IN VITRO INHIBITION OF YEAST GROWTH BY MOUSE ASCITES FLUID AND SERUM

DONALD F. SUMMERS AND H. F. HASENCLEVER

Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases,
U.S. Public Health Service, Bethesda, Maryland

Received for publication 24 June 1963

ABSTRACT

SUMMERS, DONALD F. (National Institute of Allergy and Infectious Diseases, Bethesda, Md.), AND H. F. HASENCLEVER. In vitro inhibition of yeast growth by mouse ascites fluid and serum. *J. Bacteriol.* 87:1-7. 1964.—A nondialyzable heat-stable factor(s) present in experimentally produced mouse ascites fluid and in serum from these ascitic mice was shown to inhibit the in vitro growth of several yeasts. The inhibitory activity was almost totally abolished by trypsin treatment of the ascites fluid, and was progressively diminished by repeated adsorption of the ascites fluid by heat-killed *Candida albicans* cells. A close relationship was shown to exist between growth inhibition by ascites fluid and concentration of free iron or nutrients in the growth medium. Increased concentration of nutrients or free iron caused diminution of inhibitory activity.

Beginning in the latter part of the 19th century, numerous investigators reported a variety of animal and human tissue and body-fluid factors which possess nonspecific antibacterial activity (Skarnes and Watson, 1957). More recently, several investigators observed similar factors in human and animal sera which inhibit the growth of fungi. Ayres and Anderson (1934) found that sera from patients with epidermophytosis and "phytid" eruptions completely inhibited the growth of *Epidermophyton*, whereas sera from normal patients, and sera from patients with epidermophytosis without "phytid" eruptions, did not show inhibitory activity. Peck, Rosenfeld, and Glick (1940), using *Trichophyton gypsum* (*T. mentagrophytes*) as the test fungus, demonstrated fungistatic activity in normal human sera, in sera from patients with epidermophytosis, and in pooled guinea pig serum.

Lorincz, Priestley, and Jacobs (1958) observed suppression of growth of *T. mentagrophytes* when this fungus was placed in dialysis tubing and was then implanted intraperitoneally in mice. Roth

et al. (1959), testing human sera in vitro against *T. mentagrophytes*, *T. rubrum*, and *Candida albicans*, found in the sera a dialyzable labile factor which was fungistatic.

Since there have been no previous reports of experimentally induced inhibitors of yeast growth, this paper presents data which demonstrate the occurrence of a factor or factors in experimentally induced mouse ascites fluid, and in the serum of these same mice, which inhibit the in vitro growth of several yeasts.

MATERIALS AND METHODS

Yeast strains. Two strains of *C. albicans* (B 311 and 792), one strain of *Torulopsis glabrata* (B 331A), and one large capsule strain of *Cryptococcus neoformans* (CNB) were used in this study. All of these strains were isolated in this laboratory from human sources.

Inocula. Suspensions of yeast cells were made from 48-hr cultures grown on modified Sabouraud agar [Neopeptone (Difco), 1%; glucose, 2%; agar, 2%; pH 6.8] incubated at 30 C. Cell numbers were determined by direct count in a Levy hemocytometer counting chamber. Appropriate dilutions were made with liquid media to obtain the desired concentration of cells. An inoculum of 200 yeast cells per ml of test culture was used throughout this study.

Growth conditions. Growth of *C. albicans* and *T. glabrata* was studied in varying concentrations of mouse serum or mouse ascites fluid diluted to the desired concentration with varying per cent concentrations of Trypticase Soy Broth (BBL). Growth of *C. neoformans* was studied in similar fashion, but Penassay Broth (Difco) was substituted for the Trypticase Soy Broth. Per cent concentrations of media given in the text refer to per cent of dehydrated nutrients added to make up the liquid growth media. The final volume of all test cultures was 5 ml.

Test cultures were incubated without agita-

FIG. 1. Inhibitory effect of increasing concentrations of mouse ascites fluid on growth of *Candida albicans* B 311.

tion at 37 C. Growth was followed by the quantitative plating technique, counts being made every 24 hr.

