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CHARACTERIZATION OF THE PYROGENICITY OF *CANDIDA ALBICANS*, *SACCHAROMYCES CEREVISIAE*, AND *CRYPTOCOCCUS NEOFORMANS*

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ABSTRACT

KOBAYASHI, GEORGE S. (Tulane University, New Orleans, La.), AND LORRAINE FRIEDMAN. Characterization of the pyrogenicity of *Candida albicans*, *Saccharomyces cerevisiae*, and *Cryptococcus neoformans*. J. Bacteriol. **88**:660-666. 1964.—The intravenous injection into rabbits of 10^9 yeast cells of *Candida albicans*, *Saccharomyces cerevisiae*, or *Cryptococcus neoformans* (both slightly and heavily encapsulated forms) induced a febrile response indistinguishable from that elicited by gram-negative bacterial endotoxin. There was a brisk rise in body temperature which began as early as 30 min after injection, peaked once or twice, and then returned to normal after about 10 hr. With viable *C. albicans*, the febrile response did not return to normal but remained elevated for several days and terminated at death of the animal. Of three extraction procedures employed in attempts to isolate the endotoxin-like pyrogenically active substances from *C. albicans*, only one, the phenol extraction method, was successful. Pyrogenic substances were more easily extractable from *S. cerevisiae*, but extracted cells of both species were still highly pyrogenic. It was concluded that the particulate nature of the yeast cell did not contribute to the induction of fever, for latex particles of a similar size were nonpyrogenic. Viable or heat-killed *C. albicans*, phenol extract of *C. albicans*, zymosan, and polystyrene latex particles all failed to induce in rabbits increased dermal reactivity to epinephrine.

The injection of cells or extracts of *Candida albicans* into appropriate animals results in a variety of physiological reactions such as fever (Braude, McConnell, and Douglas, 1960), shock (Salvin, 1952; Dobias, 1957; Roth and Murphy, 1957; Mourad and Friedman, 1961), leucopenia followed by leucocytosis (Braude et al., 1960), increased resistance to shock ("tolerance"; Hasenclever and Mitchell, 1962), and dermal necrosis (Maibach and Kligman, 1962). Although these reactions appear similar to some of those produced by gram-negative bacterial endotoxin (e.g., *see* Bennett and Cluff, 1957), there is one striking difference. In the case of endotoxins these phenomena are produced by micrograms and submicrogram quantities of material, whereas with cells or extracts of *C. albicans* amounts in the milligram range invariably are required to produce such effects. In the present study, we investigated pyrogenicity and increased dermal reactivity to epinephrine in rabbits, induced by various yeasts. Attempts to isolate the pyrogenically active material by various extraction procedures are summarized.

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MATERIALS AND METHODS

Cultural methods. C. albicans strain 374, virulent for mice and rabbits, was isolated from a human infection. *Saccharomyces cerevisiae* NRRL Y2572 originated from the culture collection of L. J. Wickerham of the Northern Utilization Research and Development Division of the Department of Agriculture, Peoria, Ill. *Cryptococcus neoformans* strain 103, isolated from bovine mastitis, was obtained from C. W. Emmons of the National Institutes of Health, Bethesda, Md.; it produced relatively little capsular material. *C. neoformans* strain 14 was isolated from a human infection and was highly encapsulated. All stock cultures were maintained at room temperature, between 24 and 27 C, on Sabouraud glucose agar slants, and were transferred every 2 weeks.

A synthetic medium, the preparation and composition of which are presented in detail in another paper (Kobayashi, Friedman, and Kofroth, 1964), was used for the production of all yeast

cells employed in the pyrogenic studies except *C. neoformans*. Since the latter organism did not grow well in this medium, 1 ml of Vitamin Stock Solution (100 times concentrated; Microbiological Associates, Inc., Bethesda, Md.) was added per 100 ml of medium. Casamino-yeast extract-glucose (CYE) broth (Mourad and Friedman, 1961) was employed for growth of yeast cultures used in the preparation of purified cell-wall material and for comparative growth studies of *C. neoformans*.

Growth from a 7-day-old slant of Sabouraud glucose agar was inoculated into 150 ml of the appropriate broth and incubated with constant agitation at 37 C, in the case of *Candida albicans* and *Cryptococcus neoformans*, or at room temperature for *S. cerevisiae*. Total particle counts were determined in a hemacytometer; viable cells were enumerated by plate counting.

