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Reaction of Fungal Products with Amebocyte Lysates of the Japanese Horseshoe Crab, *Tachypleus tridentatus*

DAVID S. HODES,* DENNIS HEON, ADA HASS, ALEXANDER C. HYATT, AND HORACE L. HODES

Division of Infectious Diseases, Department of Pediatrics, The Mount Sinai School of Medicine, City University of New York, New York, New York 10029

Received 19 February 1987/Accepted 5 June 1987

A commercially available endotoxin assay (CS-TAL) employing a chromogenic peptide and an amebocyte lysate from the Japanese horseshoe crab, *Tachypleus tridentatus*, gave a positive result with aqueous extracts of all 15 strains of *Candida albicans* and 1 strain each of *Candida tropicalis*, *Cryptococcus neoformans*, and a *Mucor* species that we tested. Purified glucans prepared from the *Candida* strains gave the same results. Reconstruction experiments showed that the positive results were not due to contaminating endotoxin. By contrast, assays employing amebocyte lysates of the American horseshoe crab, *Limulus polyphemus*, were inconsistent. Japanese workers have presented evidence that glucans activate the *Tachypleus* amebocyte lysate system by acting on an enzyme different from that on which endotoxin acts. Using a *Tachypleus* lysate preparation (Endospecy; Seikagaku Kogyo, Tokyo, Japan) from which this enzyme was excluded, we demonstrated a 5- to 10-fold drop in reactivity to the aqueous *Candida* extracts and glucans, whereas reactivity to endotoxin was unchanged. Normal human plasma was shown to decrease the effect of fungal extracts on CS-TAL. This inhibition was completely removed by heating the plasma. Our results suggest that *Tachypleus* systems may be of use clinically in distinguishing bacterial from fungal infections.

A number of papers published during the past 35 years have presented evidence that *Candida albicans* and other pathogenic fungi have some of the biologic properties of bacterial endotoxin (ETX) (1, 5, 7, 13). However, the quantity of material from fungi that is needed to produce these biologic effects is much greater than the quantity of ETX that is required. Also, it has been reported that fungi are unable to cause gelation of lysates of *Limulus polyphemus* (2, 14), and no compound with the chemical structure of bacterial ETX has been found in fungi.

We recently reported a study of plasma ETX levels in febrile immunocompromised children (6). The levels in plasma were measured by an accurate and sensitive system (CS-TAL) which employs a combination of a lysate of the amebocytes of the Japanese horseshoe crab, *Tachypleus tridentatus*, and a chromogenic peptide substrate. Two of the children in our study had generalized *Candida* infections. Their blood yielded pure cultures of *C. albicans*. Concurrent with these blood cultures, extracts of their plasma gave highly positive CS-TAL tests, although no gram-negative bacterial infection could be demonstrated in either child.

This experience led us to study cultured fungal cells to determine whether products extracted from them would give a positive reaction with CS-TAL. In the experiments reported here, aqueous extracts of fungal cells reacted strongly with CS-TAL. Reconstruction experiments showed that these results were not due to contaminating ETX. Glucans isolated from the extracts also gave positive results, and evidence was obtained that indicates that the action of the extracts on CS-TAL is different from that of ETX and involves a different enzyme pathway.

MATERIALS AND METHODS

Glassware and plastic ware. Glassware was sterilized by autoclaving at 121°C for 30 min and then was made ETX free by heating at 250°C for 4 h. Plastic ware consisted of sterile individually wrapped pipettes (Falcon; Becton Dickinson Labware, Oxnard, Calif.) and sterile ETX-free polystyrene test tubes (Corning Glass Works, Corning N.Y.). To determine whether the plastic ware was ETX free, parallel assays of reference U.S. standard ETX (EC-5) were made with the plastic ware and with chemically cleaned glassware that had been heated to 250°C for 4 h. There was no difference between the results obtained with plastic ware and glassware.