Ascites fluid and serum. Mice were made ascitic by the method of Lieberman, Mantel, and Douglas (1960), with repeated intraperitoneal injections of complete Freund's adjuvant. The ascites fluid collected from groups of 50 female Swiss white mice was pooled, centrifuged for 30 min at $600 \times g$ at 4 C to remove the fibrin clot, and stored at -10 C. Serum was collected from the same mice by exsanguinating the animals. Serum was stored at -10 C. Mice were injected intraperitoneally with 30 μ g of *Salmonella enteritidis* lipopolysaccharide (Difco), and the sera were collected and pooled 24 hr later. Two doses of 10 million *C. albicans* cells were injected into mice intraperitoneally at weekly intervals, and sera were collected and pooled 1 week after the last injection. Control sera were obtained from mice injected with pyrogen-free saline on the same schedule as the *C. albicans*-injected animals.

Adsorption of ascites fluid. A mixture of 5 ml of ascites fluid and 5 ml of packed heat-killed *C. albicans* strain B 311 cells was incubated at 37 C overnight. The ascites fluid was separated from the yeast cells by centrifugation, and added to fresh heat-killed yeast for multiple adsorptions.

Treatment of ascites fluid with trypsin. A mixture of 5 ml of ascites fluid and 0.2% trypsin was placed in dialysis tubing, and was dialyzed against large volumes of 0.15 M NaCl buffered with 0.05 M phosphate buffer (pH 7.4) for 60 hr.

RESULTS AND DISCUSSION

The growth of *C. albicans* B 311 in 0.5% Trypticase Soy Broth, plus increasing per cent concentrations of mouse ascites fluid, is shown in Fig. 1. It is apparent that growth inhibition begins at about 5% ascites fluid concentration, and reaches a maximum at about 10% concentration. All pools of ascites fluid tested produced maximal inhibition of about 4 to 5 logs; all serum pools tested exhibited slightly greater inhibitory activity than did the ascites fluid from the same mice. Normal mouse serum, used routinely in all studies as a control, had no detectable inhibitory activity against *C. albicans*.

C. albicans 792, tested in similar experiments, produced results identical to those obtained with *C. albicans* B 311.

The inhibitory activity of the ascites fluid was not diminished by heating at 56 C for 0.5 hr or by dialyzing for 60 hr, but no activity was detectable after heating at 80 C for 0.5 hr.

The factor(s) present in mouse ascites fluid seems to differ from that reported by Lorincz et al. (1958) and Roth et al. (1959), because 60 hr of dialysis did not cause a decrease in initial activity.

Figure 2 shows the effect of increasing the concentration of the nutrients in the Trypticase Soy Broth on the growth of *C. albicans* in the presence of 10% ascites fluid. An increase in concentration of nutrients from 0.375 to 0.75% produced a small decrease in inhibitory activity, but another twofold increase in nutrient concentration to 1.5% totally abolished activity.

These results indicated that there was a relationship between growth inhibition by ascites fluid and the amount of nutrients available to the yeast. To further investigate this relationship, the experiment shown in Fig. 3 was performed. Increasing amounts of glucose were added to

FIG. 2. Diminution of inhibitory activity of mouse ascites fluid on growth of *Candida albicans* B 311 by increasing concentrations of nutrients.

FIG. 3. Diminution of inhibitory activity of mouse ascites fluid on growth of *Candida albicans* B 311 by addition of increasing concentrations of glucose to liquid medium.

0.25% Trypticase Soy Broth containing 20% ascites fluid. As can be seen, increments in glucose concentration cause progressive loss of inhibitory activity.

Trypsin treatment of the ascites fluid resulted in almost total loss of activity (Fig. 4). This result, plus the fact that the inhibitory activity was not diminished by dialysis, offers indirect evidence that the inhibitory factor(s) is a protein. However, preliminary attempts to fractionate the ascites fluid with ammonium sulfate precipitation, cold ethanol, and Sephadex G 200 (Pharmacia, Uppsala, Sweden) all resulted in complete loss of activity.

Figure 5 shows the results of adsorption of the

FIG. 4. Loss of inhibitory activity of mouse ascites fluid after trypsin treatment of ascites fluid.

ascites fluid with large amounts of heat-killed *C. albicans* cells. Each adsorption produced a progressive loss of inhibitory activity, and, after three such adsorptions, no activity remained.