Extraction procedures. A 72-hr culture of cells grown in synthetic medium was harvested by centrifugation, washed three times with cold physiological saline, suspended in saline to a final concentration of 7 to 10% (wet w/v), placed into a 3-liter separatory funnel, and extracted by the aqueous-ether procedure of Ribi, Milner, and Perrine (1959*a*). The slightly opalescent supernatant fluid, which was obtained after centrifugation at 1,300 × *g* for 30 min, was carefully removed from the sediment by suction and dialyzed against five daily changes of distilled water at 4 C. The sedimented cells were resuspended in distilled water to a final concentration of 20% (wet w/v), and extracted by occasional stirring for 3 hr with an equal volume of 0.5 M trichloroacetic acid according to the method of Webster et al. (1955). This procedure was terminated by centrifugation at 1,300 × *g* for 30 min. The clear supernatant fluid was separated by suction and dialyzed against five daily changes of distilled water at 4 C. The extracted residue was washed repeatedly with cold distilled water until the wash water was neutral to litmus paper, and then washed four times with cold acetone and dried in vacuo. The dried cells were resuspended in distilled water, 35 ml per g (dry weight) of cells, and heated in a water bath at 65 to 68 C prior to phenol extraction (Westphal and Lüderitz, 1954). After two successive phenol extractions, the aqueous phases were recovered by centrifugation at 1,300 × *g* for 30 min, pooled, clarified by centrifugation, and dialyzed against running tap water overnight and against five daily changes of distilled water at 4 C. The extracted cells were reclaimed from the phenol by centrifugation, suspended in distilled water, dialyzed against running tap water for 48 hr, centrifuged, washed once with 95% ethanol, suspended in distilled water, and finally dialyzed against five daily changes of distilled water at 4 C.

The various fractions were shell-frozen, lyophilized, and stored under vacuum until needed.

Preparation of purified cell walls. These preparations were made in the Rocky Mountain Laboratory, National Institutes of Health, Hamilton, Mont. A 48-hr culture of *C. albicans*, grown in CYE broth, was harvested by centrifugation, washed three times with cold distilled water, suspended in saline to a concentration of 20% (wet w/v), and processed in a Ribi pressure cell (Ribi et al., 1959*b*) in a batchwise manner, about 100 ml per run, at 35,000 to 43,000 psi. Thereafter, the suspension was centrifuged at 3,500 × *g* for 15 min. The sediment was recovered, resuspended in cold saline, and centrifuged at 3,500 × *g* for 15 min; the supernatant fluid was discarded. On subsequent washings, performed similarly, only the upper half of the sediment was recovered; the lower half was discarded. The washing procedures were followed by examination of samples in an electron microscope and, when it appeared that few granules and no intact cells were present, the material was washed once with 1 M NaCl and centrifuged at 1,300 × *g* for 30 min. The final sediment was washed four times with distilled water and then lyophilized. All of these procedures were carried out at 4 C.

Animal experimentation. Male New Zealand white rabbits (Albino Farms, Red Bank, N.J.) weighing 2 to 3 kg, were housed individually in an air-conditioned room at approximately 21 C. The methods for conditioning and managing the animals used in the pyrogenicity studies are given in detail in another paper (Kobayashi et al., 1964).

The following materials were tested for pyrogenicity: extracts, viable cells, and heat-killed cells of *C. albicans;* extracts and viable cells of *S. cerevisiae;* viable *C. neoformans* cells (both slightly and heavily encapsulated strains); gram-negative bacterial endotoxin (*Escherichia coli* endotoxin of the phenol-water type prepared by C. W. DeWitt of this Department); and polystyrene latex particles, 1.171 μ in diameter.

[Polystyrene latex particles were provided by J. W. Vanderhoff of the Dow Chemical Co., Midland, Mich. These particles were acid-treated and thoroughly washed as described in a previous paper (Kobayashi and Friedman, Proc. Soc. Exptl. Biol. Med. *in press*).] Varying concentrations of the test materials were inoculated into the marginal ear vein in a volume of 1 ml. Concurrent with each animal experiment, three normal rabbits were injected with the same saline used in the preparation of the test materials. Since the temperatures of these control rabbits never fluctuated more than 0.5 F in any of the experiments, any deviation in temperature greater than this value was considered significant.

To test for increased dermal reactivity to epinephrine, 10^9 particles of viable or heat-killed *C. albicans* in a total volume of 1 ml were injected into the ear vein; immediately thereafter, 0.1 ml of epinephrine (Parke, Davis & Co., Detroit, Mich.; 1:1,000 concentration, lot no. BF 122-1) and 0.1 ml of saline were injected intradermally into different sites of previously clipped abdominal skin (Neter, Anzai, and Gorzynski, 1960). Reactions were observed at 6 and 18 hr. Instead of *C. albicans*, other groups of animals were injected with *E. coli* endotoxin, nonpyrogenic saline prepared in this laboratory, a commercially obtained nonpyrogenic saline (Baxter Laboratories, Morton Grove, Ind.), phenol-extractable material from *C. albicans*, zymosan (Fleischman's yeast zymosan, lot no. 5 B 171), or polystyrene latex particles of varying sizes.