Cultures. The 15 strains of *C. albicans* and 1 strain each of *Candida tropicalis*, *Cryptococcus neoformans*, and a *Mucor* species used in our experiments were isolated by the Mount Sinai Hospital laboratory from adult cancer patients. All the strains were passaged four times on Sabouraud glucose agar. The cultures were lyophilized or kept at 4°C on sealed Sabouraud glucose agar plates for up to 1 week and transferred to Sabouraud glucose broth as needed. The broth was made with peptone and glucose (GIBCO Laboratories, Grand Island, N.Y.) from freshly opened bottles; mixed with sterile, pyrogen-free water (Abbott Laboratories, North Chicago, Ill.) in sterile, ETX-free glassware; and autoclaved at 121°C for 18 min. Samples of the water used to make media were used as controls in our assays and were found to have from 0.02 to 0.04 U.S. standard ETX units (EU) per ml.

At the start of each experiment, the cultures to be assayed were shown to be pure by plating on Sabouraud glucose agar and sheep blood agar. Colonies picked from these plates were grown in Sabouraud glucose broth for 72 h at 37°C in sterile, ETX-free glass flasks. Cultures were then transferred to sterile 50-ml plastic calibrated centrifuge tubes and centrifuged at 1,250 × *g* at 4°C for 30 min. The sedimented cells were suspended in 35 ml of sterile, pyrogen-free water, the suspension was shaken by a vortex mixer and centrifuged at 1,250 × *g* at 4°C for 30 min, and the supernatants were discarded. The cells were washed three more times in this way. Samples of the four-times-washed cells were plated on Sabouraud glucose agar and sheep blood agar to exclude

* Corresponding author.

contaminating organisms. After it was shown that only fungi were present, the cells were extracted for assay.

Cell extracts. Cell extracts were made by two methods. In one, the cells were extracted with dilute perchloric acid. In the other, they were autoclaved in water.

The perchloric acid extraction was done as follows. A 0.2-ml sample of washed, sedimented cells was suspended in 0.4 ml of pyrogen-free water. A 0.1-ml sample of this suspension was added to 0.2 ml of 0.32 M perchloric acid and incubated for 20 min at 37°C. Precipitates produced by the perchloric acid were sedimented by centrifugation and discarded. A sample of 0.05 ml of the supernatant was neutralized by the addition of 0.05 ml of 0.18 N NaOH and used as the extract for assay.

The autoclaved extracts were made by suspending the four-times-washed cells in 20 times their volume of sterile, pyrogen-free water. This suspension was placed into an ETX-free, aluminum-foil-covered glass vial and autoclaved at 121°C for 90 min. After cooling, the suspension was centrifuged at 1,250 × *g* for 30 min. The supernatant (extract) was removed, and 0.1 ml of it was used for CS-TAL assay.

CS-TAL assays. The CS-TAL preparation used in our experiments was a combination of a chromogenic peptide, Boc–Leu–Gly–Arg–*p*-nitroanilide, and an amebocyte lysate of the Japanese horseshoe crab, *T. tridentatus*. This preparation (Toxicolor) was supplied by Seikagaku Kogyo, Tokyo, Japan. The techniques used to assay the perchloric acid and autoclaved water extracts were the same. A 0.1-ml sample of each cell extract assayed was mixed with 0.1 ml of CS-TAL reconstituted with 0.2 M Tris hydrochloride (pH 8.0) according to instructions of the manufacturer. The mixture was incubated in a water bath at 37°C for 30 min. The reaction was then stopped, and the nitroaniline produced by the action of the extract on the CS-TAL was diazotized by the addition of 0.5 ml of each of the following solutions: 0.3% ammonium sulfamate in water, 0.04% $NaNO_2$ in 0.48 M HCl, and 0.07% *N*-(1-naphthyl)ethylenediamine dihydrochloride in water. The optical density (OD) of the diazotized aniline was measured at 545 nm in a Gilford model 250 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Pyrogen-free water was tested as a control.

To test the integrity of the CS-TAL system, U.S. standard ETX was dissolved in pyrogen-free water to make concentrations of 0.018, 0.036, 0.072, 0.15, 0.22, and 0.3 EU/ml, and 0.1 ml of each of these was incubated and tested together with the cell extracts and the control. In the range of these values, the relationship of the ETX concentration to the OD reading was established as being linear. OD readings produced by the action of 0.15 EU/ml in 29 experiments gave a coefficient of variation of 10.8 ± 1% (standard error).

