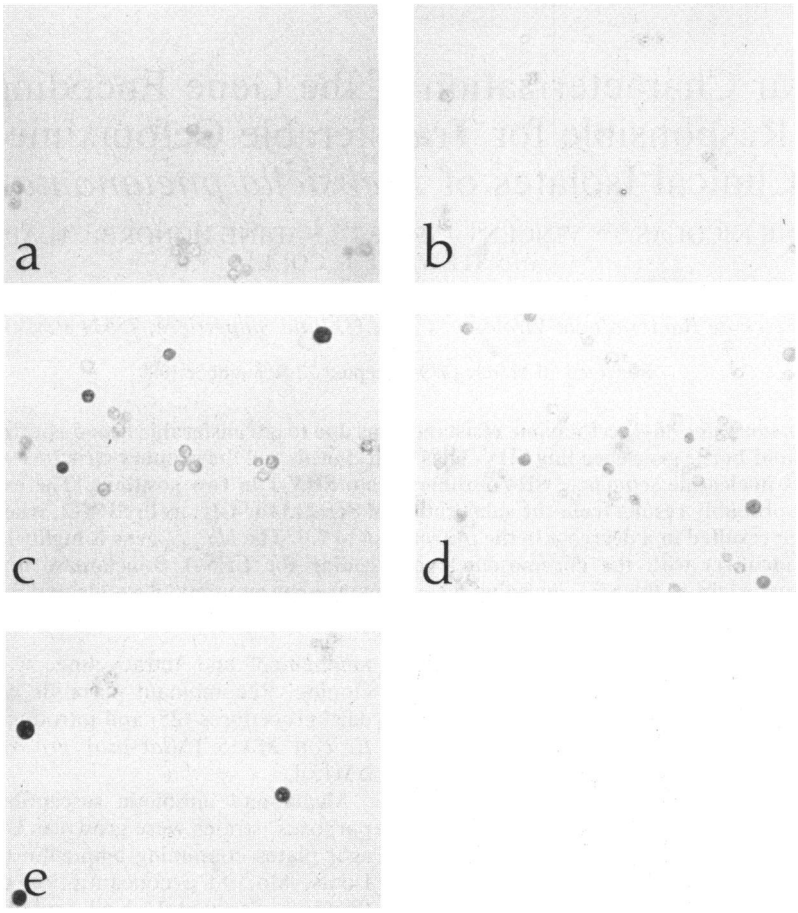

FIG. 3. Nystatin-treated, diaminobenzidine-peroxidase–stained cells from auxotroph culture shifted from cholesterol to ergosterol. Samples were taken at 0 (a), 1 (b), 2 (c), 4 (d), and 8 (e) h.

ACKNOWLEDGMENTS

This research was supported in part by grant DCB-8814387 from the National Science Foundation and Public Health Service grant DK37222 from the National Institutes of Health.

We are indebted to Stephen Kaattari for direction and discussion in the formation of hapten conjugates and development of the ELISA procedure.

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