Miscellaneous procedures. Water, doubly distilled in an all-glass still, was used for the preparation of media and reagents. All glassware, syringes, and needles were heated in a hot-air oven at 160 to 170 C overnight to destroy contaminating pyrogens. Physiological saline was prepared from each batch of water for injection into rabbits to serve as an indicator of pyrogenic contamination. Plastic syringes equipped with a rubber plunger were used for injecting the polystyrene latex particles.

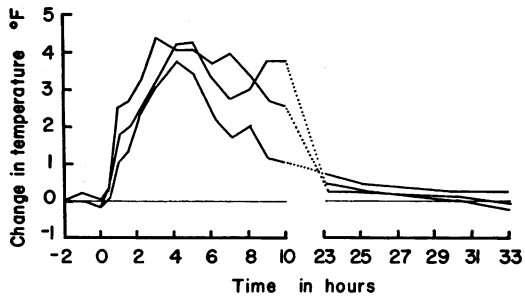

FIG. 1. *Immediate febrile responses of three rabbits intravenously inoculated with 10 μg of the endotoxin of Escherichia coli. The broken lines represent intervals without observation.*

RESULTS

Pyrogenicity in rabbits. The intravenous injection of 10 μg of bacterial endotoxin elicited pyrogenic responses typical of that material, viz., a lag of about 30 min, followed by an immediate elevation, reaching one or two peaks and then subsiding by 10 hr (Fig. 1). Fevers indistinguishable from these were observed after injection of relatively high doses (10^9) of all yeasts, viz., *C. albicans* (Fig. 2), *S. cerevisiae* (Fig. 3), and both heavily and slightly encapsulated *C. neoformans* (Fig. 4). An immediate sharp rise of temperature did not occur after inoculation of relatively low doses of *C. albicans* (10^7 viable particles). At the dose employed, all extracts of *S. cerevisiae* were

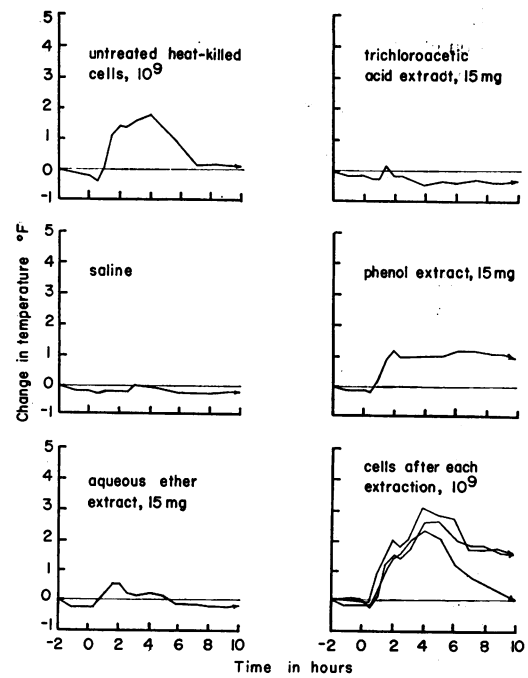

FIG. 2. *Average febrile responses of rabbits (three per group) intravenously inoculated with intact heat-killed cells, various extracts, or extracted cellular residues of Candida albicans. Arrows indicate normal body temperatures beyond 24 hr.*

pyrogenic regardless of the method of extraction (Fig. 3), but only the hot phenol extract of *C. albicans* induced the endotoxin-like fever (Fig. 2). There was no change in the pyrogenicity of the cells after extraction (Fig. 2 and 3). The only