***L. polyphemus* assays.** CS-LAL, a combination of the chromogenic peptide Ac–Ile–Arg–*p*-nitroanilide and a lysate of amebocytes of the blood of the American horseshoe crab, *L. polyphemus*, also was used in the experiments with both perchloric acid and aqueous autoclaved fungal extracts. The CS-LAL preparation was supplied by Whittaker M.A. Bioproducts, Walkersville, Md. In this assay, 0.1 ml of extract was mixed with 0.1 ml of lysate and incubated at 37°C for 30 min. The chromogenic substrate was then added, and the incubation was continued for 3 min. The reaction was stopped by the addition of 25% acetic acid. The yellow aniline compound produced by the extract-substrate reaction was read in the spectrophotometer at 405 nm. Diazotation was not performed. The controls were the same as those used in the CS-TAL experiments, and the activity of the CS-LAL system was tested by using concentrations of 0.125, 0.5, and 1.0 EU/ml.

TABLE 1. Effect on CS-TAL of perchloric acid extracts of cells of 11 *C. albicans* strains

Specimen	OD_{545}/0.1 ml of extract	
	Expt 1	Expt 2
Candida albicans		
1	1.38	
2	1.61	
3	1.32	
4	1.49	
5	1.28	
6	1.68	
7	1.46	
8		1.21
9		1.20
10		1.16
11		1.64
Escherichia coli		
1	1.05	
2	1.12	
Standard (EU/ml)		
0.0	0.09	0.09
0.018	0.25	0.26
0.036	0.41	
0.072	0.71	0.79
0.144	1.12	1.30

RESULTS

Effect of fungal extracts on CS-TAL. Extracts of all 18 strains of fungi tested gave strong reactions with CS-TAL. The effect was produced with the dilute perchloric acid cell extracts and with the autoclaved aqueous extracts. The results of two experiments in which the perchloric acid extracts of 11 strains of *C. albicans* were assayed with CS-TAL are shown in Table 1. All of the assay results were positive, with the OD_{545} produced by 0.1 ml of extract ranging between 1.16 and 1.68 (average, 1.40) for the 11 strains. Two strains of *Escherichia coli* grown in Trypticase (BBL Microbiology Systems, Cockeysville, Md.) soy broth and processed in a manner identical to that used with the fungi gave values of 1.05 and 1.12, respectively. The results of the assay of U.S. standard ETX, which was included as a test of the CS-TAL system, are also shown in Table 1.

Effect of fungal extracts on CS-LAL. Two strains of *C. albicans* were tested with CS-LAL in 10 experiments. The results were positive in three experiments, negative in three, and doubtful in four. These inconsistent results were in contrast to those of nine experiments in which the same two *Candida* strains were assayed with CS-TAL. All of the CS-TAL experiments gave strongly positive results. The reason for the inconsistent results with CS-LAL was not found. The negative and doubtful results were not limited to a single batch of CS-LAL received from the manufacturer.

Reconstruction experiments with CS-TAL. Experiments were designed to exclude the possibility that the fungal cells adsorbed or incorporated ETX from the medium and thus produced our positive results with CS-TAL. In experiment 1, ETX was added to a suspension of cells after they had completed their growth in culture but before they were washed. A 1-ml sample of a 72-h *Candida* culture was placed

into each of two ETX-free centrifuge tubes. To one tube was added 1 ml of pyrogen-free water that contained 12 EU (final concentration, 6 EU/ml). One milliliter of pyrogen-free water was added to the other tube. Both tubes were placed in a refrigerator at 5°C for 3 h, during which time they were shaken by hand three times. The tubes were washed and centrifuged four times, and the sedimented cells were then extracted by the perchloric acid method. The extracts were tested by the CS-TAL assay. Both extracts gave the same CS-TAL result, which was equivalent to 0.13 EU/ml, a value within the zone of linear relationship between ETX concentration and CS-TAL positivity. The top of this zone is approximately 0.35 EU/ml.