To test the persistence of the inhibitor in mice after induction of ascites, it was necessary to use mouse serum because the ascites fluid disappears

in 1 to 3 weeks if the mice are not tapped regularly. When serum was collected from groups of mice 1, 2, and 3 months after the initial production of ascites, and was tested for its inhibitory capacity against *C. albicans*, the serum obtained 1 and 2 months after ascites production showed no loss of inhibitory titer. However, the 3-months postascites serum showed a marked loss of inhibitory capacity, most noticeable at 48 hr, by which time the growth of the yeast in serum had reached the level of the control cultures.

To test the specificity of the inhibitory activity

FIG. 5. Progressive loss of inhibitory activity of mouse ascites fluid on growth of *Candida albicans* B 311 after repeated adsorption of ascites fluid with heat-killed *C. albicans* B 311 cells.

of mouse ascites fluid, growth of *T. glabrata* and *Cryptococcus neoformans* in the presence of ascites fluid was also studied. The results of an experiment with *T. glabrata* were quite similar to those obtained with *Candida albicans* (Fig. 6). A curve produced by 10% serum from ascitic mice is also included to show the greater inhibition usually seen with this material. If growth of any yeast tested was followed long enough, the serum from ascitic mice was often seen to have a fungicidal effect.

Figure 7 shows the results obtained when 10% ascites fluid was used to suppress the growth of *C. neoformans*. Here again, inhibition of growth was quite dependent on concentration of nutrients in the medium, with higher concentrations of nutrients causing loss of activity.

Previous workers noted that egg white would suppress the growth of a wide spectrum of fungi (Schade and Caroline, 1944; Silva and Buckley, 1962). This work also suggested that at least part of the inhibitory effect could be due to the iron-binding properties of the conalbumin fraction, because growth inhibition was partially reversed by the addition of ferrous salts. To examine the possibility that the ascites fluid may be exerting its antifungal action by binding iron, bound iron and

FIG. 6. Inhibition of growth of *Torulopsis glabrata* by mouse ascites fluid and serum from ascitic mice.

FIG. 7. Diminution of inhibitory activity of mouse ascites fluid on growth of *Cryptococcus neoformans* by increasing concentrations of nutrients.

unsaturated iron-binding capacity were determined on the ascites fluid (Schade et al., 1954); ferrous ion was added as ferrous ammonium sulfate in amounts to produce 110% saturation of the iron-binding capacity. Figure 8 shows the results of an experiment in which untreated ascites fluid is compared with an ascites to which iron has been added as described above. It is apparent that the addition of the iron completely abolishes the inhibitory activity of the ascites fluid.

Other studies showed that sera from mice injected with *S. enteritidis* endotoxin, sublethal doses of *C. albicans* cells, or nonviable *Coccidioides immitis* spherule fragments inhibited the growth of *C. albicans* in vitro. (Hasenclever and Mitchell 1963a, b). Figure 9 shows the effect of the addition of iron upon the growth of *C. albicans* in the sera of mice injected with nonlethal doses of the yeast, the sera of mice injected with endotoxin, and control sera. It is apparent that the addition of iron abolished the inhibitory activity of the test sera, but had no effect upon the growth of the microorganism in the control sera. The part that this serum function contributes to acquired resistance in mice to the acute toxicity or chronic lethal infection due to *C. albicans* remains uncertain. Martin, Jandl, and Finland (1963) reported

the enhancement of acute bacterial infections in rats and mice by the injection of iron and their inhibition by human transferrin (iron-binding protein). The possible in vivo implications of these observations are highly intriguing, but the problem of designing of adequate control conditions to permit concise interpretation of results remains to be solved.

When the results of Fig. 2 are considered, it is quite possible that the inhibitory activity of the ascites fluid was overcome by the additional iron in the more concentrated broth. Iron determinations on Trypticase soy powder indicated that, in a 0.75% solution, 1.11 μg of iron were present; 0.5 ml of ascites fluid bound 1.16 μg of iron. Therefore, when the concentration of the broth was increased to 1.5%, free iron was present for utilization by the yeast. This may indicate the mode of action under these circumstances, but it does not explain the results shown in Fig. 3. In that experiment, glucose was shown to abolish the inhibitory effect of the ascites fluid, but the glucose used did not contain detectable iron.