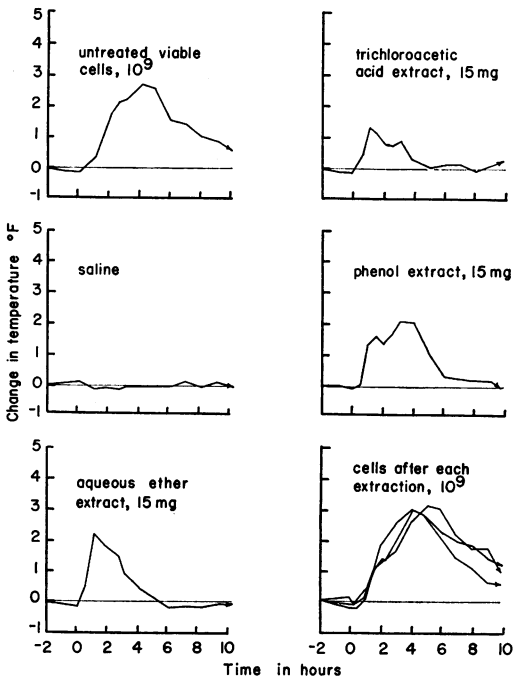

FIG. 3. *Average febrile responses of rabbits (three per group) intravenously inoculated with intact viable cells, various extracts, or extracted cellular residues of Saccharomyces cerevisiae. Arrows indicate normal body temperatures beyond 24 hr.*

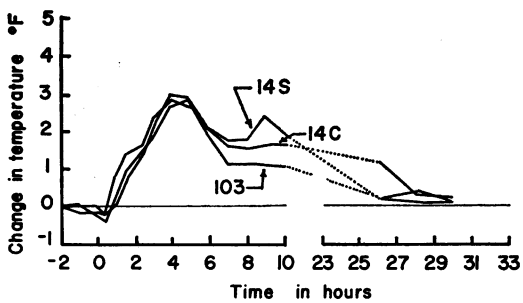

FIG. 4. *Average febrile responses of rabbits intravenously inoculated with 10⁹ particles of viable Cryptococcus neoformans strains 103 (small capsule, six rabbits), 14 C (large capsule, three rabbits) grown in nonsynthetic medium, and 14 S (large capsule, three rabbits) grown in synthetic medium. Hours 10 to 26 were without observation.*

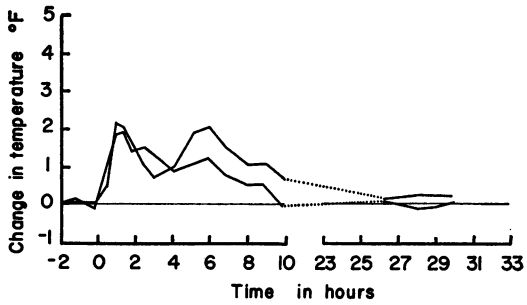

FIG. 5. *Febrile responses of two rabbits inoculated intravenously with 15 mg of purified cell-wall fragments of Candida albicans. Hours 10 to 26 were without observation.*

TABLE 1. *Test of various materials for increased dermal reactivity of rabbits to epinephrine*

Test material	No. of rabbits	Dermal reactions
Escherichia coli endotoxin, 10 μg	20[a]	Positive[b]
Candida albicans, viable, 10^9...	3[c]	Negative[d]
C. albicans heat-killed, 10^9.....	15	Negative
Phenol-extractable material, 15 mg.....	3	Negative
Polystyrene latex, 0.557 μ, 10^9..	10	Negative
Polystyrene latex, 1.171 μ, 10^9..	10	Negative
Polystyrene latex, 6 to 14 μ, 10^9.	3	Negative
Zymosan, 15 mg.....	3	Negative
Saline, 1 ml.....	14	Negative

[a] One animal dead at 24 hr.

[b] Hemorrhagic, reddish-brown to purplish lesions with subsequent necrosis.

[c] All animals dead before 120 hr.

[d] Negative: diffuse erythema occasionally noted at injection site.

cell walls injected, *C. albicans*, also were pyrogenic (Fig. 5). Latex particles were inactive.

When the inoculum of *C. albicans* was viable, there was an elevation in temperature on the second day regardless of dose. In the case of 10^9 particles, this response was a protraction of the immediate endotoxin-like fever.

Increased dermal reactivity of rabbits to epinephrine. Positive results were not given by: viable, heat-killed, or hot phenol extracts of *C. albicans;* zymosan; latex particles; saline prepared in this laboratory; or commercially prepared saline. Endotoxin obtained from gram-negative bacteria, however, gave the anticipated dermal reactions. Details of these observations are presented in Table 1.