A second experiment was performed in which the *Candida* cells were exposed to ETX in the medium throughout their 72-h incubation period. Freshly-made Sabouraud glucose broth (150 ml) was placed into each of five ETX-free flasks, sterilized by autoclaving, and preincubated for 3 days at 37°C to ensure sterility. Each flask was then inoculated with 0.5 ml of a 12-h culture of *C. albicans* in Sabouraud glucose broth. To three of the flasks was added U.S. standard ETX in quantities sufficient to give concentrations of 0.15, 2.0, and 3.0 EU/ml. The total quantities of ETX added to these three flasks were 22, 300, and 450 EU, respectively. No ETX was added to two flasks which served as controls. At the end of a 3-day incubation at 37°C, the cells were harvested and washed four times with water. The cells were then sedimented by centrifugation and suspended in 20 times their volume in pyrogen-free water, and autoclaved aqueous extracts were prepared from them as described above. Assays made by the CS-TAL method revealed no significant differences between the extracts of ETX-exposed cells and the control cells.

Experiments with a new CS-TAL preparation (Endospecy). As further proof that our results were not caused by contaminating ETX, we repeated our studies using the Endospecy test (Seikagaku). This test employs a TAL from which certain protein components have been removed, a process which, according to the manufacturer, increases specificity for ETX by eliminating cross-reactivity with certain polysaccharides (9, 11). Assays of autoclaved extracts of *Candida* cells were made simultaneously with CS-TAL and with Endospecy. The results of these experiments showed clearly that the action of the *Candida* extracts was 5 to 10 times greater with CS-TAL than it was with Endospecy.

This result could not be explained by assuming that CS-TAL was more sensitive than Endospecy to any contaminating ETX that may have been present in the extracts. U.S. standard ETX was assayed simultaneously with CS-TAL and Endospecy, and no significant difference was found.

As a further check, assays of the reconstruction experiments were also performed with Endospecy. These assays confirmed that even deliberate contamination of the *Candida* cultures with ETX could not alter the result. Extracts made from *Candida* cultures grown in ETX-added medium had the same action on CS-TAL and on Endospecy as those grown in normal medium. The data are summarized in Table 2.

Experiments with glucans extracted from *C. albicans* cells. Glucans were isolated from autoclaved aqueous *Candida* cell extracts by the method of Grimmecke and Reuter (3) and Peat et al. (12). Mannans were removed from the extract by precipitation with alkaline Fehling solution. The resulting supernatant was brought to pH 2.0 by the addition of concentrated HCl, producing a precipitate of non-polysaccharide materials which was removed by centrifugation. The supernatant was then brought to pH 6.0 by the addition of NaOH and precipitated in four volumes of ethyl alchohol. This precipitate was dried and dissolved in water. A Molisch test of this solution was positive. Spot tests with ninhydrin showed that no amino acids or amino sugars were present. In contrast, the autoclaved aqueous cell extract from which the glucans were obtained gave a positive ninhydrin reaction. The solution of glucans gave a very positive reaction with CS-TAL, but the result with Endospecy was negative (Table 2).

TABLE 2. Summary of reconstruction experiments and comparison of CS-TAL and Endospecy results

Material	ETX added to medium (EU/ml)	OD_{545}/0.1 ml of extract		Ratio of CS-TAL/ Endospecy OD_{545}
		CS-TAL	Endospecy	
Candida albicans extract	3.0	1.47	0.15	9.9
	2.0	1.12	0.13	8.6
	0.15	1.28	0.23	5.6
	0	1.48	0.15	10.0
	0	1.29	0.15	8.8
Candida glucans[a]	0	1.66	0.13	12.8
Standard ETX (EU/ml)				
0.15		0.96	0.99	0.97
0.3		1.38	1.17	1.18

[a] Isolated from autoclaved aqueous extract of *C. albicans* cells in medium without added ETX.

Effect of human plasma on CS-TAL reaction. One-third volumes of human plasma were added to *Candida* extracts that gave positive CS-TAL and negative Endospecy tests. This treatment reduced the CS-TAL reaction by 70 to 90%. The inhibition was completely removed by heating the mixture in a water bath at 100°C for 10 min. This experiment was repeated with the plasma of four healthy donors with identical results.