The effects of other cations were not studied

FIG. 8. Loss of inhibitory activity of mouse ascites fluid on growth of *Candida albicans* B 311 by addition of iron.

FIG. 9. Effect of iron on the growth of *Candida albicans* in mouse serum. Tube 1 = serum from mice injected with *C. albicans*; tube 2 = same as tube 1, plus 0.5 μg of Fe^{++} (ferrous ammonium citrate); tube 3 = serum from mice injected with endotoxin; tube 4 = same as tube 3, plus 0.5 μg of Fe^{++} ; tube 5 = serum from mice injected with pyrogen-free saline; tube 6 = same as tube 5, plus 0.5 μg of Fe^{++} . Final serum-concentration was 10%.

because Shade and Caroline (1944) had investigated them, and had found no similar activity.

Certainly, in vitro inhibition of yeast growth by mouse ascites fluid experimentally produced with Freund's adjuvant is not directly comparable to in vivo host defense mechanisms. However, the data suggest that naturally occurring non-specific stimuli could produce similar humoral inhibitors of fungi, and it may be possible to correlate these inhibitors with their ability to bind serum iron.

ACKNOWLEDGMENT

The authors wish to express their gratitude to A. L. Schade for determining the bound iron and the iron-binding capacity of the ascite fluid.

LITERATURE CITED

- AYRES, S., AND N. P. ANDERSON. 1934. Inhibition of fungi in cultures by blood serum from patients with "phytid" eruptions. *Arch. Dermatol. Syphilol.* **29**:537-547.
- HASENCLEVER, H. F., AND W. O. MITCHELL. 1963a. Endotoxin-induced tolerance to toxic manifestations of *Candida albicans*. *J. Bacteriol.* **85**:1088-1093.

- HASENCLEVER, H. F., AND W. O. MITCHELL. 1963b. Acquired immunity to candidiasis in mice. *J. Bacteriol.* **86**:401-406.
- LIEBERMAN, R., N. MANTEL, AND J. O. A. DOUGLAS. 1960. Production in mice of ascitic fluid containing antibodies induced by *Staphylococcus* or *Salmonella* adjuvant mixtures. *J. Immunol.* **54**:514-529.
- LORINCZ, A. L., J. O. PRIESTLEY, AND P. H. JACOBS. 1958. Evidence for a humoral mechanism which prevents growth of dermatophytes. *J. Invest. Dermatol.* **31**:15-17.
- MARTIN, C. M., J. H. JANDL, AND M. FINLAND. 1963. Enhancement of acute bacterial infections in rats and mice by iron and their inhibition by human transferrin. *J. Infect. Diseases* **112**:158-163.
- PECK, S. M., H. ROSENFELD, AND A. W. GLICK. 1940. Fungistatic power of blood serum. *Arch. Dermatol. Syphilol.* **42**:426-437.
- ROTH, F. J., JR., C. C. BOYD, S. SAGAMI, AND H. BLANK. 1959. An evaluation of the fungistatic activity of serum. *J. Invest. Dermatol.* **32**:549-556.
- SCHADE, A. L., AND L. CAROLINE. 1944. Raw egg white and the role of iron in growth inhibition of *Shigella dysenteriae*, *Staphylococcus aureus*, *Escherichia coli* and *Saccharomyces cerevisiae*. *Science* **100**:14-15.
- SCHADE, A. L., J. OYAMA, R. W. REINHART, AND J. R. MILLER. 1954. Bound iron and unsaturated iron-binding capacity of serum: rapid and reliable quantitative determination. *Proc. Soc. Exptl. Biol. Med.* **87**:443-448.
- SILVA, M., AND H. R. BUCKLEY. 1962. Fungi and fungous diseases. Charles C Thomas, Publisher, Springfield, Ill.
- SKARNES, R. C., AND D. W. WATSON. 1957. Antimicrobial factors of normal tissues and fluids. *Bacteriol. Rev.* **21**:273-294.