DISCUSSION

Although many facets remain to be explored, the observations in the present study suggest that the pyrogenicity of *C. albicans*, which is characterized by the endotoxin-like febrile response, is partly attributable to a substance or substances that can be extracted. It would seem, however, that the extractable toxic substances of *C. albicans* are rather tightly bound to the cells, for several conventional methods which ordinarily remove bacterial endotoxins failed, and only when the hot phenol extraction method was employed was success achieved. Furthermore, the substance that was finally isolated either was not highly active or the active principle was not present in a sufficiently high concentration, because milligram quantities were necessary to induce fever in comparison with the submicrogram amounts of bacterial endotoxin which are capable of inducing such a response. Recently, Isenberg, Allerhand, and Berkman (1963*a*) and Isenberg et al. (1963*b*) reported the phenol extraction of a toxic substance from *C. albicans*, very likely the same as that isolated in the present studies since it had endotoxin-like properties, viz., mouse lethality and the ability to cause dermal necrosis. It is tempting to characterize the toxic nature of *Candida* as endotoxic, inasmuch as other investigators have extracted materials which were dermonecrotic (Maibach and Kligman, 1962), lethal in mice (Dobias, 1957), and poorly antigenic (Isenberg et al., 1963*a*). Also, Aksoycan and LeMinor (1960) reported that *C. albicans* possess some antigenic similarities to *Salmonella* in agglutination tests with *Salmonella* O:6,7 and *Salmonella* O:4 antisera, and Vogel (1957) prepared an erythrocyte-sensitizing antigen from *C. albicans* which was lipopolysaccharide in nature. Hasenclever and Mitchell (1962) even found that previous experience with *C. albicans* rendered mice resistant to the toxic effect of *C. albicans*, but spherules of *Coccidioides immitis*, themselves not toxic, were capable of inducing the same results. It is tempting to relate pyrogenicity of the phenol-extractable material to classical endotoxin, but the facts at hand do not as yet warrant such extrapolation, even though in terms of its behavior it does share some of the characteristics of classical endotoxin. We feel the possession of such biological properties *per se* is not sufficient evidence that *C. albicans* contains classical endotoxin, because native dextrans (Bennett, 1952) and a synthetic polysaccharide derivative, methylcellulose (Wiedersheim, et al., 1953), also have endotoxin-like properties, viz., pyrogenicity. Furthermore, our pyrogenic phenol extract of *C. albicans* did not possess at least one of the properties of classical endotoxin, for it failed to cause increased dermal reactivity to epinephrine, as does classical endotoxin. Admittedly, however, this failure could have been due to insufficient concentration of active principle in the extract, because the dermal reactivity test is not sensitive to less than 1 μg of endotoxin (Thomas, 1956), whereas rabbit pyrogenicity will detect as little as 0.03 μg (Keene, Silberman, and Landy, 1961). We feel that further purification and chemical characterization must be carried out to definitely establish that the toxic substance of *C. albicans* is identical with the endotoxin of gram-negative bacilli.

It is interesting that toxic substances were more easily extracted from *S. cerevisiae* by each of the three methods employed, presumably because they are not so tightly bound to the cell. It is not surprising, however, that these extracts, as well as cells, were pyrogenic because zymosan, a substance isolated from the cell wall of *S. cerevisiae*, has been shown by Freedman and Sultzer (1961) to possess pyrogenicity. Furthermore, a proteose-lipid isolated from the same yeast is cytotoxic, in vitro and in vivo (Lund, 1953).

The present investigation has revealed, additionally, that the intravenous injection of viable *C. neoformans*, regardless of the degree of encapsulation, can cause an immediate fever in rabbits. Braude et al., (1960) failed to demonstrate such a response; they and Proknow (1962) suggested that the presence of capsular material may possibly prevent the underlying pyrogenic material from manifesting itself. In our study, the dose of *C. neoformans* used to induce the febrile response was the same as the concentration of *C. albicans* which evoked a marked immediate response; it was tenfold greater than that used by the former workers.

Since the cells of both *S. cerevisiae* and *C. albicans* which had been extracted by the aqueous-ether, trichloroacetic acid, and the phenol methods still retained pyrogenic activity, and since viable cells of *C. neoformans* were pyrogenic, an investigation of the role of the particulate nature of the cell in this response was undertaken. Polystyrene latex particles were chosen for this

study, because the particle sizes available approximated those of the yeast cells and because they were of nonbiological origin. Careful treatment of these particles to remove contaminating pyrogens, such as endotoxin, and injection of these particles in amounts comparable to those used in the study of yeast cells did not induce a febrile response. Therefore, at the concentrations we employed, the particulate nature of these cells did not contribute to the induction of pyrogenicity in rabbits. It is possible, of course, that the immediate fever produced by the suspensions of intact *C. albicans* and the other yeasts was due to contaminating gram-negative endotoxin. We attempted, however, to minimize this by employing glassware sterilized at high temperatures and nonpyrogenic distilled water at every step. Additional evidence in support of the fact that endotoxic contamination did not occur was that extraction of *C. albicans* by the aqueous-ether and the trichloroacetic acid methods yielded substances which were not pyrogenic.

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