DISCUSSION

In the experiments described here, extracts of cultures of *C. albicans* and three other fungi gave strongly positive reactions with CS-TAL. Quantitatively, the results were similar to those of *E. coli* cultures. This finding is evidence that some naturally occurring fungal product or products are capable of reacting with CS-TAL as well as is bacterial ETX.

Because it is impossible to guarantee the complete absence of bacterial ETX from growth media, we undertook to deal with the possibility that our results with fungi could have been caused by contaminating ETX. First, in a set of reconstruction experiments we deliberately added U.S. standard ETX to cultures of both growing and static fungi. When we processed these cultures with four washings with water, according to our usual procedure, the cell extracts gave reactions with CS-TAL that were not significantly different from those of the extracts of cells that had not been exposed to added ETX. Thus, it is extremely unlikely that our results were caused by contaminating ETX. Clearly, fungal cells did not adsorb ETX from the medium, or if they did, the washing they underwent removed it or the extraction process left it still adsorbed to the cells.

As further confirmation that our results were not caused by ETX contamination, we used the new CS-TAL test,

Endospecy, developed by Obayashi et al. to enhance specificity for ETX (11). The impetus for the development of this test was the finding that a β-D-glucan isolated from *Alcaligenes faecalis* produced a positive reaction with CS-TAL (8). This finding was explained as follows. The final common step in the TAL enzyme pathway is the activation of clotting enzyme, an agent which can either activate clot formation or, when presented with a chromogenic substrate, generate a colored compound which can be measured quantitatively in the spectrophotometer (4). Clotting enzyme in TAL is activated by "compound B," which is in turn activated by "compound C." Alternatively, clotting enzyme can be activated directly by "compound G," which is also present in TAL. According to Obayashi et al. (11), ETX initiates the clotting cascade exclusively via compound C, whereas the action of the synthetic 1,3-β-D-glucan and crude polysaccharide extracts from the fungi which they examined occurred only through compound G (8, 10). By omitting compound G from the mixture of the TAL enzymes used to make up Endospecy, a test more specific for ETX was produced. As shown in Table 2, our fungal extracts all gave readings from 5 to 10 times as high with CS-TAL as with Endospecy. This finding cannot be explained by differences in sensitivity to ETX of the two preparations, because they gave the same results with standard ETX (Table 2). This is a further indication that ETX contamination was not a significant factor in our data.

We tested the hypothesis that glucans, which are found in fungal cell walls, were responsible for our results. The glucans that we isolated from *C. albicans* cell extracts reproduced the positive reactions with CS-TAL, but the reactions with Endospecy were negative.

As is the case with the ETX reaction, the reaction of fungal glucans with CS-TAL is inhibited by heat-labile factors in human plasma. In performing plasma assays for fungal products, these factors must be removed.

In contrast to our results with TAL preparations, assays of fungal extracts using amebocyte lysate preparations from the American horseshoe crab, *L. polyphemus*, were inconsistent. The reason for this discrepancy is unclear. It may represent alteration of the components of the *Limulus* clotting proteins in industrial preparation or, alternatively, an inherent difference in the proteins in the amebocytes of the two genera. Our initial analyses by polyacrylamide gel electrophoresis of commercial preparations of the Japanese and American horseshoe crab amebocyte lysates revealed them to differ considerably.

In summary, our previous findings of positive CS-TAL tests of the plasma of immunocompromised children with fungal infections are underscored by our present finding that the assay reaction is triggered by fungal products, most probably cell wall glucans. It is possible that simultaneous CS-TAL and Endospecy assays can distinguish between the presence of fungal products and ETX in plasma. This would be very useful in a number of clinical situations, especially with immunosuppressed patients who have a high rate of infection with ETX-producing organisms and fungi.

The tests give promise of application to human plasma specimens. For testing plasma, the perchloric acid method (described under cell extracts in Materials and Methods) should be used. Both the CS-TAL and Endospecy tests can be performed on a single specimen by a technician within 3 h (including 1 h of incubation time). A batch of 20 samples can be completed in about 4 h. For performing both tests, 0.2 ml of plasma is required. The only large piece of equipment needed is a spectrophotometer, and positive and negative readings are easily distinguished.

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